THE VALIDATION OF PROFLUORESCENT NITROXIDES AS POTENTIAL PROBES TO MONITOR MITOCHONDRIAL OXIDATIVE STRESS

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Keywords

3-((4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium) Bromide, Adenocarcinomic Alveolar Basal Epithelial Cells, Antimycin, Antioxidant, Ataxia-Telangiectasia, Carbonyl Cyanide m-Chlorophenyl Hydrazine, Cerebellum Cells, Cervical Cancer Cells, Dulbecco’s media, Flow Cytometry, Fluorescein, Fluorescence, Fluorescent Probe, Fluorophore, Free Radicals, hTERT Immortalized Human Fibroblast Cells, Hydroxylamine, Immortalized Retinal Cells, Intraocular Pressure, Ischaemia, Isoindoline, LysoTracker Blue, Microscopy, Mitochondria, MitoSox, MitoTracker Green, Nitroxide, Oxidation, Oxidative Stress, Paramagnetic, Retinal Cells, Probe, Profluorescent, Reactive Oxygen Species, Redox, Reduction, Retina, Rhodamine, Rotenone, Sprague-Dawley Rats, Tetramethylrhodamine, Methyl Ester
Abstract

Profluorescent nitroxides (PFNs), in which redox sensitive nitroxide moieties are coupled to a fluorophore, have been reported to be effective probes to monitor cellular redox status. Based on this, several PFNs have been developed and trialled, in order to develop a biologically relevant probe that allows the assessment of cellular redox status.

Structurally modified PFNs possessing greater steric bulk around the nitroxide moiety are harder to reduce than less hindered probes. Extra steric hindrance around the radical centre within the PFN, is also helped by the extra electron-donating character of four ethyl versus four methyl groups beta to the nitroxide group. The greater steric bulk along with the extra steric hindrance in ethyl-based PFNs result in a slower reduction rate compare to methyl-based PFNs. This enables ethyl-based probes to better reflect the redox status within the systems under study as ethyl-based probes are not as rapidly reduced by the many biological reductants present in viable cells. The slower reduction rate of ethyl-based probes gives a more measured response to metabolic factors.

Fluorescein-chromophore based PFNs (tetraethylfluorescein nitroxide, TEF) are less stable to changes in the pH of the environment than rhodamine-chromophore based PFNs (tetraethylrhodamine nitroxide, TER). The rhodamine chromophore gives a constant fluorescent response over the pH range of 3-12. On the other hand, fluorescence intensity varies with pH in the case of TEF, due to structural changes that occur with fluorescein from pH 3-12.
In this study, fluorescence imaging was undertaken to investigate cellular localization of fluorescein-based and rhodamine-based PFNs. This provided the background for assessing each probe’s possible role in monitoring redox status within specific cellular compartments.

TEF undergoes predominantly lysosomal localisation while TER is present mostly in the mitochondria. As mitochondria are one of the main sites for the generation of reactive oxygen species (ROS), TER is a better probe for analysis of oxidative stress within cells.

The fluorescence-based response of TER was shown to be reversible and therefore reflects cellular redox status in real time. Based on microscopy and flow cytometry investigations, it was determined that TER responded to changes in intracellular redox environment, especially those driven by the mitochondria. To validate this, cells incubated with TER were stressed with rotenone (ROT) and antimycin (AMC). Fluorescence emission of TER decreases with increasing levels of ROT and AMC additives.

In an attempt to increase the uptake of TER type probe in the mitochondria, TER was esterified to give a probe with more positive charge. This methyl-ester tetraethyl-isoiindoline linked rhodamine molecule (ME-TER) gave significantly increased mitochondrial localization.

Flow cytometry demonstrated that ME-TER responded to changes in intracellular redox environment induced by ROT and AMC. In addition, ME-TER was shown to be able to assess redox status for a number of cell lines. ME-TER was also potentially able to assess redox status for Ataxia Telangiectasia (A-T) cell lines and within the retina of ischaemic rat model. Both A-T and ischaemic retinas are recognized as involving significant levels of oxidative stress.

As determined by cell counting and MTT (3,-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, both TER and ME-TER do not appear to affect cell viability, even over extended incubation periods up to 3 days.
Together, these results establish the feasibility of a rhodamine-based PFN probe as effective real time analytical tools to determine redox status within the mitochondria of cellular systems.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keywords</td>
<td>3</td>
</tr>
<tr>
<td>Abstract</td>
<td>4</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>7</td>
</tr>
<tr>
<td>List of Figures</td>
<td>9</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>22</td>
</tr>
<tr>
<td>Statement of Original Authorship</td>
<td>24</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>25</td>
</tr>
<tr>
<td><strong>INTRODUCTION &amp; LITERATURE REVIEW</strong></td>
<td>27</td>
</tr>
<tr>
<td><strong>CHAPTER 1: Introduction &amp; Literature Review</strong></td>
<td>28</td>
</tr>
<tr>
<td>1.1 What are free radicals?</td>
<td>28</td>
</tr>
<tr>
<td>1.2 Antioxidants</td>
<td>29</td>
</tr>
<tr>
<td>1.3 Oxidation reduction detection</td>
<td>31</td>
</tr>
<tr>
<td>1.3.1 Spectroscopic techniques</td>
<td>31</td>
</tr>
<tr>
<td>1.3.2 Chemiluminescence techniques</td>
<td>32</td>
</tr>
<tr>
<td>1.3.3 Electron Paramagnetic Resonance (EPR) or Electron Spin Resonance (ESR)</td>
<td>35</td>
</tr>
<tr>
<td>1.3.4 Fluorescence techniques</td>
<td>37</td>
</tr>
<tr>
<td>1.4 Nitroxides</td>
<td>41</td>
</tr>
<tr>
<td>1.5 Profuorescent nitroxe (PFN) probes</td>
<td>42</td>
</tr>
<tr>
<td>1.6 Underlying mechanism contributing to profuorescence</td>
<td>44</td>
</tr>
<tr>
<td>1.7 Advantages of profuorescent nitroxe (PFN) probes</td>
<td>47</td>
</tr>
<tr>
<td>1.8 Past studies of profuorescent nitroxe (PFN’s) probes in biological systems</td>
<td>48</td>
</tr>
<tr>
<td>1.9 Mitochondria- important ROS generation site within biological systems</td>
<td>55</td>
</tr>
<tr>
<td>1.9.1 Electron Paramagnetic Resonance (EPR) techniques for the analysis of mitochondrial redox status</td>
<td>57</td>
</tr>
<tr>
<td>1.9.2 Liquid chromatography tandem mass spectroscopy techniques for the analysis of mitochondrial redox status</td>
<td>59</td>
</tr>
<tr>
<td>1.9.3 Fluorescence techniques for the analysis of mitochondrial redox status</td>
<td>60</td>
</tr>
<tr>
<td><strong>Research Objectives</strong></td>
<td>65</td>
</tr>
<tr>
<td><strong>MATERIALS &amp; METHODS</strong></td>
<td>68</td>
</tr>
<tr>
<td><strong>CHAPTER 2: Materials &amp; Methods</strong></td>
<td>69</td>
</tr>
<tr>
<td>2.1 Reagents and chemicals</td>
<td>69</td>
</tr>
<tr>
<td>2.2 Cell lines and animal model</td>
<td>70</td>
</tr>
<tr>
<td>2.3 Cell culture</td>
<td>71</td>
</tr>
<tr>
<td>2.4 Cell counting</td>
<td>72</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>2.5</td>
<td>Fluorescence measurements</td>
</tr>
<tr>
<td>2.6</td>
<td>Fluorescence imaging</td>
</tr>
<tr>
<td>2.7</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>2.8</td>
<td>MTT assay</td>
</tr>
<tr>
<td>2.9</td>
<td>Statistical analysis</td>
</tr>
<tr>
<td></td>
<td><strong>RESULTS &amp; DISCUSSION</strong></td>
</tr>
<tr>
<td></td>
<td><strong>CHAPTER 3: Optimizing Mitochondrial Redox Probe</strong></td>
</tr>
<tr>
<td>3.1</td>
<td>Comparing reduction rate of tetramethyl- and tetraethyl-based PFN</td>
</tr>
<tr>
<td>3.2</td>
<td>Assessing the pH stability and intracellular localization of</td>
</tr>
<tr>
<td></td>
<td>tetraethylfluorescein nitrooxide (TEF) in cells</td>
</tr>
<tr>
<td>3.3</td>
<td>The use of rhodamine chromophore as an improvement over fluorescein</td>
</tr>
<tr>
<td>3.4</td>
<td>Assessing reversibility of TER</td>
</tr>
<tr>
<td></td>
<td><strong>CHAPTER 4: The Application of PFNs within Biological Systems</strong></td>
</tr>
<tr>
<td>4.1</td>
<td>Comparing redox status between normal and Ataxia Telangiectasia,</td>
</tr>
<tr>
<td></td>
<td>A-T diseased cells using TER as redox probe</td>
</tr>
<tr>
<td>4.2</td>
<td>Optimizing flow cytometry experiment</td>
</tr>
<tr>
<td>4.3</td>
<td>Assessing redox status within cells via flow cytometry</td>
</tr>
<tr>
<td></td>
<td><strong>CHAPTER 5: Improved Cellular Response of PFNs by Esterification</strong></td>
</tr>
<tr>
<td>5.1</td>
<td>Localization of methyl ester tetraethylrhodamine nitrooxide (ME-TER)</td>
</tr>
<tr>
<td>5.2</td>
<td>Methyl ester tetraethylrhodamine nitrooxide (ME-TER) as mitochondrial redox probe</td>
</tr>
<tr>
<td>5.3</td>
<td>Cytotoxicity of Methyl Ester Tetraethylrhodamine (ME-TER)</td>
</tr>
<tr>
<td>5.4</td>
<td>Comparing redox status between normal and Ataxia Telangiectasia, A-T diseased cells</td>
</tr>
<tr>
<td>5.5</td>
<td>Versatility of ME-TER in other cell lines</td>
</tr>
<tr>
<td>5.6</td>
<td>The use of ME-TER as potential probe to monitor the efficacy of</td>
</tr>
<tr>
<td></td>
<td>lutein as antioxidant therapy in reducing oxidative stress</td>
</tr>
<tr>
<td>5.7</td>
<td>Assessing redox status within animal model using ME-TER as redox probe</td>
</tr>
<tr>
<td></td>
<td><strong>CONCLUSION &amp; FUTURE WORK</strong></td>
</tr>
<tr>
<td></td>
<td><strong>CHAPTER 6: Conclusion &amp; Future Work</strong></td>
</tr>
<tr>
<td></td>
<td><strong>REFERENCES</strong></td>
</tr>
</tbody>
</table>
## List of Figures

<p>| Figure 1.1 | Reduction of Nitro Blue Tetrazolium (NBT(^{2+})) to formazan. | 31 |
| Figure 1.2 | Reduction of Cytochrome C protein by free radicals. | 32 |
| Figure 1.3 | Reaction pathway of luminol derived chemiluminescence. | 33 |
| Figure 1.4 | Chemical structure of 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo(1,2-(\alpha)) pyrazin-3-one (MCLA) and 6-[4-[2-<a href="5-fluoresceinyl">n((\leftarrow))-</a>thioureido]ethoxy]phenyl]-2-methylimidazo[1,2-alpha]pyrazin-3(7H)-one (FCLA). | 33 |
| Figure 1.5 | Reaction pathway of lucigenin. | 34 |
| Figure 1.6 | Examples of spin traps. | 36 |
| Figure 1.7 | Detection of free radicals using 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine (Tempone-H). | 36 |
| Figure 1.8 | Chemical structure of TAM OX063. | 37 |
| Figure 1.9 | Chemical structures of (p)-hydroxyphenylacetic acid. | 38 |
| Figure 1.10 | Dichlorodihydrofluorescein diacetate (DCFH-DA) in the detection of hydrogen peroxide (H(_2)O(_2)). GSH- Glutathione; GSSG- Glutathione Disulfide; NADH- Nicotiamide Adenine Dinucleotide. | 39 |
| Figure 1.11 | Chemical structures of A) 7-hydroxy-6-methoxy-coumarin (scopoletin) and B) 9,10-dimethylantracene (DMA). | 40 |
| Figure 1.12 | A) Pathway showing the biomolecular degradation of a phenyl-substituted nitroxide. B) Chemical structures of TMIO and TEMPO. | 41 |
| Figure 1.13 | Oxidation and reduction of a nitroxide. | 42 |
| Figure 1.14 | Coupling a fluorophore to a nitroxide to generate a profluorescent nitroxide (PFN) probe. | 43 |
| Figure 1.15 | Paramagnetic nitroxide-napthalene adduct and their diamagnetic analogues. | 44 |
| Figure 1.16 | Jablonski diagram of electron transition. | 45 |</p>
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.17</td>
<td>Electronic transition occurring upon coupling of a nitroxide radical to a fluorophore (SOMO - single occupied molecular orbital).</td>
<td>46</td>
</tr>
<tr>
<td>1.18</td>
<td>Naphthalene PFN probes.</td>
<td>48</td>
</tr>
<tr>
<td>1.19</td>
<td>Reduction pathway of 4,4,5,5-tetramethyl-2-(1-pyryl)-2-imidazoline-1-oxyl-3-oxide (FNO).</td>
<td>49</td>
</tr>
<tr>
<td>1.20</td>
<td>Piperidine nitroxides with varying substituents.</td>
<td>49</td>
</tr>
<tr>
<td>1.21</td>
<td>PFN probes used in quantitative analysis of ascorbic acid content.</td>
<td>50</td>
</tr>
<tr>
<td>1.22</td>
<td>Umbelliferone-nitroxide adducts.</td>
<td>51</td>
</tr>
<tr>
<td>1.23</td>
<td>Phenanthrene-based fluorescent nitric oxide chelotrophic traps (FNOCTs).</td>
<td>51</td>
</tr>
<tr>
<td>1.24</td>
<td>Pyrene-based fluorescent nitric oxide chelotrophic traps (FNOCTs).</td>
<td>52</td>
</tr>
<tr>
<td>1.25</td>
<td>Attachment of isoindoline profluorescent nitroxide to nucleoside.</td>
<td>53</td>
</tr>
<tr>
<td>1.26</td>
<td>Structure of most abundant urushiol.</td>
<td>53</td>
</tr>
<tr>
<td>1.27</td>
<td>Fluorescent N-alkoxyamine formed from reaction between catechol and n-butylboronic acid via Dansyl-TEMPO PFN probe.</td>
<td>54</td>
</tr>
<tr>
<td>1.28</td>
<td>PFN probes for monitoring aggregation process of amyloid-ß fibrils.</td>
<td>54</td>
</tr>
<tr>
<td>1.29</td>
<td>A) Layout of a mitochondria; B) Schematic of electron transport chain.</td>
<td>56</td>
</tr>
<tr>
<td>1.30</td>
<td>Formation of mitochondrial targeted 5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide (Mito-DEPMPO-OOH).</td>
<td>58</td>
</tr>
<tr>
<td>1.31</td>
<td>Cyclic hydroxylamine, mito TEMPO-H used in assessing reactive oxygen species (ROS) within the mitochondria.</td>
<td>58</td>
</tr>
<tr>
<td>1.32</td>
<td>Chemical structure of DMPO.</td>
<td>59</td>
</tr>
<tr>
<td>1.33</td>
<td>Reaction of MitoBoronic acid (MitoB) with hydroxyl radicals.</td>
<td>60</td>
</tr>
<tr>
<td>1.34</td>
<td>Detection of reactive oxygen species (ROS) by MitoSox.</td>
<td>61</td>
</tr>
<tr>
<td>1.35</td>
<td>Detection of hydrogen peroxide by MitoPY1.</td>
<td>62</td>
</tr>
<tr>
<td>1.36</td>
<td>Reduction of MitoRP.</td>
<td>63</td>
</tr>
<tr>
<td>Figure Number</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>---------------</td>
<td>--------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Figure 1.37</td>
<td>Tetramethyl- and tetaethyl-fluorescein nitroxide synthesized by Morrow.</td>
<td>64</td>
</tr>
<tr>
<td>Figure 2.1.1</td>
<td>Chemical structures of different PFN probes used in this study.</td>
<td>70</td>
</tr>
<tr>
<td>Figure 2.6.1</td>
<td>Chemical structures of several compounds used in Deltavision microscope imaging</td>
<td>78</td>
</tr>
<tr>
<td>Figure 2.6.2</td>
<td>Layout of a spinning disk microscopy (Figure adapted from Stehbens et al., 2012) (CCD- cooled charged-coupled device cameras).</td>
<td>80</td>
</tr>
<tr>
<td>Figure 2.7.1</td>
<td>A) A histogram representation (log scale) and B) a scatter plot of hiTERT immortalized human fibroblast cells incubated with tetraethylrhodamine nitroxide (TER) as obtained from the BD LSRFortessa flow cytometer</td>
<td>83</td>
</tr>
<tr>
<td>Figure 2.8.1</td>
<td>The conversion of water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by the enzyme mitochondria reductase to an insoluble formazan which can be measured by UV absorption.</td>
<td>85</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>A) Reduction and oxidation of nitroxide. B) Chemical structure of tetramethylfluorescein nitroxide (TMF) and tetraethylfluorescein nitroxide (TEF).</td>
<td>89</td>
</tr>
<tr>
<td>Figure 3.1.1</td>
<td>Chemical structure of 1,1,3,3-tetramethyl-isoindolin-2-yloxyl (TMIO) and 1,1,3,3-tetraethyl-isoindolin-2-yloxyl (TEIO) nitroxides.</td>
<td>90</td>
</tr>
<tr>
<td>Figure 3.1.2</td>
<td>Chemical structure of a pyrrolidine nitroxide and a tetaethyl-substituted nitroxide.</td>
<td>90</td>
</tr>
<tr>
<td>Figure 3.1.3</td>
<td>Chemical structures of 4-oxo-2,2,6,6-tetramethylpiperidine-N-oxyl (oxo-TEMPO), hydroxyl-TEMPO and 2,2,6,6-tetraethylpiperidine nitroxide radical.</td>
<td>91</td>
</tr>
<tr>
<td>Figure 3.1.4</td>
<td>Comparing the chemical structures of two fluorescein-based profluorescent (PFN) nitroxides, tetramethylfluorescein (TMF) and tetaethylfluorescein (TEF).</td>
<td>91</td>
</tr>
</tbody>
</table>
Comparing the reduction rate between tetramethylfluorescein (TMF) and tetraethylfluorescein (TEF) nitroxides using ascorbate as a reductant. A) Percentage (%) change in fluorescence intensity over time relative to fluorescence intensity at time point 0 minutes, upon reacting TMF (0.5 µM) with ascorbate (375 µM). B) Chemical structure of TMF. C) Percentage (%) change in fluorescence intensity over time relative to fluorescence intensity at time point 0 minutes, upon reacting TEF (0.5 µM) with ascorbate (375 µM). D) Chemical structure of TEF. E) Comparing reduction rate of TMF and TEF, upon reacting with the PFN probes with ascorbate. The result was displayed as percentage (%) change in fluorescence intensity per min. [Results are average of replicated experiments. Data analysed using T-test where a P value < 0.05 was obtained, indicating significant difference in percentage (%) change in fluorescence intensity per min between TMF and TEF].

Reduction of nitroxide by ascorbate.

Changes in fluorescence intensity of tetraethylfluorescein nitroxide (TEF) at varying pH.

Protolytic equilibria of fluorescein. (Figure taken from Morrow with permission).

A) Localization of tetramethylfluorescein nitroxide (TMF) and tetraethylfluorescein nitroxide (TEF) (concentration 1 µM respectively, in hTERT immortalized human fibroblast cells) co-stained with Hoechst 33258 (1.6 µM) to aid in imaging in hTERT immortalised human fibroblast cells. Cells were also incubated with the mitochondrial specific probe, MitoTracker Green. B) Chemical structure of TMF, TEF, Hoechst and MitoTracker Green.

A) Localization of tetramethylfluorescein nitroxide (TMF) (1 µM, in hTERT immortalized human fibroblast cells) co-stained with LysoTracker Red over time. (Figure taken from Morrow with permission). B) Chemical structure of TMF and LysoTracker Red.

Chemical structure of tetraethylrhodamine nitroxide (TER).
Figure 3.3.2 Comparing the reduction rate between tetramethylfluorescein (TMF), tetrakis(ethylene)rhodamine (TER) and tetraethylfluorescein (TEF) nitroxides using ascorbate as a reductant. A) Percentage change in fluorescence intensity over time relative to fluorescence intensity at time point 0 minutes, upon reacting TER (0.5 µM) with ascorbate (375 µM). B) Comparing the reduction rates of TMF, TEF and TER (0.5 µM each) upon reacting the PFN probes with ascorbate (375 µM). The result was displayed as change in fluorescence intensity per min. (Data analysed using T-test where a P value of < 0.05 was obtained, indicating a significant difference in percentage change in fluorescence intensity per min between TMF, TEF and TER).

Figure 3.3.3 Comparing change in relative fluorescence intensity of tetraethylrhodamine nitroxide (TER) and tetraethylfluorescein nitroxide (TEF) at varying pH.

Figure 3.3.4 A) Localization of tetraethylrhodamine nitroxide (TER) (1 µM) along with co-staining with MitoTracker Green (100 nM) in hTERT immortalised human fibroblast cells. B) Colocalization plot for TER (1 µM) relative to MitoTracker Green (100 nM) within mitochondria. C) Chemical structure of TER and MitoTracker Green.

Figure 3.3.5 A) Localization of tetraethylrhodamine nitroxide (TER) (1 µM) and LysoTracker Blue (50 nM) within hTERT immortalised human fibroblast cells. B) Chemical structure of TER and LysoTracker Blue.

Figure 3.3.6 Redox potential of tetraethylrhodamine nitroxide (TER).

Figure 3.4.1 A) Changes in fluorescence intensity of tetraethylrhodamine nitroxide (TER) and Rhodamine B (parent chromophore, control) upon subjection to varying oxidation-reduction cycle. B) Chemical structure of tetraethylrhodamine nitroxide (TER). C) Close-up view of changes in fluorescence intensity of TER upon subjection to varying oxidation-reduction cycle. D) Chemical structure of Rhodamine B. E) Close-up view of changes in fluorescence intensity of Rhodamine B upon subjection to varying oxidation-reduction cycle. F) Reduction and oxidation reaction of TER. G) Reacting Rhodamine B with hydrazine hydrate (N₂H₂·H₂O).
| Figure 4.1.1 | Change in TER fluorescence intensity over time upon incubating normal and A-T diseased cells with 0.1 µM of TER. A) Comparing fluorescence intensity of hTERT human normal and A-T fibroblast cells without addition of TER. Triplicate results comparing the fluorescence intensity of normal and A-T cells upon addition of 0.1 µM TER was showed in (B), (C) and (D). |
| Figure 4.1.2 | Chemical structure of mitochondrial toxins, rotenone (ROT) and antimycin (AMC) used in inducing mitochondrial oxidative stress. |
| Figure 4.1.3 | Chemical structure of MitoSox and tetramethylrhodamine methyl ester (TMRM). |
| Figure 4.2.1 | Sub-confluent hTRERT immortalized human fibroblast cells used in experiment. Image was taken using the Olympus CKx41 microscope equipped with QImaging MicroPublisher 3.3 RTV camera. |
| Figure 4.2.2 | Change in fluorescence intensity of TER treated hTERT immortalized human fibroblast cells with varying TER (1 µM) incubation time. |
| Figure 4.2.3 | Percentage reduction in fluorescence intensity of treated hTRET immortalized human fibroblast cells, F, [cells incubated with 1 µM probes for 45 or 360 minutes, followed by treatment with 1 µM of A) rotenone (ROT) or B) antimycin (AMC)] for 15 minutes relative to blank, Fc [cells incubated with 1 µM probes for 45 or 360 minutes, followed by treatment with DMSO for 15 minutes]. Each data point are mean values. |
| Figure 4.3.1 | Histogram representation of hTERT immortalized human fibroblast cells treated with antimycin (AMC) (similar trend was observed in the case of rotenone, ROT treatment) upon incubation with tetraethylrhodamine nitroxide (TER). Black - blank (DMSO treatment); Red - cells + TER; Blue - cells + TER + AMC. |
Figure 4.3.2  Percentage reduction in fluorescence intensity of treated hTRET immortalized human fibroblast cells, $F_t$ [cells incubated with 1 µM probes for 45 minutes, followed by treatment with varying concentrations of rotenone (ROT) or antimycin (AMC)] for 15 minutes relative to blank, $F_c$ [cells incubated with 1 µM probes for 45 minutes, followed by treatment with DMSO for 15 minutes] . A) Cells treated with 1 µM TER. B) Cells treated with 1 µM MitoSox. (Each data point are mean values. Data set in A were analysed using Kruskal Wallis test with pos hoc testing using the Dunn’s multiple comparison test. Data set in B were analysed using One-way ANOVA with pos hoc testing using the Dunn’s multiple comparison test. In both cases A and B, relative to blank, all treatment showed $P$ values < 0.05. Results are therefore significantly different to blank).

Figure 4.3.3  Change in fluorescence intensity of tetraethylrhodamine nitroxide (TER) upon uptake into mitochondria followed by treatment with rotenone (ROT) and antimycin (AMC).

Figure 4.3.4  Chemical structure of tetramethylrhodamine methy ester (TMRM).

Figure 4.3.5  Chemical structure of carbonyl-cyanide m-chlorophenyl hydrazone (CCCP).

Figure 4.3.6  Percentage reduction in mean fluorescence intensity of treated hTRET immortalized human fibroblast cells, $F_t$ [cells incubated with 20 nM tetramethylrhodamine methyl ester (TMRM) for 30 minutes, followed by treatment with varying concentrations of rotenone (ROT) / antimycin (AMC) / carbonyl cyanide m-chlorophenyl hydrazone (CCCP) for 15 minutes] relative to blank, $F_c$ [cells incubated with 20 nM TMRM for 30 minutes, followed by treatment with DMSO for 15 minutes] . (Each data point are mean values. Data were analysed using Kruskal Wallis test with pos hoc testing using the Dunn’s multiple comparison test. In all cases a $P$ value of < 0.05 was obtained relative to blank. Results are therefore significantly different to blank).

Figure 4.3.7  Comparing chemical structures of A) tetraethylrhodamine nitroxide (TER) and B) its non-radical alkylated derivative or methyl trap tetraethylrhodamine (MT-TER).
Figure 4.3.8 Percentage reduction in mean fluorescence intensity of treated hTRET immortalized human fibroblast cells, $F_{t}$ [cells incubated with 1 µM non-radical alkylated derivative of TER or methyl trap tetraethylrhodamine (MT-TER) for 45 minutes, followed by treatment with varying concentrations of rotenone (ROT) / antimycin (AMC) for 15 minutes] relative to blank, $F_{c}$ [cells incubated with 1 µM MT-TER for 45 minutes, followed by treatment with DMSO for 15 minutes]. (Each data point are mean values. Data were analysed using Kruskal Wallis test with pos hoc testing using the Dunn’s multiple comparison test. Relative to blank, all treatment showed a $P$ value of $> 0.05$. Results are therefore not significantly different to blank).

Figure 4.3.9 Comparing localization of non-radical alkylated derivative of TER, methyl trap tetraethylrhodamine (MT-TER) within hTRET immortalized human fibroblast cells upon treatment with varying concentration of A) rotenone, ROT (1,10 and 100 µM) and B) antimycin, AMC (1, 10 and 100 µM) as well as carbonyl cyanide m-chlorophenyl hydrazone, CCCP (100 µM).

Figure 4.3.10 MTT cell proliferation assay upon incubating cells with different compounds [tetraethylrhodamine nitroxide, TER; its non-radical alkylated derivative, methyl trap tetraethylrhodamine (MT-TER) and MitoSox] for 45 mins. Cells were left to grow over a period of 1, 12, 24, 48 and 72 hours. Results were normalized to DMSO blank as percentage change in absorbance intensity of sample, $F_{S}$ relative to DMSO blank, $F_{B}$. (GraphPad prism was used to compare times within each treatment using a two-way repeat measure ANOVA with Tukey’s multiple comparisons test used for pairwise comparisons. The standard errors to conduct pairwise comparisons between treatments were calculated manually, with the t-statistic used for the comparisons. P values of $> 0.05$ were obtained between different treatments. Hence, results were considered non-significant between treatments).
Figure 5.1  Comparing the chemical structures between A) tetraethylrhodamine nitroxide (TER) and B) methyl ester tetraethylrhodamine nitroxide (ME-TER).

Figure 5.1.1  Localization of 1 µM methyl ester tetraethylrhodamine (ME-TER) within hTERT immortalised human fibroblast cells. Cells were co-stained with MitoTracker Green (100 nM) and LysoTracker Blue (50 nM).

Figure 5.1.2  Comparing localization of 5 nM of tetraethylrhodamine nitroxide (TER) with 5 nM methyl ester tetraethylrhodamine nitroxide (ME-TER), along with the non-radical alkylated derivatives of both nitroxide probes, within hTERT immortalized human fibroblast cells.

Figure 5.1.3  A) Comparing the localization of 1 µM of tetraethylrhodamine (TER) and 5 nM of methyl ester tetraethylrhodamine nitroxide (ME-TER) within hTERT immortalized human fibroblast cells. B) Colocalization plot between ME-TER (5 nM) and MitoTracker Green (100 nM) within mitochondria.

Figure 5.2.1  Percentage reduction in fluorescence intensity of treated hTRET immortalized human fibroblast cells, Fₙ [cells incubated with 5 nM methyl ester tetraethylrhodamine nitroxide (ME-TER) for 45 minutes, followed by treatment with varying concentrations of rotenone (ROT) or antimycin (AMC)] for 15 minutes relative to blank, F₀ [cells incubated with 5 nM ME-TER for 45 minutes, followed by treatment with DMSO for 15 minutes]. (Each data point are mean values. Data were analysed using Kruskal Wallis test with pos hoc testing using the Dunn’s multiple comparison test. Relative to blank, all treatment showed a P value < 0.05, results are therefore significantly different to blank).

Figure 5.2.2  Comparing chemical structures between A) methyl ester tetraethylrhodamine nitroxide (ME-TER) and its B) non-radical alkylated derivative or methyl trap methyl ester tetraethylrhodamine (MT-ME-TER).
Figure 5.2.3 Percentage reduction in fluorescence intensity of treated hTRET immortalized human fibroblast cells, F, [cells incubated with 5 nM methyl trap methyl ester tetraethylrhodamine nitroxide (MT-ME-TER) for 45 minutes, followed by treatment with varying concentrations of rotenone (ROT) or antimycin (AMC)] for 15 minutes relative to blank, Fc [cells incubated with 5 nM MT-ME-TER for 45 minutes, followed by treatment with DMSO for 15 minutes] . (Each data point are mean values. Data were analysed using Kruskal Wallis test with pos hoc testing using the Dunn’s multiple comparison test. Relative to blank, all treatment showed P values > 0.05, results are therefore not significantly different to blank).

Figure 5.2.4 Percentage reduction in fluorescence intensity of treated hTRET immortalized human fibroblast cells, F, [cells incubated with non-radical alkylated derivative of methyl ester tetraethylrhodamine nitroxide (MT-ME-TER) for 45 minutes, followed by treatment with varying concentrations of rotenone (ROT) or antimycin (AMC) or carbonyl cyanide m-chlorophenyl hydrazone (CCCP)] for 15 minutes relative to blank, Fc [cells incubated with 5 nM MT-ME-TER for 45 minutes, followed by treatment with DMSO for 15 minutes] . (Each data point are mean values. Data were analysed using Kruskal Wallis test with pos hoc testing using the Dunn’s multiple comparison test. Relative to blank, CCCP and AMC 100 µM treatment showed P values < 0.05, results are therefore significantly different to blank).

Figure 5.2.5 Comparing localization of non-radical alkylated derivative of ME-TER, methyl trap methyl ester tetraethylrhodamine (MT-ME-TER) within hTRET immortalized human fibroblast cells upon treatment with varying concentrations of A) rotenone, ROT (1, 10 and 100 µM) and B) antimycin, AMC (1, 10 and 100 µM) as well as carbonyl cyanide m-chlorophenyl hydrazone, CCCP (100 µM).
A) hTERT immortalized human fibroblast cells were incubated with varying compounds (25 nM) for varying time point. Cells were then washed and cytotoxicity of compounds on cells were determined via MTT assay. Results were expressed as percentage absorbance change of sample, \( F_s \) relative to blank, \( F_b \) (DMSO treatment). (Each data point are mean values of replicated experiment. Data were analysed using Kruskal Wallis test with pos hoc testing using the Dunn’s multiple comparison test. Relative to blank, all treatment showed P values > 0.05, results are therefore not significantly different to blank). B) hTERT immortalized human fibroblast cells were incubated with 25nM of methyl ester tetraethylrhodamine nitroxide (ME-TER) over 24 hours. Cells were imaged every 3 minutes for over 24 hours. Image of cells before and after treatment period was shown.

Localization of tetraethylrhodamine nitroxide (ME-TER) (10 nM, 45 minutes incubation) and MitoTracker Green (100 nM, 30 minutes incubation) within A) normal and B) Ataxia Telangiectasia, A-T diseased human fibroblast cells. C) Correlation plots of mitochondrial localization of ME-TER relative to MitoTracker Green in normal human fibroblast cell line (left) and Ataxia Telangiectasia, A-T diseased human fibroblast cell line (right) were shown.

Figures showing percentage changes in fluorescence intensity of sample, \( F_s \) (compounds) relative to control, \( F_c \) (DMSO) upon treatment with A) 45 minutes incubation with 10 nM ME-TER; B) 45 minutes incubation with 5µM MitoSox; C) 30 minutes incubation with 10 nM of MT-ME-TER; D) 30 minutes incubation with 20 nM TMRM probe. Results were normalized to mitochondrial density as measured by 30 minutes incubation with Mitotracker Green (100 nM). (Result were significantly different between normal and A-T diseased cells with P values < 0.1 under different treatment conditions, as determined by T-test).
Figure 5.5.1 Localization of 10 nM methyl ester-tetraethylrhodamine nitroxide (ME-TER) and its non-radical alkylated derivative, methyl trap methyl ester tetraethylrhodamine (MT-ME-TER) along with 100 nM MitoTracker Green in A) immortalized retinal cells; B) DAOY, cerebellum cell; C) HeLa, cervical cancer cell; D) A549, adenocarcinomic alveolar basal epithelial cells. Pearson Coefficient of Correlation plots between nitroxide and MitoTracker Green were shown.

Figure 5.5.2 Percentage reduction in fluorescence intensity of treated immortalized mouse retinal cells, F_s [cells incubated with probes for 45 minutes, followed by treatment with varying concentrations of rotenone (ROT) or antimycin (AMC)] for 15 minutes relative to blank, F_c [cells incubated with probes for 45 minutes, followed by treatment with DMSO for 15 minutes]. A) Cells treated with 10 nM ME-TER. B) Cells treated with 10 nM MT-ME-TER. C) Cells treated with 1 µM MitoSox. [Each data point are mean values. Data were analysed using Kruskal Wallis test with pos hoc testing using the Dunn’s multiple comparison test where P < 0.05 means results are significantly different to blank. Results in both Figures A and C showed a P value < 0.05 (significantly different to blank), while Figure B showed a P value > 0.05 (non-significantly different to blank)].

Figure 5.6.1 Chemical structure of lutein.

Figure 5.6.2 A) Spinning disk microscopy images of 25 nM ME-TER treated cells upon subjection to different treatments; B) Spinning disk microscopy overlay images of 25 nM ME-TER with 100 nM MitoTracker Green.
Figure 5.7.1 The change in methyl ester tetraethylrhodamine nitroxide (ME-TER, 2 µM) fluorescence intensity in the retina during reperfusion following an acute ischaemic insult in Sprague-Dawley rats. A) Fluorescent fundus images. B) Quantification of ME-TER fluorescence: Comparing fluorescence intensity between control and ischaemic reperfusion (I/R) treatment. (The data was fitted with a linear model and subsequently ANOVA statistical test was carried out. Relative to control, P values < 0.05 were obtained. Results are therefore significantly different to control). (Figure was taken from Nigel\textsuperscript{175} with permission).
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>8-OHdG</td>
<td>8-oxodeoxyguanosine</td>
</tr>
<tr>
<td>A549</td>
<td>adenocarcinomic alveolar basal epithelial cells</td>
</tr>
<tr>
<td>AMC</td>
<td>antimycin</td>
</tr>
<tr>
<td>Asc</td>
<td>ascorbate</td>
</tr>
<tr>
<td>A-T</td>
<td>Ataxia Telangiectasia</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia Telangiectasia mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>CCCP</td>
<td>carbonyl cyanide m-chlorophenyl hydrazone</td>
</tr>
<tr>
<td>DAOY</td>
<td>cerebellum cells</td>
</tr>
<tr>
<td>DCFH-DA</td>
<td>2',7'-dichlorodihydrofluorescein diacetate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle media</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>ETC</td>
<td>electron transport chain</td>
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<td>FSC</td>
<td>forward scatter</td>
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<td>HeLa</td>
<td>cervical cancer cells</td>
</tr>
<tr>
<td>IOP</td>
<td>intraocular pressure</td>
</tr>
<tr>
<td>I / R</td>
<td>ischaemic / reperfusion treatment</td>
</tr>
<tr>
<td>hTERT</td>
<td>human telomerase reverse transcriptase</td>
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<tr>
<td>ME-TER</td>
<td>methyl ester tetraethylrhodamine nitroxide</td>
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<tr>
<td>Mn-SOD</td>
<td>manganese superoxide dismutase</td>
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<tr>
<td>MT-ME-TER</td>
<td>methyl trap methyl ester tetraethylrhodamine nitroxide</td>
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<td>MT-TEF</td>
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<tr>
<td>MT-TER</td>
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<tr>
<td>MTT</td>
<td>3,-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NFF</td>
<td>neonatal foreskin fibroblast cells</td>
</tr>
<tr>
<td>NMR</td>
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</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered solution</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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</tr>
<tr>
<td>PFN</td>
<td>profluorescent nitroxide</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>ROT</td>
<td>rotenone</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
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<td>SOD</td>
<td>superoxide dismutase</td>
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<td>TEF</td>
<td>tetraethylfluorescein nitroxide</td>
</tr>
<tr>
<td>TER</td>
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</tr>
<tr>
<td>TEIO</td>
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<td>TMIO</td>
<td>1,1,3,3-tetramethylisoindoline-2-yloxyl</td>
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<tr>
<td>TMR</td>
<td>tetramethylfluorescein nitroxide</td>
</tr>
<tr>
<td>TMRM</td>
<td>tetramethylrhodamine methyl ester</td>
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Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signature:  

Date:  14 JANUARY 2016
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INTRODUCTION & LITERATURE REVIEW
CHAPTER 1
Introduction & Literature Review

The significance of oxidative and reductive redox processes as an integral part of our daily life is increasingly being recognized. Redox chemistry has a significant role in key biological processes, namely cell signalling,\textsuperscript{1,2} cell fate determination\textsuperscript{3,4} and supporting the body’s defence systems.\textsuperscript{5,6} These key biological processes contribute significantly to the overall well-being of living organisms.

Free radicals generated by daily factors such as ultraviolet (UV) radiation, drug ingestion and other exogenous stress have been shown to alter the balance between oxidative and reductive processes. This may lead to physiological and pathological abnormalities such as cancer,\textsuperscript{7} vascular diseases,\textsuperscript{8} and neurodegenerative diseases.\textsuperscript{9} Understanding the chemistry of free radicals is crucial in order to assess and control the damaging effects of these reactive species.

1.1 What are free radicals?
Free radicals are chemical species that have an unpaired electron in their outer or valence orbitals. The presence of an unpaired electron renders these species highly reactive. Free radicals readily accept electrons from surrounding molecules within the biological environment such as lipids and deoxyribonucleic acid (DNA), subsequently oxidizing these molecules as the free radicals are reduced.\textsuperscript{8,10}

Free radicals that are particularly relevant to biological systems can be generalized into two categories i) reactive oxygen species (ROS) and ii) reactive nitrogen species (RNS).\textsuperscript{11,12}

ROS is a collective term used to describe oxygen containing free radicals such as superoxide (O$_2^•$), hydroxyl (OH$^•$) and peroxyl (RO$_2^•$) radicals. ROS also includes non-radicals which exist either as oxidizing agents and/or are easily converted into
radicals such as hydrogen peroxide (H$_2$O$_2$). RNS on the other hand, includes radicals such as nitric oxide (NO•), peroxynitrite (ONOO$^-$) and dinitrogen trioxide (N$_2$O$_3$).

Many studies have shown that free radicals contribute significantly to the overall health deterioration and disease progression in humans. Free radicals may be responsible for the general deterioration that comes with aging. This occurs as a result of unstable free radicals reacting with major components of the body such as lipids, proteins and carbohydrates. These unstable free radicals initiate uncontrolled chain reactions generating further radicals from the molecules being attacked, thereby damaging the normal processes of living cells. Despite their negative effects, free radicals play an important part in the body’s defence mechanisms. Free radicals are generated endogenously by neutrophiles in response to pathogens.

A balance in the amount of free radicals within any biologically variable system is crucial. Oxidative stress results when the level of free radicals exceeds the endogenous redox defences within the system. This may lead to oxidative damage, which is associated with more than 50 diseases, such as Parkinson’s disease, myocardial ischemia, diabetes, cancer, Ataxia-Telangiectasia, retinal ischaemia and in severe cases, organ dysfunction.

It is crucial that the production of free radicals within the biological system is rigorously controlled by redox defences. One such defence is a group of molecules known as antioxidants.

### 1.2 Antioxidants
Antioxidant is a collective term for molecules that preferentially oxidize in the presence of other oxidisable substrates. The oxidisable substrates could be lipids, polyunsaturated fatty acids, proteins, carbohydrates and DNA. Antioxidants are preferentially oxidised even when present at a lower concentration when compared to the oxidisable substrate.

Antioxidants therefore play a vital role in stabilizing, deactivating, delaying and preventing oxidation of molecules within biological systems or biomolecules. This prevents or at least limits oxidative damage.
Antioxidants generally play two major roles: i) acting through preventive measures or ii) acting through repairing or reduction measures. Based on their mode of action, antioxidants are classified as being either a) primary or b) secondary antioxidants.\textsuperscript{29}

a) Primary Antioxidants
Primary antioxidants act in a preventative manner prior to the occurrence of oxidative damage. This is carried out by donating electrons or hydrogen atoms, scavenging free radicals or converting free radicals into more stable products. Examples include enzymes such as superoxide dismutase\textsuperscript{30}, catalase,\textsuperscript{30} and small molecules such as vitamins, lutein, phenols and flavonoids.\textsuperscript{31}

b) Secondary Antioxidants
Secondary antioxidants function in repairing or reducing sustained oxidative damage. Secondary antioxidants retard the formation of more free radicals by sequestering metal ions. This reduces hydroperoxyl (HO\textsubscript{2}\textsuperscript{•}) radicals and H\textsubscript{2}O\textsubscript{2} formation as well as quenches O\textsubscript{2}\textsuperscript{•−} and singlet oxygen. Examples of secondary antioxidants include glutathione and oxidoreductase.\textsuperscript{32}

A method for assessing the redox status within a biological system is therefore crucial to monitor metabolic health. This would not only provide an assessment of the free radical status within biological systems, but could also serve as a means to determine the efficacy of the administration of an antioxidant treatment.

Although free radicals have been studied since the 1950’s\textsuperscript{33} the development of a method for assessing the amount of free radicals within the biological system has been deemed challenging due to several factors. Firstly, the short lifetime of most free radicals, secondly, the presence of antioxidants \textit{in vivo} and thirdly, the capability of the method to capture free radicals.\textsuperscript{34} Despite these factors, several techniques for assessing the amount of free radicals or the redox status have been explored over the last 64 years.
1.3 Oxidation reduction detection

1.3.1 Spectroscopic techniques

One of the most common and simplest redox status assessment techniques is spectroscopy using visually responsive probes. Such spectroscopic probes are substances (usually exogenous) which, upon reacting with free radicals, change their light absorption or emission characteristics. Changes in light absorption or emission characteristics can be measured by a spectrophotometer, where such changes reflect the redox status being measured.

A widely used spectroscopic technique involves measuring the changes in absorbance intensity of the system under study upon the application of Nitro Blue Tetrazolium (NBT). NBT (initially colourless to yellow) is commonly used for the detection of O$_2$• radicals. NBT is reduced by O$_2$• to a tetrazoinyl radical. The tetrazoinyl radical is subsequently reduced to the blue insoluble formazan (Figure 1.1). The change in spectroscopic properties, from NBT to formazan can be quantified using UV/Visible spectroscopy.

\[
\text{NBT} \quad \xrightarrow{2H^+} \quad \text{NBT deformazan}
\]
Other spectroscopic techniques used for assessing the concentration of free radicals involve the reduction of Fe\(^{3+}\) in Cytochrome C protein by free radicals. The reduction of Cytochrome C protein from Fe\(^{3+}\) to Fe\(^{2+}\) by free radicals gives rise to an increased absorption which can be measured by UV/visible spectroscopy (Figure 1.2).\(^\text{34}\) The reduction of Cytochrome C protein is commonly used for detecting O\(_2\)•\(^-\), which results in an increased absorbance at 550 nm.\(^\text{34,36,37}\)

\[
\text{Cyt c-Fe}^{3+} + \text{O}_2^- \rightarrow \text{Cyt c-Fe}^{2+} + \text{O}_2
\]

The use of spectroscopic probes is advantageous because of simplicity and low cost, requiring only commonly available spectrophotometers. However, the probes are usually less sensitive compared to other analytical techniques as they can suffer from non-specific reactions. For example, apart from O\(_2\)•\(^-\) radicals, NBT is also reduced by cellular reductases.\(^\text{38}\) Cytochrome C protein can also undergo re-oxidation in the presence of biological oxidants such as peroxidase thereby underestimating the actual redox level.\(^\text{34}\)

### 1.3.2 Chemiluminescence techniques

Chemiluminescence is another commonly used antioxidant detection technique. Chemiluminescence involves chemical reactions which give rise to luminescence. Similar to spectroscopic techniques, chemiluminescent probes react with free radicals, resulting in a change of light emission characteristics.

A commonly used chemiluminescence probe is 5-amino-2,3-dihydro-1,4-phthalazinedione or luminol. The use of luminol as a free radical detector was first investigated in 1960 by Totter et al.\(^\text{39}\) The technique relies on a one-electron oxidation by H\(_2\)O\(_2\) in the presence of peroxidase. The theory behind this technique is that upon oxidation of H\(_2\)O\(_2\), a luminol radical is produced. The luminal radical subsequently
reduces oxygen (O₂) to O₂• - radicals. The O₂• - radicals react with additional luminol radicals which results in the generation of an endoperoxide. Being unstable, the endoperoxide decomposes yielding nitrogen (N₂) and aminophthalate. The aminophthalate is in an electronically excited state and hence emits a photon to return to the ground state. The emission of the photon is measured as luminescence (Figure 1.3).³⁴

![Figure 1.3: Reaction pathway of luminol derived chemiluminescence.³⁴](image)

Another chemiluminescence probe commonly used is 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo (1,2-α) pyrazin-3-one or MCLA. MCLA is used mainly for detecting O₂• - radicals. MCLA has been covalently bound with fluorescein to synthesize 6-[4-[2-[[N(=O)₂]-5-fluoresceiny]thioureido]ethoxy]phenyl]-2-methylimidazo[1,2-alpha] pyrazin-3(7H)-one or FCLA (Figure 1.4). Upon interacting with the O₂• - radicals, the MCLA moiety transfers the singlet-excitation energy generated to fluorescein. This energy subsequently excites the fluorescein molecule. Upon losing energy and returning to the ground state, fluorescein emits a green luminescence.³⁴

![MCLA](image)

![FCLA](image)
Other chemiluminescence probes include bis-N-methylacridinium, commonly known as lucigenin. Lucigenin is frequently used in the detection of $O_2^{•−}$ radicals. Li et al. have demonstrated that lucigenin monitors intramitochondrial $O_2^{•−}$. Upon uptake into the mitochondria, lucigenin is reduced to a cation radical. This radical then reacts with $O_2^{•−}$ yielding an unstable dioxetane intermediate. The dioxetane intermediate subsequently decomposes producing 2 molecules of N-methylacridone which upon relaxation to the ground state, emits a photon (Figure 1.5).

Similarly, the glycoprotein derived from the chemiluminescence mollusc *Pholas dactylus*, has been adapted as a detector for free radicals. Although the mechanism contributing to the chemiluminescence remains unclear, the glycoprotein, known as Pholasin has been used in the monitoring of loss of antioxidants and changes in the type of oxidants in plasma under oxidative stress. Glebska et al. deduced that upon reacting with peroxynitrite, Pholasin forms a reversible peroxynitrite-protein adduct. The protein adduct subsequently undergoes a series of unknown reactions, leading to the formation of an excited second intermediate. The second excited intermediate decays and return to ground state via the emission of light. Changes in the light intensity, is therefore an indication of the amount of peroxynitrite present.
Chemiluminescence although being simplistic in its application, suffers from low sensitivity. In some cases such as with luminol, chemiluminescence is inhibited by other substrates such as catalase and superoxide dismutase. MCLA on the other hand, suffers from low luminescence intensity and from light, temperature and pH sensitivity. Other chemiluminescence probes are prone to auto-oxidation, giving rise to free radicals artificially as in the case of lucigenin. Another drawback associated with lucigenin is its slow response time. Lucigenin requires another cellular-reducing system such as NADPH (nicotinamide adenine dinucleotide phosphate-oxidase) oxidase to generate a chemiluminescence response which not only slows response time but may complicate the interpretation of the results.34

1.3.3 Electron Paramagnetic Resonance (EPR) or Electron Spin Resonance (ESR)
Electron Paramagnetic Resonance (EPR) or Electron Spin Resonance (ESR) enables the specific detection of the unpaired spins or paramagnetic nature within free radicals. This technique is based on the application of electromagnetic (microwave) radiation to excite electron populations from a low energy state to a high energy state. The concurrent application of an external magnetic field during microwave excitation causes the unpaired electrons to align at a low energy state or misalign at a high energy state, relative to the applied field. The net absorption of electromagnetic radiation results in the generation of a spectrum, which indicates the presence of free radicals.34

The short lifetime along with the degree of electron delocalisation, the presence of electron acceptor molecules in the environment as well as the mobility of the free radicals give rise to an issue where the free radicals do not generally accumulate in high enough amounts to be detectable by EPR.45 Hence, spin traps often have to be used to trap these species as longer lived derivatives. Spin traps are non-paramagnetic substances such as organic nitrone or nitroso compounds which react with free radicals providing radical adducts which are more stable and longer lasting. The use of spin traps therefore, results in the accumulation of semi-stable paramagnetic compounds at detectable concentrations.46
The most commonly used nitrone or nitroso spin traps are 5,5-dimethyl-1-pyrroline N-oxide (DMPO), \( N-t \)-butyl-\( \alpha \)-phenylnitrone (PBN) and 2-methyl-2-nitrosopropane (MNP) (Figure 1.6).\(^{34,46}\)

![Diagram of DMPO, PBN, and MNP reactions](image)

Figure 1.6: Examples of spin traps.\(^{34,46}\)

EPR has been used in the detection of \( \mathbf{O}_2^\bullet \) and \( \mathbf{OH}^\bullet \) radicals with the use of spin traps enabling the detection of more stable ascorbyl and tocopheroxyl radicals as well as heme-nitroxy complexes.\(^{47,48}\)

The conversion of an hydroxylamine species back into a nitroxide results in the generation of an EPR signal and has been used as a means for the detection of ROS. Using 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine (Tempone-H) as probes, this technique has been used for the detection of \( \mathbf{O}_2^\bullet \) as well as ONOO\(^-\) radicals (Figure 1.7).\(^34\)

![Diagram of Tempone-H conversion to Tempone](image)

Figure 1.7: Detection of free radicals using 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine (Tempone-H).\(^34\)
EPR has also been used to monitor the signal decay of stable radicals upon reaction of such radicals with other reactive free radicals. A commonly applied probe for this technique is TAM OX063 (Figure 1.8) which has been proposed for the detection of O₂•⁻ radicals. The rate of loss of the stable radical signal via EPR correlates to the amount of free radicals present in the system under study.

![Figure 1.8: Chemical structure of TAM OX063.](image)

Although EPR has the advantage of being specific, in that it directly interacts with the paramagnetic nature of free radicals, EPR does possess several limitations. For example with the use of nitrone spin traps, reaction can also occur with the carbon atom adjacent to the incipient nitrooxide group which is distant from the molecular orbital containing the unpaired electron. This can give rise to spectra with complicated hyperfine couplings, which in turn makes analysis of the observed spectra difficult.

Even with the use of spin traps, hydroxylamines or stable free radicals as probes, EPR still suffers from a lack of spatial resolution in assessing redox status in cells. This is probably due to the inability of the system to correct the EPR linewidth for distortion caused by uneven shapes among cells. This limitation makes it difficult to monitor processes at the subcellular level.

### 1.3.4 Fluorescence techniques

Fluorescence probes can be categorized into two types i) positive and ii) negative. Positive fluorescence probes consist of non-fluorescent or weakly fluorescent compounds, which increase in fluorescence intensity upon reacting with free radicals. Conversely, negative probes consist of inherently bright fluorescent compounds,
which upon reacting with free radicals switch off fluorescence or reduce in fluorescence intensity.\textsuperscript{51} The most commonly used approach is the positive fluorescence probe.

An example of a positive fluorescence probe is \textit{p}-hydroxyphenylacetic acid (PHPA) (Figure 1.9). PHPA has been used for the detection of \( \text{H}_2\text{O}_2 \) in the presence of peroxidase. The probe is oxidized by \( \text{H}_2\text{O}_2 \) to a dimer with absorption and emission maxima at 300 and 400 nm respectively. It has been reported that the dimer fluoresces optimally at \( \text{pH} > 10 \). However, the limitation of PHPA is the generation of spectra that overlaps with the absorption of common cellular components such as Nicotinamide Adenine Dinucleotide Phosphate (NADPH).\textsuperscript{52}

![Chemical structures of \textit{p}-hydroxyphenylacetic acid](image)

\[
\text{\textit{p}-hydroxyphenylacetic acid}
\]
\[
2\text{O}^-\text{C}_8\text{H}_4\text{OO}^- + \text{H}_2\text{O}_2 \xrightarrow{\text{Peroxidase}} (\text{O}^-\text{C}_8\text{H}_3\text{COO}^-)_2 + 2\text{H}_2\text{O}
\]

**Figure 1.9:** Chemical structures of \textit{p}-hydroxyphenylacetic acid.\textsuperscript{53}

Apart from PHPA, another example of a positive fluorescence probe is dichlorodihydrofluorescein diacetate, known as DCFH-DA (Figure 1.10). DCFH-DA is a frequently applied probe for the intracellular detection of free radicals such as \( \text{H}_2\text{O}_2 \).\textsuperscript{54} DCFH-DA is a cell permeable ester which crosses the cell membrane and is hydrolysed by intracellular esterase to the DCFH anion. Subsequent two-electron oxidation of DCFH gives rise to DCF (dichlorofluorescein) which can be monitored via fluorescence.\textsuperscript{54, 55}
Figure 1.10: Dichlorodihydrofluorescein diacetate (DCFH-DA) in the detection of hydrogen peroxide (H$_2$O$_2$). GSH- Glutathione; GSSG- Glutathione Disulfide; NADH- Nicotiamide Adenine Dinucleotide.$^{55}$

Although DCFH-DA is commonly used, this probe is of limited value because of autooxidation and the loss of probe from the intracellular to extracellular environment via diffusion.$^{56}$ DCFH-DA does not directly react with H$_2$O$_2$ and hence DCF fluorescence cannot be used as a direct measure of H$_2$O$_2$ content.$^{55}$ DCFH-DA also lacks specificity. For example when detecting H$_2$O$_2$, the DCF radical can react with oxygen giving rise to O$_2^•$ which will distort the results obtained.$^{55}$ In addition, the presence of transition metals and heme peroxidase catalyse the oxidation of DCFH-DA which further falsifies fluorescence results.$^{55}$

7-Hydroxy-6-methoxy-coumarin (scopoletin) and 9,10-dimethylanthracene (DMA) (Figure 1.11) are examples of commonly used negative fluorescence probes.$^{51}$ Scopoletin have been widely used for the detection of H$_2$O$_2$ in leukocytes$^{56}$ and eosinophils$^{57}$ while DMA used for O$_2^•$ detection.$^{51}$ The reduction in fluorescence observed in scopoletin results from oxidation of the probe while that of DMA results from the generation of a non-fluorescent endoperoxide. Assessment of redox status by both negative probes is hence based on an inverse fluorescence measurement.
Figure 1.11: Chemical structures of A) 7-hydroxy-6-methoxy-coumarin (scopoletin) and B) 9,10-dimethylanthracene (DMA).\textsuperscript{51}

Similar to their positive counterparts, these negative fluorescence probes also present several drawbacks. Scopoletin has excitation and emission spectra which are susceptible to autofluorescence from biological samples. Apart from that it has a low fluorescent emission and hence requires significant signal amplification leading to increased background signal. Scopoletin also suffers from pH and temperature sensitivity.\textsuperscript{51} DMA, on the other hand, is not located in a preferential depth within the membrane\textsuperscript{58} and hence may have issues crossing the cell membrane to assess intracellular redox status. These limitations have possibly led to the low adoption of negative fluorogenic probe technology.

Current fluorescence techniques are limited by sensitivity to pH variation (changes in fluorescence intensity upon subjection to varying pH conditions) and photobleaching (loss of fluorophore molecules upon illumination). However, because the fluorescence technique is able to provide a sensitive response with a wide range of free radicals it is a preferred technique. Fluorescence detection is versatile through incorporation of biological relevant systems such as microtiter plate readers and microscopes as well as advanced and more sensitive detection systems such as flow cytometry. The use of confocal microscopy makes studies of cellular topography relating to free radical generation possible. In addition, fluorescence techniques have been frequently used in cell culture and tissue due to their ability to provide biologically relevant excitation and emission profiles.\textsuperscript{51,59}
Recent research has led to harnessing the paramagnetic nature of radicals, one of which is nitroxide, NO• in the development of improved fluorescence techniques.

1.4 Nitroxides

N,N-disubstituted nitric oxides or nitroxides [R(NO•)R′] are stable free radicals. Two main factors contribute to their stability: 1) resistance towards dimerization and 2) the nature of the substituents attached to the radical centre.

Dimerization requires the formation of a weak R₂NO-ONR₂ bond which is thermodynamically unstable. However, if an aromatic ring is attached to the nitrogen atom of the nitroxide moiety, this results in bimolecular degradation. Delocalization of the radical into the aromatic system stabilizes the molecule by creating a new reaction centre for biomolecular degradation by increasing the radical character of the carbon atoms within the aromatic ring. Most stable nitroxides, hence possess bulky functional groups such as the alkyl groups of 1,1,3,3-tetramethylisoindolin-2-yloxyl (TMIO) and 2,2,6,6-tetramethylpiperidin-1-yloxyl (TEMPO) (Figure 1.12).⁶⁰

![Diagram showing the biomolecular degradation of a phenyl-substituted nitroxide and chemical structures of TMIO and TEMPO.](image)

**Figure 1.12:** A) Pathway showing the biomolecular degradation of a phenyl-substituted nitroxide. B) Chemical structures of TMIO and TEMPO.⁶⁰

The application of nitroxides to assess redox status of biological systems is increasingly being recognized. A nitroxide may undergo a one-electron reduction giving rise to a hydroxylamine after the addition of H⁺. On the other hand, nitroxides can also undergo a one-electron oxidation giving rise to oxoammonium ions (Figure 1.13).⁵⁰,⁵⁹,⁶¹
The ability of nitroxides to shuttle between redox states has enabled nitroxides to be modified into redox sensing probes. The coupling of nitroxides to fluorophores has created a new class of analytical tool given the name profluorescent nitroxides or PFNs.

### 1.5 Profluorescent nitoxide (PFN) probes

Due to the potential of nitroxides to act as both reductants and oxidants, the idea of combining a fluorophore with a nitroxide in a single molecule as a redox probe, was first described in 1965 by Stryer and Griffith.\textsuperscript{62} The significance of this technology was subsequently realized by Likhtenstein et al.\textsuperscript{63} and Bystryak et al.\textsuperscript{64}

A paramagnetic nitroxide moiety covalently linked in close proximity to a fluorophore efficiently quenches the excited state of the fluorophore, suppressing fluorescence. The suppression of fluorescence occurs as a result of inter- and intra- molecular quenching of the singlet excited state of the fluorophore. The unpaired electron within the nitroxide is capable of quenching the excited singlet state which normally leads to fluorescence. Consequently, reduction of the nitroxide to a diamagnetic hydroxylamine, oxidation to an oxammonium ion, or radical trapping removes this quenching effect, and hence restores the fluorescence emission of the fluorophore (Figure 1.14).\textsuperscript{59}
Figure 1.14: Coupling a fluorophore to a nitroxide to generate a profluorescent nitroxide (PFN) probe. ⁵⁹

Several terms have been used to describe the system of combining a nitroxide with a fluorophore such as fluorophore-nitronyl probes, ⁶⁵ double spin and fluorescence sensors, ⁶⁶ prefluorescent ⁶⁷ and profluorescent probes. ⁶⁸ Among the terms used, the most appropriate term would be that with the prefix “pro” as this describes the fluorescence of a fluorophore being masked or hidden. ⁵⁹ Such a term also aligns with other pro-system terms used in literature such as pro-drug. Coupled to a nitroxide, the profluorescent system can be conveniently termed a profluorescent nitroxide or PFN probe.

Blough et al. have provided significant evidence showing the profluorescent nature of a nitroxide coupled to a fluorophore. Upon comparing a series of paramagnetic nitroxide-naphthalene adducts with their diamagnetic analogues (Figure 1.15), the fluorescence quantum yields of the paramagnetic derivatives were recorded by Blough et al. to be 2.9-6.0 or 30-55 fold (depending on the solvent). Such quantum yield is lower than the corresponding diamagnetic derivatives. Alternatively, reduction of the nitroxide moiety to the diamagnetic hydroxylamine restores the fluorescence yield to a level that parallels the effect of nitroxide radical loss from the system. ⁶⁹
Further studies by Green and Blough et al. reported that the absorption and emission energies of attached fluorophores are not affected by the presence of the nitroxide. In addition, the residual emission that is observed from the PFN arises from the excited fluorophore and not from charge recombination.\(^6^1\) An understanding of the underlying mechanism contributing to the suppression of fluorescence is therefore of importance.

### 1.6 Underlying mechanism contributing to profluorescence

The underlying mechanism contributing to the observed reduction in fluorescence intensity of the PFN, is based mainly on the electron exchange between the nitroxide and the fluorophore moiety, which occurs both inter- and intra-molecularly.\(^7^0,7^1,7^2\)

Fluorescence involves the excitation of a singlet ground state \((S_0)\) electron to one of the vibration levels \((S_2)\) of the excited state, upon receiving energy from a photon (Figure 1.16).
The excited electron subsequently returns to ground state via a two-step process. The first step is through the dissipation of energy to the surrounding environment via vibrational relaxation (VR). This is followed by an internal conversion (IC) until the energy within the molecule reaches the lowest vibrational level of the first excited singlet state, S\(_1\). The second step on the other hand, involves several mechanisms depending on the difference in energy between the ground (S\(_0\)) and excited state (S\(_1\)). In cases where the energy difference is large, energy is either lost via emission of a photon through a process commonly known as fluorescence (F). Alternatively, energy can be lost by intersystem crossing (ISC), which is a process involving changes in the spin angular momentum of the excited electron. If the energy difference is relatively small, energy loss can occur via non-radiative internal conversion.\(^{59}\)

In the typical case of fluorescence (F), the emitted photon is detected within the visible or ultraviolet spectrum range. In the case of ISC, it results in changes in multiplicity, giving rise to a triplet excited state (T\(_1\)).\(^{59}\)

The relaxation of the excited electron from the first excited triplet state, may result in the emission of a photon through a process known as phosphorescence. Similar to fluorescence, phosphorescence involves the emission of light, but over a longer time.
scale and longer wavelength compared to fluorescence. In most cases however, the energy loss occurs in a non-radiative manner, arising from various chemical and physical interactions of the molecule with the surrounding owing to the long lifetime of the triplet state.\textsuperscript{59}

ISC therefore reduces fluorescence emission, and it is via facilitating this electronic interaction that paramagnetic species quench fluorescence when coupled to fluorophores. In summary, upon coupling to fluorophores, the unpaired spin of the nitroxide radical interacts with the electron of the conjugated system within the fluorophore. This in turn results in a change in multiplicity, giving rise to the triplet excited state.\textsuperscript{59,73}

Owing to the antiparallel spin of the radical, the singlet ground state and the lowest singlet excited state, \textit{S}_0 and \textit{S}_1, become doublet states, \textit{D}_0 and \textit{D}_n respectively (Figure 1.17). The triplet state, \textit{T}_1 (Figure 1.16) is represented by \textit{D}_1 (Figure 1.17).  

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure117.png}
\caption{Electronic transition occurring upon coupling of a nitroxide radical to a fluorophore.\textsuperscript{59} (SOMO- single occupied molecular orbital)}
\end{figure}
Such a change in electronic transition, indicates that the previously $S_1$ to $T_1$ transition which occurs during ISC, and subsequently the $T_1$ to $S_0$ transition becomes spin-allowable within double state transitions. This then results in the lost of fluorescence.\textsuperscript{59,73}

The extent to which fluorescence quenching occurs is related to the proximity of the nitroxide radical to the fluorophore within the coupled molecule. Effective quenching is known to only occur within the distance of 0-25\textdegree. In most synthesized PFN probes, this is achieved via a short spacer unit coupling both the radical and the fluorophore.\textsuperscript{74}

Such an electron-transition quenching mechanism makes the PFN a powerful analytical tool to follow redox chemistry. The use of PFN as redox probes allows several unique analytical capabilities.

**1.7 Advantages of profluorescent nitroxide (PFN) probes**

Combining the properties of a nitroxide and fluorophore provides advantages such as:

1) Allowing the measurement of EPR and fluorescence information simultaneously from the system being studied.

2) Monitoring redox processes in systems of low optical density and those giving a weak EPR signal. This is due to the higher sensitivity of fluorescence spectroscopy, which may be deemed to be two or three orders more sensitive compared to optical absorption spectroscopy and EPR.

3) Enabling the photochemical and photophysical nature of a system to be studied by comparing EPR and fluorescence data.

A wide range of studies have been undertaken involving the use of PFN probes. These studies range from physical applications, such as monitoring the formation of polymers as well as the degradation of aging materials\textsuperscript{68,69,70} and the detection of radical in pollutants,\textsuperscript{71,72} through to the analysis of biological systems.

Exploring the applications of PFN’s to assess biological systems forms the basis of this research project. Since the discovery of PFN probes, past studies have discussed the one-electron reduction of nitroxide to hydroxylamine involving biological relevant
reductants such as uric acid, quercetin, glutathione as well as cysteine. Among all the reductants, the reduction of the PFN probes by ascorbate has been most studied.

**1.8 Past studies of profluorescent nitroxides (PFN’s) probes in biological systems**

In one of the earliest studies in this field, Blough and Simpson reported of the reduction of naphthalene probes by ascorbate (Figure 1.18). These researchers reported an increase in fluorescence quantum yield along with a reduction in EPR signal intensity.  

![Figure 1.18: Naphthalene PFN probes](image)

Similar studies involving the reduction of a PFN with ascorbate were carried out by Medvedeva et al. almost two decades later. Medvedeva et al. synthesized a pyrene-nitroxide adduct, 4,4,5,5-tetramethyl-2-(1-pyryl)-2-imidazoline-1-oxyl-3-oxide or FNO. FNO demonstrated the capability to detect O$_2^•$ and to quantitatively estimate the antioxidant activity of compounds such as ascorbic acid and quercetin within biological systems (Figure 1.19). Similar to the findings of Blough and Simpson, the reduction of FNO gave a reduction in EPR signal intensity, accompanied by an increase in fluorescent quantum yield. Medvedeva et al. also reported that using the FNO as probe, the presence of O$_2^•$ radical could be determined when generated at rates as low as 10 nM per minute.
Recent experiments by Yamasaki et al. involving piperidine nitroxides, agree with previous results by Blough, Simpson and Medvedeva. In an attempt to study the structure-reactivity relationship of piperidine nitroxides, Yamasaki et al. documented the rate of reduction in EPR signal intensity upon reacting several piperidine nitroxides (Figure 1.20) with ascorbate. It was concluded that the redox potentials of piperidine nitroxides were influenced by the electronic effect of substituents at 2 and 6 positions of the piperidine ring.78

Lozinsky et al. proposed the use of two PFN probes (Figure 1.21) for quantitative analysis of ascorbic acid in biological and chemical solutions. By performing a series of reactions between the PFN probes and ascorbic acid, a calibration curve for analysis was obtained. Based on the calibration curve, the ascorbic acid concentration of samples was determined. Lozinsky et al. reported that the PFN probes allow for determination of ascorbic acid content ranging from 0.01 to 0.8 mM and higher.79
Further experiments by Lozinsky et al.\(^7^9\) reported that the reduction of the dansyl PFN probe (Figure 1.21A) was influenced by large biomolecules. Bovine serum albumin (BSA) was observed to affect the kinetics of ascorbate reducing the dansyl PFN probe. It was postulated that such an observation was a result of the adsorption of both, the PFN probe and ascorbate into the protein molecule. This in turn enabled a more efficient reduction process.\(^8^0\)

Similarly, Saphier et al. reported that a piperidine-based PFN probe, 5-dimethylaminonaphthalene-1-sulfonyl-4-amino-2,2,6,6-tetramethyl-1-piperidine-oxyl is adsorbed to the outer surface of erythrocytes (red blood cells).\(^8^1\) Within the scope of human blood sample, Saphier et al. further reported that the only natural reducing agent that reacts with the piperidine-based probe is ascorbate.

The first observation of an increase in fluorescence intensity and a decrease in EPR signal in umbelliferone-nitroxide PFN probes (Figure 1.22) upon reduction by ascorbic acid was reported by Sato et al. Both umbelliferone-nitroxide PFNs A and B, have longer wavelength (443 and 450 nm) compared to those developed by Medvedeva et al., Yamasaki, et al., Lozinsky et al. and Saphier et al. These longer wavelength PFNs, have been used for assessing both the presence of OH• radicals in biosystems and Fe(II) in solution.\(^8^2\)
On the basis of Nitric Oxide Cheletropic Traps (NOCTs), Sustmann et al. successfully developed a system which traps nitric oxide (NO) into cyclic nitroxide radicals which in turn give rise to a PFN in situ. Several derivatives of phenanthrene-based cheletropic traps were developed and were given the term fluorescent nitric oxide chelotropic traps (FNOCTs) (Figure 1.23).

Further changes on the FNOCTs traps were carried out by Sustmann et al. The previous phenanthrene fluorophore within FNOCTs was replaced with a pyrene group (Figure 1.24). Among the different FNOCTs in Figure 1.23, FNOCT A which has a
higher NO trapping rate, has been used in NO trapping studies in cell culture systems and in tissue model, namely pig aorta.\textsuperscript{86}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig124.png}
\caption{Pyrene-based fluorescent nitric oxide chelotrophic traps (FNOCTs).\textsuperscript{86}}
\end{figure}

On a genetic biomolecular level, a PFN has been applied in the development of a nucleoside. Barhate et al.\textsuperscript{87} and Cekan et al.\textsuperscript{88} reported of the development of Nucleoside C, consisting of the attachment of an isoindoline nitroxide to a nucleoside (Figure 1.25). Nucleoside C is able to base pair with the guanine nucleotide within deoxyribonucleic acid (DNA). The nitroxide moiety within Nucleoside C is reduced by sodium sulphide (Na\textsubscript{2}S) in DNA to the corresponding hydroxylamine, yielding a fluorescent probe that enables the study of the base pairing and hence structure of the DNA double helix.
Derivatives of PFNs have also been reported as a means for the detection of urushiol from poison oak. Urushiol is the collective term given to compounds consisting of 3-substituted catechols, which have a long linear hydrophobic, C15 or C17 alkyl group chain (Figure 1.26). Urushiol is the causative agent of dermatitis, a type of skin inflammation, which in severe cases leads to fatal systemic anaphylaxis.

Braslau et al. developed a PFN probe which reacts with \( B-n \)-butylcatecholboronate ester (Figure 1.27). The ester is formed \textit{in situ} as a result of the reaction between the catechol urushiol and \( n \)-butylboronic acid. The PFN probe is reduced by the ester to an N-alkoxyamine which is fluorescent.
Figure 1.27: Fluorescent N-alkoxyamine formed from reaction between catechol and $n$-butylboronic acid via Dansyl-TEMPO PFN probe. \(^{89}\)

In addition, Mito et al. have synthesized two PFN probes (Figure 1.28) in an attempt to monitor the aggregation process of amyloid-$\beta$ fibrils, the main constituents suspected to lead to Alzheimer’s disease. It was reported that among the two PFN probes, the fluorescence of PFN probe A increased in a concentration-dependent manner after incubation with 100 $\mu$M amyloid-$\beta$ peptide for 24 hours. PFN probe B on the other hand, showed a decrease in fluorescence intensity with increasing concentration of probe after incubation with 100 $\mu$M amyloid-$\beta$ peptide. The difference in observation was proposed to possibly be due to the distance between the nitroxyl radical and the fluorophore. \(^{90}\)

![PFN probes for monitoring aggregation process of amyloid-$\beta$ fibrils.](image)

Synthetic chemistry has enabled alteration and advancement to be made to the chemical structure of these probes, tethering a variety of spacers to the fluorophore and/or nitroxide segments which alters the absorption, fluorescence and EPR signals. Such alteration has enabled a better understanding of redox processes occurring in biological environments. \(^{59}\) One such environment is within the mitochondria. Being one of the main sites of ROS generation, monitoring the redox status within the mitochondria is of utmost importance as it determines the overall well-being of the biological system.
1.9 Mitochondria- important ROS generation site within biological systems

The mitochondrion is a small organelle (0.5-1μm in diameter) located within eukaryotic cells (cells with nuclear membranes). This membrane bound organelle can be divided into three compartments, i) outer membrane, ii) intermembrane space and iii) inner membrane. Each compartment functions synergistically in the generation of energy, in the form of Adenosine Trisphosphate (ATP) via a process known as oxidative phosphorylation.

As the name suggests, oxidative phosphorylation involves the oxidation of nutrients taken up by cells and subsequently phosphorylation of an Adenosine Diphosphate (ADP) unit. Oxidative phosphorylation involves the transfer of electrons along several protein complexes (Complex I, II, III and IV), collectively known as the electron transport chain (ETC) (Figure 1.29).
Catabolic processes within the biological system breaks down food molecules to form Nicotiamide Adenosine Dinucleotide (NADH), succinate and ADP. Electrons flow from NADH to Complex I (NADH Dehydrogenase). The flow of these electrons is accompanied by the flow of positively charged hydrogen ions (H$^+$) into the intermembrane space.$^{91}$

Succinate on the other hand, loses electrons to Complex II forming fumarate. The electrons are subsequently transferred to Complex III (Cytochome C Reductase) via Ubiquinone (Coenzyme Q). These electrons are then transferred to Complex IV
(Cytochrome C Oxidase) via Cytochrome C, involving the pumping of $H^+$ from the matrix into the intermembrane space. The pumping of $H^+$ generates an electrochemical proton gradient. This proton gradient aids in the generation of ATP, powered by the flow of $H^+$ down the gradient via ATP synthase. The electrons from Complex IV subsequently interact with oxygen forming water molecules. 

Although the ETC is essential to cellular energy generation, the transduction of electrons which occur during ATP generation may be a significant source of damaging ROS, especially $O_2•^-$ and $H_2O_2$ as the electrons escape from the ETC. Such phenomena has been described as ROS “leakage”. 

Complex I and Complex III are the main sites for ROS production and therefore ROS leakage. Complex I and Complex III generates strongly oxidizing compounds such as reduced flavin mononucleotide and semiquinone, which has redox potential below than that of $O_2•^-$. Having redox probes that target or are able to detect redox changes within the mitochondria, focusing on both Complex I and III is therefore of significant value.

Several techniques have been explored to investigate mitochondrial redox status. Such techniques range from the use of EPR, to liquid chromatography tandem mass spectroscopy and fluorescence analysis.

### 1.9.1 Electron Paramagnetic Resonance (EPR) techniques for the analysis of mitochondrial redox status

It was reported that Hardy et al. (2007) successfully modified a cyclic nitrone with an attached triphenylphosphonium group, to form Mito-5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide (DEPMPO). Mito-DEPMPO covalently bonds with $O_2•^-$ producing a stable product, Mito-DEPMPO-OOH which is EPR detectable (Figure 1.30). Despite the mitochondrial specificity of Mito-DEPMPO, the probe suffers from several drawbacks. A high concentration of Mito-DEPMPO is required, around 50 mM for good detectable EPR signal. Such large amounts may affect normal functioning of the mitochondria due to accumulation of lipophilic cations within the mitochondrial membrane. This in turn affects the proton gradient within the ETC, which in turn affects ATP generation.
Similarly, Dikalov et al. reported of the use of the cyclic hydroxylamine mito TEMPO-H (1-hydroxy-4-[2-triphenylphosphonio-acetamido]-2,2,6,6-tetramethylpiperidine) as a means of assessing ROS within the mitochondria (Figure 1.31). The hydroxylamine in its reduced state is oxidized by ROS into EPR detectable stable nitrooxide. An increase in EPR signal, hence serves as a measure of possible oxidative stress within the biological system under study. Superoxide dismutase activity however, has been reported to inhibit mito TEMPO-H signal caused by the mitochondrial toxin, rotenone.\textsuperscript{96,97,98}

![Figure 1.30: Formation of mitochondrial targeted 5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide (Mito-DEPMPO-OOH).\textsuperscript{94}]

![Figure 1.31: Cyclic hydroxylamine, mito TEMPO-H used in assessing reactive oxygen species (ROS) within the mitochondria.\textsuperscript{96,97,98}]

One of the earliest methods for detecting $\text{O}_2^\cdot$ is via spin trapping with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) (Figure 1.32).\textsuperscript{99} Immunospin trapping techniques which
couple spin trap and immunology have been used to detect free radical adduct formation in the mitochondria.\textsuperscript{100} Using immunospin trapping, DMPO adducts formed from reaction with O$_2$•$^-$ within the ETC have been detected.\textsuperscript{101} DMPO reacts with protein radicals resulting in the formation of DMPO-protein radicals. These DMPO-protein radicals reacts with antibodies which can subsequently be identified via immunostaining, western blot analysis and immunofluorescence.\textsuperscript{99} Despite the versatility of this technique, it is limited to the detection of radical protein adducts and the development of antibodies to react with the DMPO-protein radicals can be costly.\textsuperscript{55,102}

![Diagram of DMPO structure](image)

Figure 1.32: Chemical structure of DMPO.\textsuperscript{103}

Owing to their advantage of a direct interaction with ROS in assessing redox status within the mitochondria, these EPR probes minimize possible artifacts generated from multistep reactions. However, a down-side to this technique is that it requires the use of relatively expensive and complex EPR instrumentation.

Apart from EPR, liquid chromatography techniques have also been explored in assessing redox status within mitochondria.

1.9.2 Liquid chromatography tandem mass spectroscopy techniques for the analysis of mitochondrial redox status

Cocheme et al. (2011) described a liquid chromatography tandem mass spectrometry probe targeting the mitochondria by the name MitoBoronic acid or MitoB.\textsuperscript{104} As suggested by its name, MitoB consists of an arylboronic acid conjugated to a triphenylphosphium group. This species reacts with hydrogen peroxide (H$_2$O$_2$) and peroxynitrite (ONOO$^-$) to form a phenol product, MitoP (Figure 1.33). Quantification of MitoP/MitoB ratio by liquid chromatography tandem mass spectroscopy provides quantification of the H$_2$O$_2$ and ONOO$^-$ levels within the system studied. Although this
technique of quantifying MitoP/MitoB is sensitive, it requires rather laborious effort and specialized instrumentation.

\[ \text{MitoB} \xrightarrow{\text{HOO}^-} \text{MitoP} \]

Figure 1.33: Reaction of MitoBoronic acid (MitoB) with hydroxyl radicals.\textsuperscript{104}

Owing to its sensitivity and easily assessable instrumentation, much focus has been placed in developing fluorescent techniques for assessing redox status within the mitochondria.

### 1.9.3 Fluorescence techniques for the analysis of mitochondrial redox status

MitoSox, which consists of dihydroethidium with an attached triphenylphosphonium group, is a commonly used fluorescent probe.\textsuperscript{105,106,107} This probe was initially developed for \( \text{O}_2^- \) detection. However, it has been reported that two products are formed from the use of MitoSox, i) 2-hydroxyethidium which is formed from reaction with \( \text{O}_2^- \) and ii) ethidium, formed from non-specific redox reactions (Figure 1.34).\textsuperscript{108}

MitoSox has been used to assess redox status within patients suffering from Wolf-Hirschborn syndrome (a genetic disorder which results in a complex set of developmental phenotypes ranging from growth restriction to intellectual disability).\textsuperscript{109} MitoSox has also been used to monitor oxidative stress in myocardial ischemia-reperfusion injuries,\textsuperscript{110} to detect ROS in obesity,\textsuperscript{111} to monitor the production of ROS in B cell immune response,\textsuperscript{112} to assess genetic oxidative stress in cancer\textsuperscript{113,114} and to monitor regulation of oxidative stress in cells resulting from radiation stress.\textsuperscript{115}

Despite being a commonly used probe, MitoSox suffers from several drawbacks. MitoSox is somewhat unstable. It suffers from potential interference from heme
enzymes and is prone to auto-oxidation as well as being light sensitive. In addition, the chemical transformation involves a free radical intermediate which can be scavenged by antioxidants, thereby compromising results.

Analogous to MitoSox, boronate based probes have also been developed. An example of such a boronate probe involves the conjugation of a boronate based peroxo-yellow 1 moiety with a triphenylphosphonium group. This probe known as MitoPY1 has been used to detect H$_2$O$_2$ within the mitochondria (Figure 1.35). Boronate suppresses the fluorescence of the peroxo-yellow 1 fluorophore. Upon nucleophilic attack the boronate is cleaved, restoring the fluorescence of the fluorophore. Boronate based probes however, suffer from a lack of specificity as they have to compete with fast reacting substrates such as peroxiredoxin and glutathione peroxidase for H$_2$O$_2$. Change in the fluorescence intensity of MitoPY1 is therefore an estimate of the degree of oxidative stress within the studied biological system.
Although much effort has been undertaken to investigate mitochondrial redox status, many of the described techniques and probes are unable to provide an on-going or reversible dynamic real time assessment of redox status.

Attempts to address this short-coming, have led to the development of redox sensitive proteins. These proteins involve the integration of genetic codes within plasmid constructs, via adenoviruses into the genome of host cells. The genetic codes within the plasmid are transcribed and translated into redox sensitive proteins within host cells. A subsequent change in fluorescence intensity of the redox proteins within host cells is hence an indication of intracellular redox status. \(^55\)

One such protein is the mitochondrial targeted protein is pHyPer-dMito. pHyPer-dMito is derived from the yellow fluorescent protein, cpYFP. It has been used in assessing H\(_2\)O\(_2\) within human uterine cancer cells (HeLa). \(^121\)

Other examples include a class of redox sensitive protein derived from green fluorescent proteins conjugated to redox sensitive reporter (roGFP). Meyer et al. reported a redox sensitive reporter (roGFP2) conjugated with a H\(_2\)O\(_2\) sensor (Orp1). \(^122\) H\(_2\)O\(_2\) oxidizes Orp1, upon which Orp1 is reduced by roGFP2. The oxidized species of
roGFP2 subsequently emits fluorescence which can be measured as an indication of oxidative stress.\textsuperscript{123}

These redox sensitive proteins have the advantage of allowing real time measurement of varying ROS fluxes within cells, as they are constantly being produced within cells. The drawback of the application of these proteins is the possibility of over-production of redox sensitive proteins within cells. Over production may in turn act as a precursor to initiate an increase in ROS generation, giving false positive results.\textsuperscript{55} A possible solution to this drawback is the application of redox sensitive proteins along with other small molecule fluorescence probes.\textsuperscript{124}

Recently, Hirosawa et al. (2012) reported a TEMPO-conjugated proflourescent probe, MitoRP which enables dynamic reversible monitoring of mitochondrial redox status. The TEMPO (2,2,6,6-tetramethyl-piperidin-1-yl)oxyl moiety is coupled to the fluorophore coumarin 343 (Figure 1.36). The monocationic coumarin fluorophore aids in the localization of the probe into the mitochondria. The probe, however, was reported to only show specificity towards monitoring dynamic changes of electron flow within Complex I of the ETC and not other protein complexes.\textsuperscript{125}

![Figure 1.36: Reduction of MitoRP.\textsuperscript{125}](image)

Despite the many techniques explored and developed for assessing mitochondrial redox status, much remains to be discovered. A need to develop reversible and easily applicable small molecule fluorescent probes is of much importance.

Previous work by Morrow\textsuperscript{126} has successfully developed two isoindoline profluorescent fluorescein nitroxides, which will be referred to as tetramethyl- and tetraethyl-fluorescein profluorescent nitroxides in this study (Figure 1.37). Initial
studies on the tetraethylfluorescein nitroxide (TEF) by Morrow\textsuperscript{126} reported that in the presence of the nitroxide radical, the fluorescence of the chromophore fluorescein was suppressed from $\phi_0$ 0.94 to 0.11, an almost 90\% suppression.

![Tetramethylfluorescein Nitroxide (TMF) and Tetraethylfluorescein Nitroxide (TEF)](image)

Using flow cytometry, Morrow showed that both TMF and TEF have the capability to assess overall cellular redox environment. It was reported that normal fibroblast cells treated with rotenone and 2-deoxyglucose, resulted in an oxidizing cellular environment which was displayed by a lack of reduction of both TMF and TEF. Both TMF and TEF were also shown to be more effective than the widely used redox sensing probe, 2',7'-dichlorohydrofluorescein diacetate.\textsuperscript{126}

The study presented here serves as a follow up to the initial study by Morrow, focusing on comparing and assessing the application of varying PFN probes in monitoring biological redox status, especially in the mitochondria.
Research Objectives

In summary, the previously described spectrophotometry and chemiluminescence techniques for assessing biological redox status, although inexpensive and simple, suffer from several limitations namely low sensitivity and undergo non-specific reactions. EPR on the other hand, although specific in detecting unpaired spin within free radicals, is expensive and involve complex instrumentation setup and application. In addition, most of the redox sensing techniques up-to-date are not reversible in their detection and hence are not able to provide a dynamic assessment of the redox status within biological systems.

The main objective of this study is therefore to validate an inexpensive, simple, sensitive, and reliable as well as mitochondrial specific fluorescence based redox sensing techniques based on the use of PFN probe. In achieving this objective, the capabilities of fluorescein- and rhodamine-based profluorescent nitroxide (PFN) as potential probes to monitor the redox state of the mitochondria will be explored. Several factors will be assessed namely:

i) The reduction rate of the PFN.
ii) The influence of changing pH conditions on the fluorescence characteristics of the PFN.
iii) The localization of the PFN in cells (intracellular localization of the PFN).
iv) The reversible characteristics of the PFN.
v) The applicability of the PFN within biological systems.

Probes which are easily reduced or oxidized can deliver a rapid response to the overall redox status within the biological system studied. However, such a response can be a limitation when assessing the redox status within highly redox active intracellular organelles such as the mitochondria. The rapid response of the probe gives rise to difficulties in differentiating the probe response to additives and changing redox conditions before reaching the mitochondria. Ideally the best probes for such cellular environments should not react prior to reaching the organelle. This enables any response detected to reflect the conditions within the targeted organelle better, where in this case the highly reactive mitochondria.
In this study, the effect of increasing steric bulk of ethyl- over methyl-groups around the radical centre on reduction rate of PFN will be assessed. The reduction rate of tetramethylfluorescein (TMF) and tetraethylfluorescein (TEF) (Figure 1.37) will be compared through fluorescence measurement of solutions containing TMF or TEF, upon reacting the PFN solutions with ascorbate.

The pH stability of the chromophore molecules attached to the nitroxde moiety in PFN probes will also be assessed. As fluorescence intensity has been reported to change with the surrounding pH, a probe which exhibits stable fluorescence over a biologically relevant pH range is therefore desired. The pH stability of fluorescein- compared to rhodamine-based PFN probes will also be determined in order to validate the usefulness of the probe.

Another important criteria in validating biological relevant redox probes, is the localization of the PFN probe within cells or the intracellular localization of the PFN probe. It is crucial that the PFN probe localizes to redox relevant sites such as the mitochondria. Following the initial development of fluorescein- and rhodamine -based PFNs by Morrow, the localization of both these PFNs will be explored. Rhodamine chromophores are known to exhibit great photostability, high water solubility, possess excitation and emission profiles free of interference from biological molecules and capable of localizing into the mitochondria.

In addition, the reversibility of PFN will also be explored. Such reversible detection methodologies would enable a dynamic assessment of the redox status within biological systems. Redox probe having such reversible detection capability has the potential to be used to assess the efficacy of antioxidant therapies provided to patient by comparing the oxidative stress level within diseased models both prior and after antioxidant treatment.

Flow cytometry as well as microscopic techniques will also be used to determine the capability of the PFN probes to assess intracellular redox status on a variety of cell
lines including Ataxia-Telangiectasia (A-T) diseased cells, which are known to be under oxidative stress.

In summary the key objectives are:

1) To compare the reduction rate of methyl- and ethyl- based PFN probes.
2) To compare the pH stability of fluorescein- and rhodamine-based PFN probes.
3) To monitor the localization of PFN probes within biological cells.
4) To assess the reversibility characteristics of PFN in providing a dynamic assessment of biological redox condition.
5) To assess the capability of the developed PFN probes to detect redox changes within biological systems and oxidative stress associated diseases, namely Ataxia Telangiectasia (A-T).
MATERIALS & METHODS
CHAPTER 2
Materials and Methods

A general outline of the reagents and chemicals, initial cell preparation procedures and statistical analysis carried out in this study are described below. Any deviations from these described procedures are outlined in the experimental section of the subsequent results and discussion chapter. Concentrations of the probes and drugs used were based on previous work carried out by Morrow.\textsuperscript{126}

2.1 Reagents and chemicals

All reagents and chemicals are of analytical grade and were purchased from Ajax Finechem, BDH Chemicals, Sigma-Aldrich or Invitrogen. All experimental work described were carried out in Queensland University of Technology (QUT), Queensland Medical Research (QIMR), Queensland Eye Institute (QEI) and University of Queensland Centre for Clinical Research (UQCCR).

Acknowledgement goes to Dr. Benjamin Morrow and Dr. Benjamin Chalmers for developing and synthesizing the PFN probes used in this study. The PFN probes were tetramethylfluorescein (TMF), tetraethylfluorescein (TEF), tetraethylrhodamine (TER), methyl ester tetraethylrhodamine (ME-TER) nitroxides along with their respective non-radical alkylated derivatives (Figure 2.1.1). Synthesis steps were developed based on work by Morrow, et al.\textsuperscript{50,126} The probes were kept as solids at -20°C with minimum light exposure.
Figure 2.1.1: Chemical structures of different PFN probes used in this study.

2.2 Cell lines and animal model

The cell lines used in this study were as listed below:

a. Immortalized Human Telomerase Reverse Transcriptase (hTERT) fibroblast cells
b. Normal human fibroblast cells
c. Ataxia Telangiectasia diseased human fibroblast cells
d. Human cerebellum cells (DAOY)

e. Human cervical cancer cells (HeLa)

f. Adenocarcinomic human alveolar basal epithelial cells (A549)

g. Rat immortalized retinal cells

Cell lines a to f were supplied by Lavin research group from QIMR, while cell line g was supplied through the Barnett research group from QEI/UQCCR. Cells were cultured either from a biopsy human sample or purchased. In all cases, stock solution of cells were cultured and maintained in Dulbecco’s modified eagle medium (DMEM) added with fetal calf serum (final concentration 12% v/v) and penicillin / streptomycin (final concentration 1% v/v). hTERT fibroblast cells were used to compliment a follow up study to previous work by Morrow.\textsuperscript{126}

Studies on Sprague-Dawley rats were carried out by Miss Cassie Rayner from UQCCR using the methyl ester tetraethylrhodamine (ME-TER) probe. Further details of these animal experiments are described in the experimental section of the subsequent chapter.

2.3 Cell culture

All cells were incubated and maintained at 37\(^\circ\)C, under incubation condition of 37\(^\circ\)C, with 95% air and 5% carbon dioxide (CO\(_2\)). Cell lines a to g mentioned previously were cultured in Dulbecco’s Modified Eagle medium (DMEM). The medium DMEM was supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v). All cell lines were subcultured when they reached 80-90% confluency by washing the cells with 1-5 mL of 10% v/v versene (depending on the size of the flask) followed by 0.5-5 mL of 0.5% v/v trypsin (depending on the size of the flask). The cells were left to detach for approximately 1 minute under incubation condition of 37\(^\circ\)C, with 95% air and 5% CO\(_2\). 10 mL of DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)] were subsequently added to deactivate the trypsin. Cells were subcultured from the trypsinized stock according to the required dilution using DMEM [supplemented with fetal calf serum
(final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v).

Cell morphology as well as viability was monitored by microscopic examination and cells were assessed for possible Mycoplasma infection.

In assessing Mycoplasma infection, cells were cultured unto a coverslip within a petri dish at an initial concentration of approximately 5x10^4 cells per mL for 7 days. The cells were cultured within DMEM supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v). The coverslip containing cells were carefully removed from the petri dish and the cells were fixed by fully submerging the coverslip containing cells into 3% paraformdehyde (PFA) for 15 mins. The fixed cells were then washed twice with 1xPBS and subsequently stained by adding 3 µL of VectorShield (DAPI) dye. The coverslip of cells were then fixed unto a glass slide and the fluorescence of the cells were imaged under a fluorescence microscope excited at wavelength 372-408 nm and emission detected at wavelength 417-453 nm. Imaging was carried out at room temperature (27°C, normal atmospheric condition). Based on assessment, cells used were free from Mycoplasma contamination.

2.4 Cell counting

Cells were treated with 1 mL of 10% v/v versene, followed by 0.5 mL of 0.5% v/v trypsin and left to detach for approximately 1 minute from the flask under incubation condition of 37°C, with 9% air and 5% CO₂ into a 5 mL FALCON tube. Trypsinized cells were centrifuged (1200xg for 4 mins) and the supernatant removed. The cells were resuspended in 1x phosphate buffer solution (PBS) and taking into consideration the dilution factor, cell solution were stained with 0.4 % Trypan Blue in a 1:1 ratio. Total cell numbers were determined via the use of Countess Automated Cell Counter. The Countess Automated Cell Counter is a benchtop automated cell counter which uses optics and image analysis to quantify cell number within a cell sample. The use of an automated cell counter was chosen over the standard haemocytometer to improve accuracy of cell count.
In quantifying the cells number using the Countess Automated Cell Counter, 10 µL of cell sample mixed with 10 µL of 0.4% Trypan Blue. 10 µL of the mixture was then added to the cell counter slide and the image of the cells optimize by using the focus knob to adjust the image for analysis and the number of cells quantified.

2.5 Fluorescence measurements

Fluorescence was measured using the Cary Eclipse fluorometer. Depending of the probes, each treatment was excited and fluorescence emission measured at different wavelengths.

<table>
<thead>
<tr>
<th></th>
<th>Excitation wavelength</th>
<th>Emission wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetramethylfluorescein</td>
<td>491 nm</td>
<td>519 nm</td>
</tr>
<tr>
<td>Tetraethylfluorescein</td>
<td>491 nm</td>
<td>519 nm</td>
</tr>
<tr>
<td>Tetraethylrhodamine</td>
<td>556 nm</td>
<td>575 nm</td>
</tr>
<tr>
<td>Non-radical alkylated derivative of Tetraethylrhodamine nitroxide</td>
<td>556 nm</td>
<td>575 nm</td>
</tr>
</tbody>
</table>

Measurements were carried out at room temperature with voltage set at 550V and samples were mixed to ensure a homogenous solution prior measuring the fluorescence intensity of the solution.

Fluorescence measurement was used:

i) To assess the reduction rate of methyl- and ethyl-based PFN probes.
ii) To compare the pH stability of fluorescein- and rhodamine-based PFN probes.
iii) To assess the reversibility characteristics of PFN in providing a dynamic assessment of biological redox condition.
Assessing the reduction rate of PFN probes

Reduction rates were determined by measuring the change in fluorescence intensity (using Cary Eclipse fluorometer) over time upon subjecting the PFN probes, TMF, TEF and TER (each of concentration 0.5 µM, prepared from a 1 mM dimethyl sulfoxide, DMSO stock solution) to reduction by ascorbate at a concentration of 375 µM. The concentration of ascorbate was added at an excess of 750 times to that of the PFN probes (1:750) in a final volume of 4 mL of 1x PBS.

Experiments were carried out in argon-bubbled 1xPBS (pH 7) to minimize residual oxygen from re-oxidizing hydroxylamine back to nitroxide. The PFN probes were excited at wavelength 491 nm and fluorescence emission was measured at 519 nm in the case of TMF and TEF. However, in the case of TER, an excitation at wavelength 556 nm, followed by measuring the emission fluorescence intensity at wavelength 575 nm was applied. Changes in fluorescence intensity of the PFN solutions were recorded over time by recording the change in fluorescence intensity of PFN every 3 minutes over 600 minutes within the fluorometer. The reduction rates of the PFN probes were calculated (based on the slope of the graph of percentage fluorescence intensity against time) and compared.

Comparing the pH stability of fluorescein- and rhodamine-based PFN probes

Solutions of varying pH were prepared from a 10 M hydrochloric acid (HCl) solution through serial dilution. 10 M HCl was diluted 100-fold to give a solution of pH 1. Subsequently, an aliquot of the pH 1 solution was further diluted 10-fold to give a solution of pH 2. An aliquot of the pH 2 solution was further diluted 10-fold to give a solution of pH 3. Successive 10-fold dilutions were carried out until a pH 7 solution was obtained.

On the other hand, a 10 M sodium hydroxide (NaOH) solution was diluted 100-fold to give a solution of pH 14. An aliquot of the pH 14 solution was further diluted 10-fold, providing a solution of pH 13. Successive 10-fold dilutions were carried out until a pH 8 solution was obtained.
Stock solutions of TEF and TER at a concentration of 1 mM were prepared in DMSO and added to the different pH solutions, to give a final probe concentration of 0.5 µM. The prepared solutions were mixed and the fluorescence intensity of the mixture was obtained via the fluorometer. An excitation wavelength of 491 nm and an emission wavelength of 519 nm were applied to assess the fluorescence intensity of TEF in varying pH solutions, while an excitation wavelength of 556 nm and an emission wavelength of 575 nm were applied to assess the fluorescence intensity of TER.

Assessing the reversible characteristics of PFN in providing a dynamic assessment of biological redox condition

1 µM of TER and Rhodamine B was prepared in a cuvette from 4 mM DMSO stock in a final volume of 4 mL 1xPBS solution (Concentration was selected based on previous study to previous work by Morrow.126). The PBS solution was previously bubbled with N₂ to minimize residual O₂, hence preventing initial oxidation. Fluorescence intensity of TER solution was measured in a fluorometer (Cary Eclipse) at time point 0 minute (excite: 556nm, emit: 575nm). The reductant, hydrazine hydrate (N₂H₂H₂O) was subsequently added in excess (200 mM) and fluorescence intensity was measured immediately. Fluorescence of TER was then measured every 3 minutes, for a total of 15 minutes. Reduction by N₂H₂H₂O was carried out in a sealed environment to avoid oxidation by atmospheric O₂.

The solution was subsequently oxidized by bubbling O₂ into the solution for approximately 15 minutes. Concurrent changes in fluorescence intensity within the solution were measured every 3 minutes within the fluorometer. The whole reduction and oxidation cycle was repeated while the changes in fluorescence intensity within the solution were measured accordingly. The observed fluorescence changes were displayed as a plot of change in fluorescence intensity (a.u.) at 580 nm against time (minutes).

2.6 Fluorescence imaging

Fluorescence imaging was carried out based on the use of i) Deltavision Microscope and ii) Spinning Disk Microscope. In both cases, live cells were imaged under an atmospheric condition of 37°C and 5% CO₂ in air. Depending on the probes in which
the cells were incubated with, each treatment was excited and fluorescence emission measured at different wavelengths.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Excitation wavelength</th>
<th>Emission wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetramethylfluorescein nitroxide (TMF)</td>
<td>447-503 nm</td>
<td>487-559 nm</td>
</tr>
<tr>
<td>Tetraethylfluorescein nitroxide (TER)</td>
<td>447-503 nm</td>
<td>487-559 nm</td>
</tr>
<tr>
<td>MitoTracker Green (commercially available Mitochondria dye)</td>
<td>447-503 nm</td>
<td>487-559 nm</td>
</tr>
<tr>
<td>LysoTracker Blue (commercially available lysosome dye)</td>
<td>372-408 nm</td>
<td>417-453 nm</td>
</tr>
<tr>
<td>Tetraethylrhodamine nitroxide (TER)</td>
<td>515-569 nm</td>
<td>549-639 nm</td>
</tr>
<tr>
<td>Non-radical alkylated derivative of TER (MT-TER)</td>
<td>515-569 nm</td>
<td>549-639 nm</td>
</tr>
<tr>
<td>Methyl Ester Tetraethylrhodamine Nitroxide</td>
<td>515-569 nm</td>
<td>549-639 nm</td>
</tr>
<tr>
<td>Non-radical alkylated derivative of MT-ME-TERe</td>
<td>515-569 nm</td>
<td>549-639 nm</td>
</tr>
</tbody>
</table>
Samples were mixed to ensure a homogenous solution by carefully pipetting the solution several times, while taking care to avoid detaching cells from the culture dish.

i) Deltavision microscope imaging

1.5x10^5 cells per mL of hTERT immortalized human fibroblast cells were cultured overnight in IBIDI slides in DMEM containing fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v) and phenol red.

The cells were subsequently incubated with different compounds [1 µM TMF, 1 µM TEF, 1 µM TER and MT-TER (Figure 2.1.1), 1 µM or 5 nM ME-TER and MT-TER, 1.6 µM Hoechst 33258, 100 nM MitoTracker Green or 50 nM LysoTracker Blue (Figure 2.6.1)] by replacing previous medium within each well with 2 mL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)], containing the different compounds.
The cells were incubated for 45 minutes (an incubation period of 45 minutes was chosen to coincide with previous studies by Morrow\textsuperscript{126}) at 37\textdegree{}C under an atmosphere of 5\% CO\textsubscript{2} in air. Upon incubation, the cells were washed once with 2 mL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12\% v/v) and penicillin streptomycin antibiotics (final concentration 1\% v/v)]. A new batch of 2 mL phenol red free DMEM [supplemented with fetal calf serum (final concentration 12\% v/v) and penicillin streptomycin antibiotics (final concentration 1\% v/v)] was added to
each well prior imaging. Imaging conditions were maintained at 37°C under an atmosphere of 5% CO₂ in air.

ii) Spinning disk microscopy

Conventional fluorescence microscopy involves exciting the sample under observation with a broad stream of light. The fluorescence given off by the sample, both within and out of focal plane were subsequently collected and imaged. In the case of confocal fluorescence microscopy, a pinhole is used to block out light which is out of focus. This improves the resolution of image.

A commonly used confocal fluorescence microscopic technique is the confocal laser scanning microscope. Such a laser scanning microscope uses a laser of distinct wavelength governed by the nature of the sample to rapidly scan the sample at several different points. The light returning from each point is quantified by a photomultiplier tube and a digital image is reconstructed.

Although the confocal laser scanning microscope is able to provide a fast response, the microscope however suffers from a trade off between image resolution and imaging speed. In order to obtain a significantly good resolution in a short period of time, relatively intense laser power has to be used. Such intense power can result in photobleaching and even phototoxicity in live cell imaging.

Another technology that has the potential to overcome this limitation is a spinning disk instrument where the microscope is equipped with multiple pin holes. A spinning disk containing rapidly rotating pinhole disk enables thousands of light points to scan the sample simultaneously and can achieve frame rates up to 1000 frames per second (Figure 2.6.2). This enables the use of a lower intensity laser power and multiple point detection simultaneously, giving high resolution images in a short period of time.
Due to its sensitivity and low phototoxicity, spinning disk microscopic techniques was used in this study to: A) assess the effect of PFN probes on cells by incubating cells with PFN probe ME-TER and imaging the condition of the cells every 3 minutes over time; B) to assess the efficacy of lutein as an antioxidant therapy in reducing oxidative stress. The CV1000 spinning disk microscope was used in this study.

A) Assessing the effect of PFN on cells
The effect of ME-TER on cells was assessed. 4x10^5 cells per mL of hTERT immortalized human fibroblast cells were cultured in IBIDI slides overnight in DMEM media [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)]. The cells were subsequently incubated with 25 nM of ME-TER (prepared in 2 mL of phenol red free DMEM media supplemented with fetal calf serum (final concentration 12% v/v) and...
penicillin streptomycin (final concentration 1% v/v) and localization of probe was imaged under the CV1000 spinning disk microscope every 3 minutes for over 24 hours. Imaging conditions were maintained at 37°C and 5% CO₂. The probe was excited at wavelength 561 nm and emission detected at wavelength 582 nm.

B) Assessing the efficacy of lutein as an antioxidant therapy in reducing oxidative stress

4x10⁵ cells per mL of hTERT immortalized human fibroblast cells were cultured in IBIDI slides overnight in DMEM media [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)]. The cells were subsequently incubated with 300 µL of 25 nM ME-TER (prepared from a 10 mM DMSO stock, in 2 mL of phenol red free DMEM media supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v) for 45 minutes. The media was then replaced with a fresh batch of media (300 µL) that does not contain ME-TER (but supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v). The ME-TER treated cells were subsequently imaged and the change in fluorescence intensity was imaged every minute for 3 minutes.

During imaging, the cells were then subjected to different treatments as follow. Different compounds were added to the cells within IBIDI slides between imaging intervals.

a) 45 minutes incubation with 25 nM of ME-TER (as described), followed by a 20 µM AMC treatment (prepared from a 100 mM DMSO stock) in a final volume of 300 µL. In addition to ME-TER, cells were also incubated with 100 nM MitoTracker Green (prepared from a 100 µM DMSO stock, in 2 mL of phenol red free DMEM media supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v) for 30 minutes.

b) 45 minutes incubation with 25 nM of ME-TER (as described), followed by a 2 mM lutein treatment (prepared from a 0.6 M DMSO stock) in a final volume of 300 µL.
c) 45 minutes incubation with 25 nM of ME-TER (as described), followed by a 20 µM AMC treatment (prepared from a 100 mM DMSO stock) and 2 mM lutein treatment (prepared from a 0.6 M DMSO stock) in a final volume of 300 µL.

d) A blank solution was prepared where upon 45 minutes incubation with 25 nM of ME-TER (as described), cells were incubated with DMSO in place of AMC and lutein, in a final volume of 300 µL.

Fluorescence intensity of ME-TER treated cells subjected to the different treatment were imaged and compared accordingly. An excitation at wavelength 515-569 nm and emission detection at wavelength 549-639 nm was applied in the case of ME-TER, while in the case of MitoTracker Green, an excitation at wavelength 447-503 nm and emission detection at wavelength 487-559 nm was applied. Imaging conditions were maintained at 37°C under an atmosphere of 5% CO₂ in air in phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin (final concentration 1% v/v)].

2.7 Flow cytometry

Flow cytometry enables the fluorescence of each cell in a cell suspension to be measured. PFN treated individual cells are excited based on the PFN specific wavelength and subsequently passed through a detector where the intensity of the light emitted are measured. On average, the fluorescence intensity of 10000 cells can be measured within minutes. This technique therefore provides a fast as well as accurate cell fluorescence measurement.

The flow cytometer used was BD LSRFortessa. Changes in PFN fluorescence intensity upon subjecting PFN treated cells to different treatment were analysed via flow cytometry. In all cases, live cells were pass through the detector at a flow rate of 60 µL per min. Measurements were carried out at room temperature and cells were suspended in 500 mL of 1xPBS within 1 mL tubes. Cells were kept in dark to avoid photobleaching of PFN probe prior measurement. Between each run, the system was rinsed and cleaned with bleach (70% v/v) to avoid cross-contamination. Details of each fluorescence measurement carried out were outline in the subsequently chapters.
A histogram representation and a typical scatter plot obtained from the flow cytometer, displaying cells incubated with TER is shown below.

![Histogram and Scatter Plot](image)

**Figure 2.7.1:** A) A histogram representation (log scale) and B) a scatter plot of hTERT immortalized human fibroblast cells incubated with tetraethylrhodamine nitroxide (TER) as obtained from the BD LSRFortessa flow cytometer.

In analysing flow cytometry data, light scattering and fluorescence data were collected from a total of 10000 cells. Light scattered in the forward direction, in line with the beam (FSC, X-axis on Figure 2.7.1B) relates to cell size. Light scattered in the side direction, 90° to the incident beam (SSC, Y-axis on Figure 2.8.1B) is an indication of the surface roughness of the cells.\(^ {131}\)

Each data point on the scatter plot (Figure 2.7.1B) represents data from a single cell. The eclipse surrounding the set of data points represents the cell population of interest as these cells are of the correct size and shape. Data points nearer to the Y axis on the other hand, represent cellular debris and are, therefore, excluded from analysis. Such methodology of excluding cellular debris is commonly known as “gating”. The mean fluorescence data obtained from gating of cells was used to plot the histogram (Figure 2.7.1A). Unless otherwise stated, all fluorescence data were gated and in each histogram, the fluorescence intensity were given on the X-axis (log scale) while the number of cell count were given on the Y-axis.
A stock culture of hTERT immortalized human fibroblast cells were prepared as described in 2.3. From the stock culture hTERT immortalized human fibroblast cells were seeded at an initial concentration of approximately 5x10^4 cells per mL in 6-well plates. The cells were cultured in DMEM, supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v) and phenol red over a period of approximately 48 hours (final cell concentration approximately 2x10^5 cells per mL). Within different wells, the cells were treated differently as follows. Details of each experiment will be described further in the experimental section in subsequent chapter.

i) Rotenone (ROT) treatment
The cells were initially incubated with different probes [TER or MT-TER (1 µM for 45 / 360 minutes), MitoSox (1 µM for 45 minutes), ME-TER or MT-ME-TER (1 µM / 5 nm / 10 nm for 45 minutes), TMRM (20 nM for 30 minutes)] at 37°C, 5% CO₂, in 1 mL of DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v) and phenol red]. The medium containing probes was subsequently removed by pipette and a fresh batch of DMEM containing varying concentration of ROT (0.01, 0.1, 1, 10, 100 µM) in a final volume of 1 mL was added to each of the 6 well respectively. The cells were incubated with ROT for 15 minutes at 37°C under an atmosphere of 5% CO₂ in air.

ii) Antimycin (AMC) treatment
Similar experimental procedures to that of ROT treatment was carried out in the case of AMC treatment. Upon treatment with TER, the cells were incubated with AMC (0, 0.01, 0.1, 1, 10 and 100 µM) instead of ROT, in a final volume of 1 mL of DMEM for 15 minutes.

Blanks were carried out by replacing ROT or AMC with DMSO in a final volume of 1 mL of DMEM, followed by 15 minutes incubation at 37°C under an atmosphere of 5% CO₂ in air.

Upon treatment, the cells were washed with 1 mL of 10% v/v versene, treated with a stock solution of trypsin (adding 0.5 mL of 0.5% v/v trypsin) and centrifuged (speed-1200xg, time- 4 mins and temperature- 37°C). Supernatant was removed and cells
were resuspended in 1 mL 1xPBS. Fluorescence readings for approximately 10000 cells were subsequently obtained via the BD LSRFortessa flow cytometer. The cells incubated with TER were excited around wavelength 556 nm and emission collected at wavelength 575 nm.

2.8 MTT assay

An understanding on the cytotoxic effect of TER provides a greater understanding on the applicability of the PFN probe. A commonly used assay for assessing the cytotoxicity of compounds is the MTT or (3,-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

MTT (3,-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assay is a widely used assay for assessing cytotoxicity of a compound in cells. It involves the conversion of the water soluble MTT (3,-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by the enzyme mitochondria reductase to an insoluble formazan which can be measured by UV absorption.\textsuperscript{132}

![Diagram of MTT conversion](image_url)

Figure 2.8.1: The conversion of water soluble MTT (3,-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by the enzyme mitochondria reductase to an insoluble formazan which can be measured by UV absorption.

hTRET immortalized human fibroblast cells were cultured in 96-well plate at a concentration of 2x10^4 cells per mL over a period of 24 hours. The cells were then incubated with compounds namely, TER, MT-TER and MitoSox (1 µM each) as well as ME-TER, MT-ME-TER (25 nM each). DMSO was used in place of the compounds as blank.
Cells were incubated with the compounds for 45 minutes, washed and replaced with 100 µL of fresh phenol red free DMEM supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin (final concentration 1% v/v).

A 12 mM stock solution of MTT was prepared by adding 1 mL of 1xPBS to a 5 mg vial of MTT (commercially available). 10 µL of the 12 mM MTT stock solution was added to each well within the 96-well plate. A negative control was prepared by adding of 10 µL of the 12 mM MTT stock solution to 100 µL media.

The mixtures were incubated at 37°C for 4 hours. After which, 85 µL of the solution was removed and 50 µL of DMSO was added to each well. The mixtures were thoroughly mixed and incubated for a further 10 minutes at 37°C.

The mixtures within the 96-well plate were subsequently mixed to form a homogenous solution and absorbance within each well was obtained at 540 nm using the BioTek Synergy plate reader.

2.9 Statistical analysis

Statistical analysis was carried out using the GraphPad version 6 program. One-way ANOVA or Kruskal Wallis test with pos hoc testing using the Dunnett’s multiple comparison test as well as T-test were used to analyse the results in this study. Data used in statistical analysis were mean values of replicated experiments.
RESULTS & DISCUSSION
Assessing Bioreduction of Redox Probes

The rate at which redox probes react with the changing oxidation-reduction conditions within cells determines the potential application of the probe. Redox probes which are easily reduced provide a rapid response to the redox status within the biological system studied. Although a rapid response can be desirable for certain conditions, this can prove to be a limitation when applied to a highly redox active intracellular organelles such as the mitochondria. An ideal mitochondrial redox probe should not react prior to reaching the organelle. This will ensure that any response given off by the probe reflects the redox status within the mitochondria.

In an attempt to validate a mitochondrial redox sensing PFN, the rate at which nitroxides are reduced to hydroxylamines (Figure 3.1A) was assessed. As previously described, the groups attached to the nitroxide radical centre play an important role in determining the rate of reduction of the PFN.\textsuperscript{133,134} The attachment of a large group around the radical centre increases steric bulk around the radical centre. This increased in steric bulk hinders or slows down the reduction of the radical. Two PFNs of varying groups of different sizes attached to the nitroxide radical centre, tetramethylfluorescein (TMF) and tetraethylfluorescein (TEF) nitoxides (Figure 3.1B) were subjected to ascorbate reduction. The increase in fluorescence intensity of the PFNs upon reduction were subsequently measured and compared.
3.1 Comparing reduction rate of tetramethyl- and tetraethyl-based PFN probes

Past studies have reported that ethyl-based probes have a slower reduction rate compared to methyl-based probes. Marx et al. carried out an EPR comparative study of the reduction rate between 1,1,3,3-tetramethylisoindolin-2-yloxyl (TMIO) and 1,1,3,3-tetraethylisoindolin-2-yloxyl (TEIO) (Figure 3.1.1) with ascorbic acid. It was reported that at a concentration of 25 equivalents of ascorbic acid, TEIO 2a did not undergo any reduction as observed in the case of TMIO 1a. In addition, upon reacting TMIO 1b and TEIO 2b (both probes at a concentration of 1x10^{-3} M) with ascorbic acid (at a concentration of 1x10^{-1} M), the initial concentration of TMIO 1b decreased by 50 % within 0.5 minutes while TEIO 2b has a much longer half life of 13 minutes.\textsuperscript{135}
A similar result was reported by Bobko et al. upon comparing the reduction rate of a pyrrolidine nitroxide with a tetraethyl-substituted nitroxide both reacted with ascorbate in de-oxygenated aqueous solution (Figure 3.1.2). EPR results carried out by Bobko et al. reported that the tetraethyl-substituted nitroxide demonstrated a remarkable ability to resist reduction up to a 100 fold excess of ascorbate.134

Kinoshita et al. successfully synthesized a 2,2,6,6-tetraethylpiperidine nitroxide radical (Figure 3.1.3). Compared to other tetramethyl-substituted compounds such as 4-oxo-2,2,6,6-tetramethylpiperidine-N-oxyl (oxo-TEMPO) and hydroxyl-TEMPO, the 2,2,6,6-tetraethylpiperidine nitroxide radical showed increased stability towards reduction by ascorbic acid. Using EPR, the reduction rate constant (derived from the slope of a graph where the area under the low-field EPR signal of the first derivative spectrum was plotted against time after the addition of redox probe) of the tetramethyl-substituted compounds was compared to that of the tetraethyl piperidine radical.136
Both oxo-TEMPO and hydroxyl-TEMPO were found to give a similar rate of reduction. The rate constants were found to be $0.427 \pm 0.034$ per min and $0.381 \pm 0.0076$ per min, respectively. These rates are much higher than that of 2,2,6,6-tetraethylpiperidine nitroxide radical ($0.003 \pm 0.0005$ per min). This indicates that the tetramethyl-substituted compounds have a faster reduction rate compared to that of the tetraethyl-substituted nitroxide radical.

![Chemical structures of oxo-TEMPO, hydroxyl-TEMPO, and 2,2,6,6-tetraethylpiperidine nitroxide radical](image)

Figure 3.1.3: Chemical structures of 4-oxo-2,2,6,6-tetramethylpiperidine-N-oxyl (oxo-TEMPO), hydroxyl-TEMPO and 2,2,6,6-tetraethylpiperidine nitroxide radical.136

Using the biologically relevant reductant ascorbate, the reduction rates of the PFN probes, tetramethylfluorescein (TMF) and tetraethylfluorescein (TEF) (Figure 3.1.4) were compared. A $0.5 \mu M$ solution of PFN probes in DMSO/PBS buffer was treated with a 750 fold excess of ascorbate in PBS buffer and the fluorescence emission at 519 nm was measured every 3 minutes for 10 hours.

![Comparing the chemical structures of TMF and TEF](image)

Figure 3.1.4: Comparing the chemical structures of two fluorescein-based proflourescent (PFN) nitroxides, tetramethylfluorescein (TMF) and tetraethylfluorescein (TEF).
Experimental
The experimental procedures used in this study are fully described in Materials and Methods, Section 2.5 (page 73).

Results and discussion

A

![Graph A](image)

B

![Structure B](image)

Tetramethylfluorescein Nitroxide (TMF)

C

![Graph C](image)

D

![Structure D](image)

Tetraethylfluorescein Nitroxide (TEF)
Figure 3.1.5: Comparing the reduction rate between tetramethylfluorescein (TMF) and
tetraethylfluorescein (TEF) nitroxides using ascorbate as a reductant. A) Percentage (%) change in fluorescence intensity over time relative to fluorescence intensity at time point 0 minutes, upon reacting TMF (0.5 µM) with ascorbate (375 µM). B) Chemical structure of TMF. C) Percentage (%) change in fluorescence intensity over time relative to fluorescence intensity at time point 0 minutes, upon reacting TEF (0.5 µM) with ascorbate (375 µM). D) Chemical structure of TEF. E) Comparing reduction rate of TMF and TEF, upon reacting with the PFN probes with ascorbate. The result was displayed as percentage (%) change in fluorescence intensity per min. [Results are average of replicated experiments. Data analysed using T-test where a P value < 0.05 was obtained, indicating significant difference in percentage (%) change in fluorescence intensity per min between TMF and TEF].

As previously mentioned (Figure 1.14), the attachment of a paramagnetic nitroxide moiety in close proximity to the chromophore efficiently quenches the excited state of the chromophore, suppressing its fluorescence. Consequently, the reduction of the nitroxide by ascorbate to a diamagnetic hydroxylamine (Figure 3.1.6), removes this quenching effect and restores the fluorescence of fluorescein.\textsuperscript{50,59} The rate at which the fluorescence of the PFN increases, therefore provides an indication of the reduction rate of the PFN.
Figure 3.1.6: Reduction of nitroxide by ascorbate.

As can be seen in Figure 3.1.5A and C, the fluorescence intensity of TMF reaches peak approximately 50 minutes into the reduction process. The fluorescence of TEF on the other hand, took over 600 minutes to reach its peak. These observations suggest TMF was being reduced at a faster rate compared to TEF. In addition, the fluorescence response of TMF reflects a slow reoxidation by oxygen or air where the fluorescence of TMF remained relatively stable after peaking at approximately 50 minutes up to 600 minutes.

Triplicate results showed that the reduction of TMF occurs at an average rate, 0.37±0.03 per min compared to that of TEF, an average rate of 0.05±0.02 per min (Figure 3.1.5E). The observed result agrees with that reported by Marx et al.\(^{135}\) and Bobko et al.\(^{134}\), concluding that the reduction of methyl-based probes occur at a faster rate compared to ethyl-based system. The slower reduction rate of ethyl-based probes hence makes these probes a more appropriate redox probe for assessing mitochondrial redox status as such probe will not react prior reaching the mitochondria. This will ensure that any response given off by the probe reflects the redox status within the mitochondria.

A possible reason for the decreased reduction rate of TEF compared to TMF is thought to be due to the enhanced steric bulk around the nitroxide radical centre within TEF. This is contributed by the greater size of the ethyl groups around the radical centre within TEF compared to its methyl counterpart, TMF (Figure 3.1.4). The enhanced steric bulk in TEF hinders or slows down the rate at which the radical centre reacts. The ethyl groups in TEF also increase the electron donating effect around the nitroxide centre within TEF. Such an increase in electron density increases shielding around the
radical centre. Hence, both increased steric bulk and shielding effects within TEF, led to a decreased reduction rate of the PFN.\textsuperscript{126,136,137,138}

Observations and results obtained from measuring the fluorescence response of ascorbate treated TMF and TEF (Figure 3.1.5) suggest that apart from EPR techniques used previously by Marx et al.\textsuperscript{135}, Bobko et al.\textsuperscript{134} and Kinoshita et al.\textsuperscript{136}, fluorescence measurement is a convenient means to follow the reduction response of PFN.

In conclusion, upon comparing the reduction rate between TMF and TEF, ethyl-based probes have a slower reduction rate compared to methyl-based probes. The slower reduction rate of TEF makes it possibly a more relevant redox probe for assessing redox status within the redox active mitochondria where TEF will not react prior reaching the mitochondria. This will ensure that any response given off by the probe reflects the redox status within the mitochondria.

Apart from reduction rate, other factors such as effect of pH on fluorescence of PFN probe as well as localization of the probe serve as critical factors in determining the relevance of TEF as a mitochondrial redox probe. These factors will be explored in the next section.

3.2 Assessing the pH stability and intracellular localization of tetraethylfluorescein nitrooxide (TEF) in cells

Compared to TMF, TEF has a slower reduction rate, making it potentially a more appropriate probe for assessing mitochondrial redox status. However, apart from reduction rate, other factors such as the pH stability of TEF and the actual localization of TEF into the mitochondria still need to be determined to assess the value of TEF as a mitochondrial redox sensing probe.
In assessing the pH stability of TEF, an understanding of biological pH in the locations where the probe is present is of importance. The steady state maintainence of pH 7.4 is critical for normal cellular function. However, it has been reported that disorders such as mucolipidosis (a metabolic disorder), neuronal ceroid lipofuscinoses (a neurodegenerative disorder) and cancer can impact on the pH of certain cells and tissues.

In validating PFN probes to be used to assess redox status within both normal and diseased cells, pH stability of the PFN is crucial. Previous work by Morrow et al. reported that changes in pH affect the fluorescence intensity of TMF. Studies were hence carried out to assess the pH stability of TEF by measuring the change in fluorescence intensity of TEF under different pH conditions.

The possible localization of TEF into the mitochondria was also assessed. This serves as a crucial factor to quality the use of TEF as a mitochondrial redox probe.

Previous studies concluded that the localization of probes into the mitochondria is largely dependent on the charge present on the probe. Hardy et al. (2007) reported the successful use of a cyclic nitro with an attached cationic triphenylphosphonium group to form Mito-5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide (DEPMPO). DEPMPO was used to access mitochondrial redox status via EPR (Figure 1.30). Similarly, Dikalov et al. reported of the use of the cyclic hydroxylamine mito TEMPO-H (1-hydroxy-4-[2-triphenylphosphonio-acetamido]-2,2,6,6 tetramethylpiperidine). Being cationic in nature, mito TEMPO-H was reported to successfully assess ROS level within the mitochondria via EPR (Figure 1.31).

Further studies by Cocheme and Murphy et al. (2011) through the use of MitoB which consists of an arylboronic acid conjugated to a triphenylphosphium group (Figure 1.33) confirmed the localization of cationic probes into the mitochondria. Recently, Hirosawa et al. (2012) reported the mitochondrial localization of the probe MitoRP which consist of a TEMPO (2,2,6,6-tetramethyl-piperidin-1-yl)oxyl moiety coupled to the monocationic chromophore coumarin 343 (Figure 1.36).
The intracellular localization of both TMF and TEF nitroxides was observed via Deltavision fluorescence microscopy. hTERT immortalized human fibroblast cells were used in an attempt to study the behaviour of the PFN probes within human biological conditions. Details of the experiment to assess the pH stability of TEF and the localization of TMF and TEF are described below.

**Experimental**

**Assessing the pH stability of tetraethylfluorescein nitroxide (TEF)**

A 10 M hydrochloric acid (HCl) solution was prepared and diluted 100-fold to give a solution of pH 1. Subsequently, an aliquot of the pH 1 solution was further diluted 10-fold to give a solution of pH 2. Similarly, an aliquot of the pH 2 solution was further diluted 10-fold to give a solution of pH 3. Successive 10-fold dilutions were carried out until a pH 7 solution was obtained.

On the other hand, a 10 M sodium hydroxide (NaOH) solution was prepared and diluted 100-fold to give a solution of pH 14. An aliquot of the pH 14 solution was further diluted 10-fold, providing a solution of pH 13. Successive 10-fold dilutions were carried out until a pH 8 solution was obtained.

Stock solutions of TEF at a concentration of 1 mM were prepared in DMSO and added to the different pH solutions, to give a final probe concentration of 0.5 µM. The prepared solutions were mixed and the fluorescence intensity of the mixture was obtained via fluorometer. The actual pH of the solution upon addition of probe was subsequently measured using a pH meter. The experiment was carried out in triplicate and the results obtained presented as graphs of relative fluorescence intensity against measured pH values. Fluorescence intensities were measured using the Cary Eclipse fluorometer on which an excitation wavelength of 491 nm and an emission wavelength of 519 nm were applied to assess the fluorescence intensity of TEF in varying pH solutions.

**Assessing the intracellular localization of probes**

hTERT immortalized human fibroblast cells at a concentration of 1.5x10^5 cells per mL were cultured overnight in IBIDI slides. The cells were cultured in 2 mL of DMEM
within each well, containing fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v) and phenol red.

The cells were subsequently incubated with TMF or TEF by replacing previous medium within each well with 2 mL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)] containing 1 µM of either TMF or TEF. The cells were incubated for 45 minutes (an incubation period of 45 minutes was chosen to coincide with previous studies by Morrow\textsuperscript{126} at 37\textdegree{}C under an atmosphere of 5% CO\textsubscript{2} in air. Care was taken to avoid rigorous movement in handling the slides to avoid detachment of cells from the slide.

Upon completion of the incubation period, the cells were washed once with 2 mL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)] and were subsequently co-stained with the nucleus specific probe, known as Hoechst 33258 to aid in the imaging of cells. This was achieved by adding 2 mL of phenol red free medium [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)] containing 1.6 µM of Hoechst to the cells in each well and the cells were incubated for 15 minutes at 37\textdegree{}C under an atmosphere of 5% CO\textsubscript{2} in air.

Similarly, another batch of cells was incubated with the commercially available mitochondrial specific probe, MitoTracker Green. Cells were incubated with 100 nM of MitoTracker Green in 2 mL of phenol red free medium [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)]. The cells were incubated for 30 minutes at 37\textdegree{}C under an atmosphere of 5% CO\textsubscript{2} in air.

The cells were subsequently washed once with 2 mL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)] and a new batch of 2 mL phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and
penicillin streptomycin antibiotics (final concentration 1% v/v)] was added to each well prior imaging.

The localization of the PFN probes was imaged under an Applied Precision Deltavision fluorescence microscope. The PFN probes were excited at wavelength 447-503 nm and emission detected at wavelength 487-559 nm. In the case of Hoechst 33258, an excitation wavelength of 372-408 nm and an emission wavelength of 417-453 nm were applied, while in the case of MitoTracker Green, an excitation wavelength of 447-503 nm and an emission wavelength of 487-559 nm was applied. Imaging conditions were maintained at 37°C under an atmosphere of 5% CO₂ in air.

Results and discussion

![Graph](image_url)

Figure 3.2.1: Changes in fluorescence intensity of tetraethylfluorescein nitroxide (TEF) at varying pH.

Based on Figure 3.2.1, TEF shows significant fluctuations in fluorescence intensity over a pH range of 3-12. At pH ≤ 6, there is hardly any fluorescence emission.

Previous work by Morrow⁵⁰,¹²⁶ showed that the coupling of a nitroxide moiety with the chromophore fluorescein does not affect the pH stability of the overall compound. The fluorescence changes observed were primarily due to the nature of the chromophore fluorescein.
Fluorescein has been reported to exist in different forms under different pH conditions, namely cationic, neutral, anionic as well as dianionic derivatives (Figure 3.2.2). Each of these derivatives varies in their fluorescence characteristics and the conversion between these species is largely pH dependent.\textsuperscript{146,147}

![Diagram of fluorescein derivatives with pK values](image)

Figure 3.2.2: Protolytic equilibria of fluorescein. (Figure taken from Morrow\textsuperscript{126} with permission).

Analysis derived from Figure 3.2.1 hence concluded that TEF is not stable to changes in the pH of the environment. Slight fluctuation in pH results in a significant change in the fluorescence intensity of TEF.

Subsequent localization experiment as displayed on Figure 3.2.3 revealed that both TMF and TEF seemed to localize within the lysosome. This was due to the fact that the localization patterns observed in the case of TMF and TEF are different from that of MitoTracker Green. Such observations support previous work by Morrow,\textsuperscript{126} who observed the lysosomal localization of TMF via co-staining of cells with LysoTracker Red (Figure 3.2.4). Morrow et al. reported that TMF was initially taken up by endocytotic vesicles (within 15 minutes) before migrating to the lysosomes (within 45 minutes and 120 minutes). A similar localization pattern was observed in the case of TEF compared to TMF (Figure 3.2.3A) suggesting similar lysosomal localization of TEF. The internal environment of lysosomes has a relatively low pH (ranging from 4-5) and based on Figure 3.2.1, the fluorescent is decreased under such acidic conditions. Whilst it is likely that these probes accumulate in the lysosomes as part of
the excretion from the cell, it is important to note that lower observed fluorescence can occur despite accumulation within cellular organelles of the fluorescein based PFN probes depending on the pH of the microenvironment. Further work would be necessary to definitively confirm co-localization of these probes within lysosomes. However, these results do not provide any evidence that there is localization within mitochondria with these probes.

**A**

![Images of TMF, TEF, and MitoTracker Green probes](image)

**Probe / Hoechst**

**B**

![Chemical structures of TMF, TEF, Hoeschst, and MitoTracker Green](image)
Figure 3.2.3: A) Localization of tetramethylfluorescein nitroxide (TMF) and tetraethylfluorescein nitroxide (TEF) (concentration 1 µM respectively, in hTERT immortalized human fibroblast cells) co-stained with Hoechst 33258 (1.6 µM) to aid in imaging in hTERT immortalised human fibroblast cells. Cells were also incubated with the mitochondrial specific probe, MitoTracker Green. B) Chemical structure of TMF, TEF, Hoechst and MitoTracker Green. (Triplicate life cell images were taken, only one image is being displayed).
Figure 3.2.4: A) Localization of tetramethylfluorescein nitroxide (TMF) (1 µM, in hTERT immortalized human fibroblast cells) co-stained with LysoTracker Red over time. (Figure taken from Morrow\textsuperscript{126} with permission). B) Chemical structure of TMF and LysoTracker Red.

Results from these experiments indicate that fluorescein-based probes (both the tetramethyl- and tetraethyl-systems) suffer from pH instability. The drastic change in fluorescein fluorescence intensity with slight changes in pH, limits their potential as redox probes for assessing the changing pH condition within biological system.

In addition, although both TMF and TEF are able to provide an assessment of the redox status within intracellular lysosomes, lysosomes are not active sites for redox processes in cells. A PFN probe which localizes to the main redox active site, the mitochondria, is desirable. A possible candidate was the tetraethylrhodamine nitoxide (TER) (Figure 3.3.1). The commercially available chromophore, rhodamine has been used as an effective probe for staining mitochondria since the 1980’s.\textsuperscript{148} Coupling a tetraethylisoindoline nitroxide with rhodamine could therefore potentially give rise to an effective redox probe for assessing the redox condition within the mitochondria.

### 3.3 The use of rhodamine chromophore as an improvement over fluorescein

As discussed in Chapter 3.2, TEF has a slow reduction rate making it a potential probe for assessing mitochondrial redox status. However, further studies revealed that TEF suffers from pH instability and does not localize into the mitochondria. An alternative mitochondrial redox sensing probe is therefore required. This probe should potentially
be ethyl-based, stable to changes in the pH of the environment and show significant localization into the mitochondria. Tetraethylrhodamine nitroxide (TER) was deemed to be the possible candidate. Since the 1980’s, rhodamine has been used as an effective probe for staining mitochondria. Chen et al.\textsuperscript{148} reported the use of rhodamine 123 in monitoring mitochondrial distribution within cells following transformation of cells with Rous sarcoma virus. TER, a tetraethylisoindoline nitroxide coupled with rhodamine could potentially be an effective mitochondrial redox probe.

TER consists of an ethyl-based PFN system which incorporates the chromophore rhodamine with an isoindoline nitroxide (Figure 3.3.1). Being an ethyl-based system, TER therefore has a reduction rate similar to that of TEF. The incorporation of rhodamine would potentially render TER a mitochondrially targeted redox probe.

![Figure 3.3.1: Chemical structure of tetraethylrhodamine nitroxide (TER).](image)

Effort was therefore carried out to assess the pH stability and cellular localization of TER. In an attempt to assesss the properties of TER, similar experimental procedures to determine the reduction rate, pH stability and intracellular localization as described previously for TEF were carried out. Details of the experimental procedures are listed below.

**Experimental**

a) Assessing the reduction rate of tetraethylrhodamine nitroxide (TER)

TER at a final concentration of 0.5 µM was prepared from a 1 mM DMSO stock solution in a final volume of 4 mL of 1xPBS (argon-bubbled PBS). The solution
containing 0.5 µM of TER was subsequently subjected to reduction by the addition of 375 µM of ascorbate (an excess of 750 times to that of TER).

The solution containing TER was excited at the wavelength of 556 nm and emission collected at wavelength 575 nm. Changes in fluorescence intensity of TER within the solution were recorded over time. The reduction rate of TER was subsequently determined based on the slope of the graph of percentage fluorescence intensity against time.

b) Assessing pH stability and cellular localization of tetraethylrhodamine nitroxide (TER)

Assessing pH stability
The preparation of solutions with varying pH was carried out as described on page 97. As in the case of TEF, stock solutions of TER at a concentration of 1 mM were prepared in DMSO. An aliquot of the TER stock solution was added to the different pH solutions to give a final probe concentration of 0.5 µM. Solution was mixed and the fluorescence intensity of the mixture was obtained along with its pH. Fluorescence intensities were measured using the Cary Eclipse fluorometer based on an excitation wavelength of 556 nm and emission wavelength of 575 nm. Triplicates were carried out and the results presented as graphs of relative fluorescence intensity against pH values.

Localization of TER
In place of TMF and TEF, hTERT human fibroblast cells were incubated with 1 µM of TER for 45 minutes. Instead of Hoechst, co-staining with the mitochondrial specific probe, MitoTracker Green was carried out.

hTERT immortalized human fibroblast cells at a concentration of 1.5x10^5 cells per mL were cultured overnight in IBIDI slides as previously described on page 97. The cells were subsequently treated with 1 µM of TER by replacing previous medium within each well with 2 mL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)]. The cells were incubated with TER for 45 minutes at 37°C with 5% CO₂ in air.
Upon incubation, the cells were washed once with 2 mL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)] and were co-stained with 100 nM of MitoTracker Green in 2 mL of phenol red free medium [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)]. The cells were incubated for a further 30 minutes at 37°C under an atmosphere of 5% CO₂ in air.

Subsequently, the cells were washed once with 2 mL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)] and a new batch of 2 mL phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)] was added to each well prior imaging.

An excitation wavelength of 515-569 nm and an emission wavelength of 549-639 nm was applied for imaging the localization of the TER under the Applied Precision DeltaVision fluorescence microscope. In the case of MitoTracker Green, an excitation wavelength of 447-503 nm and an emission wavelength of 487-559 nm were applied. Imaging conditions were maintained at 37°C under an atmosphere of 5% CO₂ in air.

Results and discussion
Figure 3.3.2: Comparing the reduction rate between tetramethylfluorescein (TMF), tetraethylfluorescein (TEF) and tetraethylrhodamine (TER) nitroxides using ascorbate as a reductant. **A**) Percentage change in fluorescence intensity over time relative to fluorescence intensity at time point 0 minutes, upon reacting TER (0.5 µM) with ascorbate (375 µM). **B**) Comparing the reduction rates of TMF, TEF and TER (0.5 µM each) upon reacting the PFN probes with ascorbate (375 µM). The result was displayed as change in fluorescence intensity per min. (Data analysed using T-test where a P value of < 0.05 was obtained, indicating a significant difference in percentage change in fluorescence intensity per min between TMF, TEF and TER).

Figure 3.3.2A (measured at a higher gain than Figure 3.1.5) showed that reacting TER with ascorbate increases the fluorescence of the PFN. As previously described, the reduction of the nitroxide by ascorbate to hydroxylamine, removes the quenching effect which arose from the coupling of the PFN with the chromophore moiety. The removal of the quenching effect, restores the fluorescence of chromphore,\textsuperscript{50,59} which in the case of TER, the fluorescence of rhodamine. The rate at which the fluorescence of TER reaches peak upon reacting with ascorbate, provides an indication of the reduction rate of TER.

Figure 3.3.2A shows that the fluorescence intensity of TER takes over 400 minutes to reach peak fluorescence and comparing the rate of reduction between all three PFN, TMF, TEF and TER (Figure 3.3.2B), reveals that TER has the slowest reduction rate among all three PFN probes. TMF has a reduction rate of 0.37 ± 0.03 per min, followed by TEF 0.05 ± 0.02 per min and lastly TER with a reduction rate of 0.0017 ± 0.0007 per min. The reduction rate of TER re-confirms that ethyl-based probes have a slower reduction rate compare to methyl-based systems due to reasons aforementioned.

Although the slow reduction rate potentially qualifies TER as a probe for assessing the mitochondrial redox status, the pH stability as well as the actual localization of TER into the mitochondria remains to be discovered. In terms of pH stability, it can be observed that compared to TEF, TER gave a relatively constant fluorescent response over the pH range of 3-12 (Figure 3.3.3). Rhodamine shows consistent fluorescence
response over a pH range that encompasses cellular systems (pH 6-7). Fluorescein fluorescence response on the other hand, shows a significant drop as the pH decreases from neutral (pH = 7) to slightly acidic (pH = 6). As rhodamine is less affected by pH change, PFN probes based on this structure would, therefore, provide better response than fluorescein analogues. Hence, this makes TER the more favourable PFN probe relative to TEF.

![Graph comparing fluorescence response of TER and TEF](image)

**Figure 3.3.3:** Comparing change in relative fluorescence intensity of tetraethylrhodamine nitroxide (TER) and tetraethylfluorescein nitroxide (TEF) at varying pH.

Subsequent experiment to assess the intracellular localization of TER (Figure 3.3.4) shows that TER localizes predominantly within the mitochondria. An overlayed image for TER treated and MitoTracker Green treated cells (Figure 3.3.4A) suggests localization of TER within the mitochondria. However, the localization of TER is not specific only towards the mitochondria as TER was observed to localize within globular structures which lacked staining by MitoTracker Green. This finding was confirmed by a coefficient of correlation colocalization plot (Figure 3.3.4B). The colocalization plot shows how two intensities (in this case TER and MitoTracker Green) correlate on a pixel-by-pixel basis (where each spot represents a pixel). The plot was derived from the image analysis program SoftWorx where a complete localization would result in a coefficient correlation of 1 or 100% correlation. Based on the coefficient of correlation of TER relative to MitoTracker Green, a value of 0.7269 was obtained. It can therefore be concluded that approximately 73% of the total
amount of TER localizes into the cell, coincides with that of MitoTracker Green, leaving 27% of the probe within the globular structures.
Figure 3.3.4: A) Localization of tetraethylrhodamine nitroxide (TER) (1 µM) along with co-staining with MitoTracker Green (100 nM) in hTERT immortalised human fibroblast cells. B) Colocalization plot for TER (1 µM) relative to MitoTracker Green (100 nM) within mitochondria. C) Chemical structure of TER and MitoTracker Green. (Triplicate life cell images were taken, only one image is being displayed).

The globular structures were suspected to be lysosomes. Subsequent co-staining experiment between TER with the lysosome specific probe, LysoTracker Blue was hence carried out. Details of the LysoTracker Blue co-staining experiment are as follows.

**Experimental**

Similar experimental procedures to that of co-staining hTERT cells with TER and MitoTracker Green were carried out. hTERT immortalized human fibroblast cells were cultured in IBIDI slides as described on page 97.

The cells within IBIDI slides were subsequently incubated with 1 µM of TER by replacing previous medium within each well with 2 mL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)]. The cells were incubated with TER for 45 minutes at 37°C under an atmosphere of 5% CO₂ in air.

Upon incubation, the cells were washed once with 2 mL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)], followed by co-staining with 50 nM of LysoTracker Blue in 2 mL of phenol red free medium [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)] for 30 minutes at 37°C under an atmosphere of 5% CO₂ in air.

The LysoTracker Blue treated cells were subsequently washed once with 2 mL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)] and a new batch of 2 mL phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)] and incubated at 37°C under an atmosphere of 5% CO₂ in air for 30 minutes.
concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)] was added to each well prior imaging.

The localization of the different probes was imaged under the Applied Precision Deltavision fluorescence microscope. An excitation at wavelength 515-569 nm and emission detected at wavelength 549-639 nm was applied for imaging localization of TER while in the case of LysoTracker Blue, an excitation wavelength of 372-408 nm and an emission wavelength of 417-453 nm were applied. Imaging conditions were maintained at 37°C under an atmosphere of 5% CO₂ in air.

Results and discussion
An overlayed image for TER treated and LysoTracker Blue treated cells reveals that the localization of TER coincides with lysosome partially (Figure 3.3.5A). Other globular structures are suspected to be endosomes. Despite the slight discrepancy, most of the fluorescence given off from cells incubated with TER (70% as determined from the coefficient of correlation between TER and MitoTracker Green, Figure 3.3.5B) were predominantly from the mitochondria. This therefore entitles TER as a potential probe for assessing mitochondrial redox status.
Figure 3.3.5: A) Localization of tetraethylrhodamine nitroxide (TER) (1 µM) and LysoTracker Blue (50 nM) within hTERT immortalised human fibroblast cells. B) Chemical structure of TER and LysoTracker Blue. (Triplicate life cell images were taken, only one image is being displayed).

The localization of TER into the mitochondria is attributed by the positive charge present within the molecule (Figure 3.3.6).

Figure 3.3.6: Redox potential of tetraethylrhodamine nitroxide (TER).

The uptake of TER, a cationic molecule into the mitochondria agrees with findings from several past studies as described previously. Hardy et al. (2007) reported the successful use of DEPMPO or Mito-5-dietoxyphosphoryl-5-methyl-1-pyrroline N-oxide, a cyclic nitron with an attached cationic triphenylphosphonium group, to determine mitochondrial redox status via EPR (Figure 1.30). Similarly Dikalov et al. 96,97,98 reported the use of the cationic mito TEMPO-H or (1-hydroxy-4-[2-triphenylphosphonio-acetamido]-2,2,6,6 tetramethypiperidine) to assess ROS within the mitochondria via EPR (Figure 1.31).
Subsequent studies by Cocheme and Murphy et al. (2011) on MitoB, which consists of a cationic triphenylphosphium group conjugated to an arylboronic acid (Figure 1.33) shows significant uptake of MitoB into the mitochondria as determined by mass spectroscopy. Recent work by Hirosawa et al. (2012) on MitoRP, a TEMPO (2,2,6,6-tetramethyl-piperidin-1-yl)oxyl moiety coupled to the monocationic chromophore coumarin 343 (Figure 1.36), further confirms the localization of cationic probes into the mitochondria. Using isolated mitochondria, Hirosawa et al. (2012) demonstrated the capability of MitoRP to monitor electron flow within the mitochondria via fluorescence measurement.

As described in the introduction, the generation of energy within the mitochondria involves a process known as the oxidative phosphorylation. Oxidative phosphorylation involves the transfer of electrons along several protein complexes, collectively known as the electron transport chain (ETC) (Figure 1.29). The transfer of electrons generates a proton gradient which results from the pumping of H⁺ ions out of the mitochondrial matrix. The net negative charge across the mitochondrial membrane generated during ETC, attracts the positively charged TER. Findings from the experiments described in this chapter hence concluded that TER have a slow reduction rate, is stable to changes in the pH of the environment and localizes predominantly in the mitochondria. These criteria make TER an ideal probe for assessing mitochondrial redox status. One criterion however remains in qualifying TER as a viable probe for assessing mitochondrial redox status. Due to the changing redox condition within biological systems, a redox probe with reversible sensing capacity is desirable. In an attempt to determine the capacity of TER to provide dynamic mitochondrial redox measurement, the reversible sensing capacity of TER was assessed.

3.4 Assessing reversibility of TER
The ability of the nitroxide to act as both a reductant and an oxidant, should theoretically enable TER to respond reversibly as the oxidation or reduction conditions within the system changes. Such change is indicated by a change in fluorescence intensity of the PFN. An experiment was carried out to assess the reversible nature of TER by subjecting the probe to varying oxidation and reduction cycles. The parent
chromophore analogue, Rhodamine B was used as a control. The details of the experiment are described as below.

Experimental

The experimental procedures used in this study are fully described in Materials and Methods, Section 2.5 (page 73).

Results and Discussion
Figure 3.4.1: A) Changes in fluorescence intensity of tetraethylrhodamine nitroxide (TER) and Rhodamine B (parent chromophore, control) upon subjection to varying oxidation-reduction cycle. B) Chemical structure of tetraethylrhodamine nitroxide (TER). C) Close-up view of changes in fluorescence intensity of TER upon subjection to varying oxidation-reduction cycle. D) Chemical structure of Rhodamine B. E) Close-up view of changes in fluorescence intensity of Rhodamine B upon subjection to varying oxidation-reduction cycle. F) Reduction and oxidation reaction of TER. G) Reacting Rhodamine B with hydrazine hydrate ($N_2H_4\cdot H_2O$).

Based on Figure 3.4.1C, TER displayed a cycle of increasing and decreasing fluorescence intensity upon being subjected to reduction and oxidation. Such cycle of
increasing and decreasing fluorescence was missing from the control, Rhodamine B (Figure 3.4.1E). The difference in the change of fluorescence observed between TER and Rhodamine B was due to the difference in chemical structures between TER and Rhodamine B.

Rhodamine B consists of just a chromophore which lacks the isoindoline nitroxide attachment as observed in TER (Figure 3.4.1B and 3.4.1D). The presence of the isoindoline nitroxide moiety, quenches the fluorescence of the chromophore rhodamine. Reaction with N₂H₂.H₂O reduces the nitroxide to hydroxylamine, causing the nitroxide to lose its radical nature. The lost of the radical nature of the nitroxide, removes the fluorescence quenching effect on the rhodamine chromophore thereby restoring the fluorescence of TER. Subsequent oxidation of the reduced TER by O₂, oxidizes the hydroxylamine back to the nitroxide. The oxidation of the hydroxylamine back to the nitroxide, reinstates the fluorescence quenching effect on the rhodamine chromophore. The fluorescence quenching effect decreases the fluorescence of TER. These observations indicated the reversible nature of TER.

N₂H₂.H₂O being a strong basic reductant, causes a general loss of fluorescence over time as indicated by a gradual decrease in peak fluorescence intensity of both TER and Rhodamine B over time (Figure 3.4.1A and E).

It can therefore be concluded that TER being an ethyl-based PFN has a slower reduction rate relative to methyl-based PFN namely TMF. In addition, TER is stable to changes in the pH of the environment compared to TEF and TMF due to the attachment of a rhodamine chromophore instead of fluorescein to the isoindoline nitroxide in TER. The attachment of the chromophore rhodamine also aids in the localization of TER into the mitochondria. Being a nitroxide-based redox sensing probe, TER shows reversible nature as indicated by a change in fluorescence intensity of the probe in varying oxidizing and reducing cycles.

The aforementioned characteristics of TER, entitles the potential use of TER as a mitochondrial redox sensing PFN. Experiments were therefore undertaken in the subsequent chapter, to assess the capability of TER to monitor changes in the
mitochondrial redox status within hTERT immortalized human fibroblast cells, based on flow cytometry techniques.
CHAPTER 4
The Application of PFNs within Biological Systems

Findings from previous chapters have shown that TER exhibits characteristics that favor its potential use as a probe to assess mitochondrial redox status. TER undergoes a slow reduction rate. Such slow reduction rate prevents TER from being easily reduced by other biological reductants prior reaching the mitochondria. In addition, TER is stable to changes in the pH of the environment, shows mitochondrial localization and is reversible in its detection. This therefore enables TER to provide a dynamic measurement of the redox status within the active mitochondria.

Despite studies from previous chapters which confer that TER exhibit promising characteristics as a potential mitochondrial redox probe, the actual capability of TER to assess mitochondrial redox condition remains crucial. This chapter hence serve as a preliminary studies to assess the ability of TER to measure redox changes within the mitochondria, was carried out by comparing the fluorescence change of normal and oxidative stressed diseased cells, both treated with TER, incubated within the fluorometer over time.

4.1 Comparing redox status between normal and Ataxia Telangiectasia, A-T diseased cells using TER as redox probe

Ataxia Telangiectasia, A-T is a neurodegenerative disease characterized by poor motor coordination and small dilated blood vessels. The disease results from the lack of a functioning ATM (Ataxia Telangiectasis Mutated) protein. This protein is involved in regulating cell cycle, maintaining chromosomal integrity as well as controlling deoxyribonucleic acid (DNA) damage responses within the cells.\textsuperscript{151} ATM is required for DNA repair during DNA replication. Although not fully understood, a defect in the gene results in heightened levels of oxidative stress, possibly due to an increase in the accumulation of free radical during DNA replication.\textsuperscript{24} A-T patients suffer from abnormalities particularly in the development of neurons. Such abnormalities result in poor motor coordination among A-T patients.\textsuperscript{152,153}
The potential use of TER along with a fluorometer to assess mitochondrial redox status within biological systems presents a simple and inexpensive method to monitor mitochondrial redox status. TER treated normal and A-T disease cells were incubated within the fluorometer and the change in fluorescence intensity was measured over time. Details of the experiment are as follows.

**Experimental**

Immortalized hTERT human normal and A-T disease fibroblast cells were cultured in a 25 mL flask to a final concentration of approximately $1\times10^6$ cells per mL. The cells were cultured over 4 days at $37^\circ$C under an atmosphere of 5% CO$_2$ in air. RPMI (Roswell Park Memorial Institute) medium, supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v) and phenol red was used to culture the cells. 1 mL of each cell line was pipetted into 4 different cuvettes and subjected to one of the following treatments.

- **Cuvette 1:** Normal cells + DMSO blank (negative control)
- **Cuvette 2:** A-T cells + DMSO blank (negative control)
- **Cuvette 3:** Normal cells + 0.1 µM of TER
- **Cuvette 4:** A-T cells + 0.1 µM of TER

TER was prepared from a 4 mM DMSO stock and added to the cells to a final concentration of 0.1 µM (a low probe concentration was used to minimize intermolecular quenching of fluorescence between TER molecules). The cuvettes were left within the fluorometer with conditions maintained at $37^\circ$C and 5% CO$_2$ bubbled into the surrounding within the machine at a rate of approximately 1 bubble every 2 seconds. Fluorescence readings were obtained every 30 minutes via the use of the fluorometer (Cary Eclipse, excite: 556 nm, emit: 575 nm) and the cell solutions were mixed throughout measurements.

A graph of change in TER fluorescence intensity of normal and A-T treated cells against time was subsequently plotted.
Results and Discussion

A

DMSO Control

Fluorescence Intensity (a.u.)

Time (hours)

B

TER Treatment 1

Fluorescence Intensity (a.u.)

Time (hours)
Figure 4.1.1: Change in TER fluorescence intensity over time upon incubating normal and A-T diseased cells with 0.1 µM of TER. A) Comparing fluorescence intensity of hTERT human normal and A-T fibroblast cells without addition of TER. Triplicate results comparing the fluorescence intensity of normal and A-T cells upon addition of 0.1 µM TER was showed in (B), (C) and (D).

Figure 4.1.1A shows that without TER treatment, both normal and A-T cells have similar fluorescence response. However, with TER treatment and under similar experimental setup, varying responses were obtained upon comparing fluorescence response of TER treated cells in Figure 4.1.1B, C and D. In Figures 4.1.1 B and D, A-T cells were observed to have a higher fluorescence compared normal cells. However,
in Figure 4.1.1C, both normal and A-T cells were observed to have similar fluorescence response over time. Such varying response renders the results from this experimental setup in-conclusive.

Possible reason for such an observation could be due to experimental setup. Due to possible contamination and inefficient control of experimental condition, it was observed that at the end of the experimental period (approximately 80 hours), the cells were clumping (possibly signifying changes in the extracellular matrix or cell membranes). Such experimental setup is therefore, not a good representation of the actual oxidative condition within biological systems. However, results from this preliminary study shows that TER has the ability to be used over extended period of time to assess mitochondrial redox status.

As an alternative method, flow cytometry technique was adapted. Flow cytometry has long been used in studies from measuring apoptosis of cell thymocytes\textsuperscript{154} and determination of lymphocyte division\textsuperscript{155} to study of immune cells\textsuperscript{156}. Flow cytometry enables the fluorescence of each cell in a cell suspension to be measured where individual cells treated with TER are excited at the specific wavelength and subsequently passed through a detector where the intensity of the light emitted are measured.

The ability to incorporate the application of PFN probes with flow cytometry, promises a sensitive and accurate tool where redox measurements in terms of fluorescence intensity of PFN within tens of thousands of cells can be made in relatively short period of time.

Having established the potential use of TER as a mitochondrial redox probe, attempts were made to assess the capability of TER to monitor redox changes within the mitochondria. This was achieved by altering mitochondrial redox status using mitochondrial toxins. Given that Complex I and III within the mitochondrial ETC are the main sources of basal reactive ROS production (as discussed in the introduction, Section 1.8),\textsuperscript{157,158} the ROS production within both complexes were altered. Rotenone (ROT) was used to induce ROS production within Complex I, while antimycin (AMC) to induce ROS production within Complex III (Figure 4.1.2). It was reported that
Complex I produces ROS into the matrix of the mitochondria while Complex III produces ROS in both the matrix as well as the intermembrane space of the mitochondria.\textsuperscript{159}

![Chemical structure of mitochondrial toxins, rotenone (ROT) and antimycin (AMC) used in inducing mitochondrial oxidative stress.](image)

Figure 4.1.2: Chemical structure of mitochondrial toxins, rotenone (ROT) and antimycin (AMC) used in inducing mitochondrial oxidative stress.

The capability of TER to assess mitochondrial redox status was compared to that of MitoSox, which serve as a control. As described in the introduction, MitoSox consists of a dihydroethidium with an attached triphenylphosphonium group (Figure 4.1.3). MitoSox is a commonly available and used commercial fluorescent redox probe.\textsuperscript{105,106,107}

Apart from MitoSox, a second control was set in place using tetramethylrhodamine methyl ester (TMRM). TMRM is a widely used commercial probe to assess mitochondrial membrane potential (Figure 4.1.3) and was used alongside TER and MitoSox as a means to assess the effect of ROT and AMC treatment on mitochondrial membrane potential.
In assessing the capability of TER to monitor mitochondrial redox status, hTERT immortalized human fibroblast cells were incubated with TER or MitoSox or TMRM, followed by treatment with either ROT or AMC. The changes in fluorescence intensity of TER upon the different treatment were recorded and presented as graphs of percentage change in fluorescence intensity relative to non-treated sample, against concentration of mitochondrial toxins.

Initially experiments were carried out to optimize the incubation time of PFN probe to be used in assessing the ability to incorporate the application of PFN probes with flow cytometry techniques. Details of the experiment are described below.

### 4.2 Optimizing flow cytometry experiment

hTERT immortalized human fibroblast cells were incubated with 1 µM TER over range of time period as described below. Upon incubation, the fluorescence of the TER treated cells was recorded via flow cytometry.

**Experimental**

A stock culture of hTERT immortalized human fibroblast cells was prepared as described in research objective. From the stock culture, hTERT immortalized human fibroblast cells were seeded at an initial concentration of approximately 5x10^4 cells per mL in 6-well plates. The cells were cultured in DMEM, supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final
concentration 1% v/v) and phenol red over a period of approximately 48 hours (final cell concentration approximately 2\times10^5 cells per mL).

The cells were initially incubated with TER (1 µM) for 15, 30, 45, 120, 240, 360, 720 and 1440 minutes at 37°C, 5% CO₂, in 1 mL of DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v) and phenol red]. Upon treatment, the cells were washed with 1 mL of 10% v/v versene, treated with a stock solution of trypsin (adding 0.5 mL of 0.5% v/v trypsin) and centrifuged (speed- 1200rpm, time- 4 mins and temperature-37°C). Supernatant was removed and cells were resuspended in 1 mL 1xPBS. Fluorescence readings for approximately 10000 cells were subsequently obtained via the BD LSFRFortessa flow cytometer. Cells incubated with TER were excited around wavelength 556 nm and emission collected at wavelength 575 nm.

Results and discussion
In attempt to provide an environment that mirrors the actual conditions within biological systems, experiments to monitor the mitochondrial redox sensing capability of TER were carried out on sub-confluent cells (cell concentration of approximately 2\times10^5 cells per mL) (Figure 4.2.1). The sub-confluent state ensures cells have adequate space for replication and are actively respiring.

Changes in TER fluorescence intensity upon subjecting TER treated cells to different ROT and AMC treatment were analysed via flow cytometry.
Based on Figure 4.2.2, a general trend where increasing incubation period, increases fluorescence intensity of TER treated cells was observed. Such a trend was expected as increasing TER incubation period among cells, increases the uptake of TER probe into the mitochondria of the cells. This in turn increases the fluorescence intensity of the cells.

The fluorescence intensity reaches peak fluorescence at time point 360 minutes or 6 hours. In an attempt to minimize incubation time, studies were subsequently carried out to compare the fluorescence changes between incubating hTERT immortalized cells for 45 minutes and subsequently treating the TER treated cells with ROT and AMC to induce ROS production within the mitochondria. Experimental setup and details are described as below.

**Experimental**

A stock culture of hTERT immortalized human fibroblast cells were prepared as described in Cell Preparation and Analysis chapter. From the stock culture hTERT immortalized human fibroblast cells were seeded at an initial concentration of approximately $5 \times 10^4$ cells per mL in 6-well plates. The cells were cultured in DMEM, supplemented with fetal calf serum (final concentration 12% v/v), penicillin
streptomycin antibiotics (final concentration 1% v/v) and phenol red over a period of approximately 48 hours (final cell concentration approximately $2 \times 10^5$ cells per mL). Within different wells, the cells were treated differently as follows:

i) Rotenone (ROT) treatment
The cells were initially incubated with TER (1 µM) either for 45 minutes or 360 minutes (6 hours) at 37°C, 5% CO₂, in 1 mL of DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v) and phenol red]. The medium containing TER was subsequently removed by pipette and a fresh batch of DMEM containing 1 µM of ROT in a final volume of 1 mL was added to each of the 6 well respectively. The cells were incubated with ROT for 15 minutes at 37°C under an atmosphere of 5% CO₂ in air.

ii) Antimycin (AMC) treatment
Similar experimental procedures to that of ROT treatment was carried out in the case of AMC treatment. Upon treatment with TER, the cells were incubated with 1 µM of AMC (0, 0.01, 0.1, 1, 10 and 100 µM) instead of ROT, in a final volume of 1 mL of DMEM for 15 minutes.

Blanks were carried out by replacing ROT or AMC with DMSO in a final volume of 1 mL of DMEM, followed by 15 minutes incubation at 37°C under an atmosphere of 5% CO₂ in air.

Upon treatment, the cells were washed with 1 mL of 10% v/v versene, treated with a stock solution of trypsin (adding 0.5 mL of 0.5% v/v trypsin) and centrifuged (speed-1200xg, time- 4 mins and temperature- 37°C). Supernatant was removed and cells were resuspended in 1 mL 1xPBS. Fluorescence readings for approximately 10000 cells were subsequently obtained via the BD LSRFortessa flow cytometer. The cells incubated with TER were excited around wavelength 556 nm and emission collected at wavelength 575 nm.
**Results and discussion**

Figure 4.2.3.: Percentage reduction in fluorescence intensity of treated hTRET immortalized human fibroblast cells, $F_i$ [cells incubated with 1 μM probes for 45 or 360 minutes, followed by treatment with 1 μM of A) rotenone (ROT) or B) antimycin (AMC)] for 15 minutes relative to blank, $F_c$ [cells incubated with 1 μM probes for 45 or 360 minutes, followed by treatment with DMSO for 15 minutes]. (Each data point are mean values. Result were not significantly different with P values > 0.05 under different treatment conditions, as determined by T-test).

Figure 4.2.3 shows that under both experimental setup namely 45 minutes incubation or 360 minutes incubation with TER, followed by 15 minutes treatment with ROT and AMC in both cases, similar reduction in TER fluorescence response was detected.
Subsequent experimental setup was hence based on 45 minutes incubation time with TER, followed by 15 minutes incubation with mitochondrial ROS inducer, ROT and AMC.

In an attempt to further assess the capability of TER along with flow cytometry techniques to assess mitochondrial redox status, TER treated hTERT immortalized human fibroblast cells were subjected to increasing concentration of ROT and AMC. Changes in fluorescence response with increasing ROT and AMC dose response was analysed. Details of the described experiment are detailed below.

### 4.3 Assessing redox status within cells via flow cytometry

The incorporation of PFN with flow cytometry technique promises an efficient yet accurate method for assessing the redox status within biological system via fluorescence. Upon optimizing the flow cytometry setup in the previous section (Section 4.2), redox status within cells subjected to increasing ROT and AMC dose response was analysed.

**Experimental**

hTERT immortalized human fibroblast cells were cultured in 6-well plates as previously described (page 124). The cells were cultured in DMEM, supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v) and phenol red over a period of approximately 48 hours (final cell concentration approximately $2 \times 10^5$ cells per mL). Within different wells, the cells were treated differently as follows:

i) Rotenone (ROT) treatment

The cells were initially incubated with TER (1 µM) or MitoSox (1 µM) for 45 minutes at 37°C, 5% CO$_2$, in 1 mL of DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v) and phenol red]. The medium containing TER was subsequently removed by pipette and a fresh batch of DMEM containing varying concentrations of ROT (0, 0.01, 0.1, 1, 10 and 100 µM) in a final volume of 1 mL was added to each of the 6 wells respectively. The cells were incubated with ROT for 15 minutes at 37°C under an atmosphere of 5% CO$_2$ in air.
ii) Antimycin (AMC) treatment

Similar experimental procedures to that of ROT treatment was carried out in the case of AMC treatment. Upon treatment with TER, the cells were incubated with varying concentrations of AMC (0, 0.01, 0.1, 1, 10 and 100 µM) instead of ROT, in a final volume of 1 mL of DMEM for 15 minutes.

Blanks were carried out by replacing ROT or AMC with DMSO in a final volume of 1 mL of DMEM, followed by 15 minutes incubation at 37°C under an atmosphere of 5% CO₂ in air.

Upon treatment, the cells were washed with 1 mL of 10% v/v versene, treated with a stock solution of trypsin (adding 0.5 mL of 0.5% v/v trypsin) and centrifuged (speed-1200xg, time- 4 mins and temperature- 37°C). Supernatant was removed and cells were resuspended in 1 mL 1xPBS. Fluorescence readings for approximately 10000 cells were subsequently obtained via the BD LSRSFortessa flow cytometer. Cells incubated with TER was excited around wavelength 556 nm and emission collected at wavelength 575 nm, while cells incubated with MitoSox was excited around wavelength 510 nm and emission collected at wavelength 580 nm.

**Results and discussion**

During oxidative phosphorylation, electrons escape from the ETC. This results in the generation of free radicals especially O₂•−. Superoxide (O₂•−) is converted to H₂O₂ by manganese superoxide dismutase (Mn-SOD). Subsequently, some of the H₂O₂ is detoxified by glutathione peroxidise to water (H₂O), while others are converted to the highly reactive hydroxyl radicals (HO•) via Fenton reaction.⁹³,¹⁶⁰

The O₂•− generated during the ETC can be categorized into two main pools i) the mitochondrial matrix and ii) the mitochondrial intermembrane space. Each O₂•− pool arises from electron leakage within Complex I and Complex III of the ETC respectively.¹⁰⁵

Altering the concentration of O₂•− generated from both Complex I and III will affect the overall redox status within the mitochondria. Two mitochondrial toxins were used to induce oxidative stress in cells, rotenone (ROT) and antimycin (AMC) (Figure
4.1.2). ROT affects ROS generation within Complex I while AMC affects that of Complex III. ROT inhibits the transfer of electrons from the iron-sulfur centre of Complex I to ubiquinones (Coenzyme Q). This subsequently alters the flow of electrons within the whole electron chain complex. Such alteration gives rise for the reduction of oxygen ($O_2$) to ROS, instead of the generation of water molecules. In the case of AMC, it binds to Complex III preventing the transfer of electrons from ubiquinol (reduced ubiquinones) to Complex IV. This in turn disrupts the electron transfer as in the case of ROT treatment, resulting in ROS production.

Both ROT and AMC treatments of cells pre-incubated with TER results in a reduction of fluorescence as displayed in Figures 4.3.1 and 4.3.2.

Figure 4.3.1: Histogram representation of hTERT immortalized human fibroblast cells treated with antimycin (AMC) (similar trend was observed in the case of rotenone, ROT treatment) upon incubation with tetraethylrhodamine nitroxide (TER). Black - blank (DMSO treatment); Red - cells + TER; Blue - cells + TER + AMC.
Figure 4.3.2: Percentage reduction in fluorescence intensity of treated hTERT immortalized human fibroblast cells, $F_i$ [cells incubated with 1 µM probes for 45 minutes, followed by treatment with varying concentrations of rotenone (ROT) or antimycin (AMC)] for 15 minutes relative to blank, $F_c$ [cells incubated with 1 µM probes for 45 minutes, followed by treatment with DMSO for 15 minutes]. A) Cells treated with 1 µM TER. B) Cells treated with 1 µM MitoSox. (Each data point are mean values. Data set in A were analysed using Kruskal Wallis test with pos hoc testing using the Dunn’s multiple comparison test. Data set in B were analysed using One-way ANOVA with pos hoc testing using the Dunn’s multiple comparison test. In both cases A and B, relative to blank, all treatment showed P values < 0.05. Results are therefore significantly different to blank).

MitoSox upon reacting with ROS forms hydroxyl triphenylphosphonium ethidium which fluoresces with an emission wavelength of 580 nm. MitoSox was used as a control to confirm if increasing the concentration of ROT and AMC increases ROS production within the mitochondria. Figure 4.3.2B displayed MitoSox acting as a positive fluorescence probe where an increasing ROT and AMC concentration, increases MitoSox fluorescence intensity. The increasing fluorescence intensity of MitoSox hence confirmed that ROS production within the mitochondria of hTERT cells increases with increasing ROT and AMC treatment.

Due to the reducing environment within the mitochondria, the localized TER nitroxides are reduced to hydroxylamines (Figure 4.3.3). The reduction gives rise to an initial increase in fluorescence intensity upon the uptake of TER nitroxides into the
mitochondria. Subsequent treatment with ROT or AMC, increases ROS production within the mitochondria\textsuperscript{105,162} where this was confirmed via the increasing fluorescence intensity of MitoSox with increasing concentration of ROT and AMC. The ROS in turn oxidises the hydroxylamines to nitroxides, resulting in a drop in TER fluorescence intensity. Dose response studies by increasing the concentration of ROT and AMC, therefore results in simultaneous reduction in TER fluorescence intensity (Figure 4.3.2). Results therefore shows that TER acts as a negative probe (opposite to MitoSox which acts as a positive probe), where increasing ROT and AMC concentration, decreases TER fluorescence intensity.

Figure 4.3.3: Change in fluorescence intensity of tetraethylrhodamine nitroxide (TER) upon uptake into mitochondria followed by treatment with rotenone (ROT) and antimycin (AMC).

Recent studies by Zorov et al.\textsuperscript{164} suggest that the opening of mitochondrial permeability transition pore (mPTP) plays an important role in the regulation of mitochondrial ROS. In situations of increasing ROS, mPTP opens and releases a burst of ROS from within the mitochondria. This mPTP opening-assoiated ROS release or
termed ROS-induced ROS release (RIRR) is reversible in nature. It serves as a defensive response to minimize the accumulation of potential toxic levels of ROS within the mitochondria. Although such a defense mechanism is vital, in conditions of excessive ROS, the prolonged opening of mPTP may disrupt the mitochondrial membrane in addition to an increase burst of ROS. Both increase burst of ROS and disruption of the mitochondrial membrane potentially affects the functionality of the mitochondria.

In an attempt to assess the mitochondrial membrane potential under the described ROT and AMC treatment, cells were incubated with tetramethylrhodamine methyl ester (TMRM) (Figure 4.3.4).

![Chemical structure of tetramethylrhodamine methyl ester (TMRM).](image)

TMRM is a widely used commercial probe to assess mitochondrial membrane potential and was used alongside TER and MitoSox as a means to assess the effect of ROT and AMC treatment on the mitochondrial membrane potential in the treated cells.

TMRM being positively charged is taken into the mitochondria. As aforementioned, increasing ROS production may lead to a loss of mitochondrial membrane potential. Such loss in membrane potential will result in the leakage of TMRM out of the mitochondria and subsequently out of the cell. A reduction in TMRM fluorescence intensity, hence is an indication of a loss of mitochondrial membrane potential.

In addition of TMRM, carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (Figure 4.3.5) was used as a positive control to induce disruption of the mitochondrial membrane potential. CCCP being an ionophore is capable of disrupting the electrochemical gradient generated during the ETC within the mitochondria. The
disruption of the electrochemical gradient subsequently results in a loss of membrane potential.\textsuperscript{165}

\[ \text{Figure 4.3.5: Chemical structure of carbonyl-cyanide m-chlorophenyl hydrazone (CCCP).} \]

Experimental procedures to assess the mitochondrial membrane potential within ROT and AMC treated cells were detailed as below.

**Experimental**

hTERT immortalized human fibroblast cells at an initial concentration of approximately $5 \times 10^4$ cells per mL were cultured in 6-well plates as described on page 124.

Similar experimental procedures to that of TER and MitoSox treatment, followed by ROT and AMC treatment were carried in assessing the effect of ROT and AMC treatment on the membrane potential of hTERT immortalized human fibroblast cells. However, instead of treatment with TER and MitoSox, the cells were incubated with TMRM as described below.

i) Rothenone (ROT) treatment

The cells were initially incubated with TMRM (20 nM) for 30 minutes in 1 mL of DMEM [supplemented with fetal calf serum (final concentration 12\% v/v), penicillin streptomycin antibiotics (final concentration 1\% v/v) and phenol red]. The medium containing TMRM was subsequently removed by pipette and a fresh batch of DMEM containing varying concentrations of ROT (0, 0.01, 0.1, 1, 10 and 100 µM) in a final volume of 1 mL was added to each of the 6 well respectively. The cells were incubated with ROT for 15 minutes at 37\(^{\circ}\)C under an atmosphere of 5\% CO\(_2\) in air.
ii) Antimycin (AMC) treatment

Similar experimental procedures to that of ROT treatment was carried out in the case of AMC treatment. Upon treatment with TMRM, the cells were incubated with varying concentrations of AMC (0, 0.01, 0.1, 1, 10 and 100 µM) instead of ROT, in a final volume of 1 mL of DMEM for 15 minutes.

iii) Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) treatment

Similar experimental procedures to that of ROT treatment was carried out in the case of CCCP treatment. Upon treatment with TMRM, the cells were incubated with CCCP (100 µM) instead of ROT, in a final volume of 1 mL of DMEM for 15 minutes.

Blanks were carried out by replacing the ROT or AMC or CCCP treatment with DMSO.

Upon treatment, cells were washed with 1 mL of 10% v/v versene, treated with a stock solution of trypsin (adding 0.5 mL of 0.5% v/v trypsin) and centrifuged (speed-1200xg, time- 4 mins and temperature- 37°C). Supernatant was removed and cells resuspended in 1 mL 1xPBS containing 20 nM TMRM. Fluorescence readings were subsequently obtained for 10000 cells via flow cytometry (BD LSRFortessa).
Results and discussion

Figure 4.3.6: Percentage reduction in mean fluorescence intensity of treated hTRET immortalized human fibroblast cells, $F_i$ [cells incubated with 20 nM tetramethylrhodamine methyl ester (TMRM) for 30 minutes, followed by treatment with varying concentrations of rotenone (ROT) / antimycin (AMC) / carbonyl cyanide m-chlorophenyl hydrazone (CCCP) for 15 minutes] relative to blank, $F_c$ [cells incubated with 20 nM TMRM for 30 minutes, followed by treatment with DMSO for 15 minutes]. (Each data point are mean values. Data were analysed using Kruskal Wallis test with pos hoc testing using the Dunn’s multiple comparison test. In all cases a P value of < 0.05 was obtained relative to blank, 0%. Results are therefore significantly different to blank).

Figure 4.3.6 shows a decrease in TMRM fluorescence intensity with increasing ROT and AMC concentration. Results from CCCP treatment, which serve as a positive control to incur loss of membrane potential, also shows a reduction in TMRM fluorescence intensity. This observation incidates a loss of membrane potential with increasing ROT and AMC concentrations.
The loss of membrane potential with increasing ROT and AMC concentrations, hence raised the question that the previous drop in TER fluorescence intensity under these ROT and AMC treatments, may possibly be due to the leaking of the TER out of the cells. In an attempt to assess such deduction, the non-radical alkylated derivative of TER, also known as methyl trap tetraethylrhodamine (MT-TER) (Figure 4.3.7) was used.

![Chemical structures](image)

**Figure 4.3.7:** Comparing chemical structures of A) tetraethylrhodamine nitroxide (TER) and B) its non-radical alkylated derivative or methyl trap tetraethylrhodamine (MT-TER).

MT-TER has similar structure to TER but has an extra coupled methyl group (Figure 4.3.7). The coupling of an extra methyl group at the nitroxide radical centre of MT-TER, renders MT-TER non-reactive and has its fluorescence fully switched on. Any decrease in fluorescence intensity observed upon incubating cells with MT-TER followed by subsequent treatment with ROT and AMC, therefore confirms the leaching of TER out of cells under the ROT and AMC treatment conditions. Experimental procedures carried out for assessing the mitochondrial membrane potential using MT-TER are as follows.

**Experimental**

Similar cell preparation steps along with similar experimental procedures as detailed on page 129 for the treatment of TER treated cells with ROT and AMC treatment was carried out. However, instead of TER, hTERT immortalized human fibroblast cells were incubated with MT-TER. Details of the experimental procedures are described below.
i) Rotenone (ROT) treatment
The cells were initially incubated with ME-TER (1 µM) for 30 minutes in 1 mL of DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v) and phenol red]. The medium containing ME-TER was subsequently removed by pipette and a fresh batch of DMEM containing varying concentrations of ROT (0, 0.01, 0.1, 1, 10 and 100 µM) in a final volume of 1 mL was added to each of the 6 well respectively. The cells were incubated with ROT for 15 minutes at 37°C under an atmosphere of 5% CO₂ in air.

ii) Antimycin (AMC) treatment
Similar experimental procedures to that of ROT treatment was carried out in the case of AMC treatment. Upon treatment with ME-TER, the cells were incubated with varying concentrations of AMC (0, 0.01, 0.1, 1, 10 and 100 µM) instead of ROT, in a final volume of 1 mL of DMEM for 15 minutes.

Blanks were carried out by replacing the ROT or AMC or CCCP treatment with DMSO.

Upon treatment, cells were washed with 1 mL of 10% v/v versene, treated with a stock solution of trypsin (adding 0.5 mL of 0.5% v/v trypsin) and centrifuged (speed-1200xg, time- 4 mins and temperature- 37°C). Supernatant was removed and cells resuspended in 1 mL 1xPBS. Fluorescence readings were subsequently obtained for 10000 cells via flow cytometry (BD LSRFortessa).
Results and discussion

Figure 4.3.8: Percentage change in mean fluorescence intensity of treated hTRET immortalized human fibroblast cells, $F_s$ [cells incubated with 1 µM non-radical alkylated derivative of TER or methyl trap tetraethylrhodamine (MT-TER) for 45 minutes, followed by treatment with varying concentrations of rotenone (ROT) / antimycin (AMC) for 15 minutes] relative to blank, $F_c$ [cells incubated with 1 µM MT-TER for 45 minutes, followed by treatment with DMSO for 15 minutes]. (Each data point are mean values. Data were analysed using Kruskal Wallis test with pos hoc testing using the Dunn’s multiple comparison test. Relative to blank, all treatment showed a P value of > 0.05. Results are therefore not significantly different to blank).

Based on Figure 4.3.8, increasing ROT and AMC concentration does not result in subsequent decrease in fluorescence intensity of MT-TER, as observed in the case of TMRM (Figure 4.3.6). This indicates that changes in TER fluorescence intensity previously observed upon treatment with ROT and AMC were a result of changes in mitochondrial redox condition and not due to a leakage of TER out of cells.
As a further confirmation, imaging experiment of hTERT immortalized human fibroblast cells incubated with MT-TER, followed by treatment with ROT, AMC and CCCP of varying concentrations was carried out as follows.

**Experimental**

hTERT immortalized human fibroblast cells were prepared in IBIDI slides as described on page 97. The cells were subjected to different treatment as follows.

i) Incubation with MT-TER

1 µM of MT-TER was incubated with hTERT cells for 45 minutes in 200 µL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v)] at 37°C under an atmosphere of 5% CO₂ in air. The cells were subsequently washed once with 200 µL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v)].

ii) Incubation with mitochondrial specific probe, MitoTracker Green

Upon incubation with MT-TER, the cells were subsequently incubated with 100 nM of MitoTracker Green probe in 200 µL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v)] for 30 minutes at 37°C under an atmosphere of 5% CO₂ in air. After which, the cells were washed once with phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v)] and subjected to ROT or AMC or CCCP treatment.

iii) ROT / AMC / CCCP treatments

The previously treated with MT-TER and MitoTracker Green were subsequently treated with ROT or AMC (1, 10 and 100 µM) or CCCP (100 µM) in 200 µL phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin (final concentration 1% v/v)] for 15 minutes at 37°C under an atmosphere of 5% CO₂ in air.

Blanks were carried out by replacing ROT or AMC or CCCP treatment with DMSO.
Upon ROT/AMC /CCCP treatments, cells were washed once with phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin (final concentration 1% v/v)]. The localization of MT-TER and MitoTracker Green was imaged under an Applied Precision Deltavision microscope (excited at wavelength 515-569 nm and emission detected at wavelength 549-639 nm in the case of MT-TER and excited at wavelength 447-503 nm and emission detected at wavelength 487-559 nm in the case of MitoTracker Green). Imaging conditions were maintained at 37°C under an atmosphere of 5% CO₂ in air in phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin (final concentration 1% v/v)].
Results and discussion

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Figure 4.3.9: Comparing localization of non-radical alkylated derivative of TER, methyl trap tetrathyrlrhodamine (MT-TER) within hTRET immortalized human fibroblast cells upon treatment with varying concentration of A) rotenone, ROT (1,10 and 100 µM) and B) antimycin, AMC (1, 10 and 100 µM) as well as carbonyl cyanide m-chlorophenyl hydrazone, CCCP (100 µM). (Triplicate life cell images were taken, only one image is being displayed).

Figure 4.3.9 shows that MT-TER remains within cells despite treatment with varying concentrations of ROT and AMC as compared to control. This differs from that observed with CCCP treatment. CCCP disrupts the cell membrane, resulting in the leakage of both MitoTracker Green and MT-TER out of the mitochondria and subsequently out of the cell. Analysis observation made from Figure 4.3.9 hence confirms that the changes in fluorescence intensity upon treatment with TER was not due to leakage of probe out of the cells. Instead, it was due to changes in redox status within mitochondria. This renders TER as an effective probe for assessing mitochondrial redox status.

Previous studies by Hirosawa (2012) reported of a TEMPO-conjugated profluorescent probe for monitoring redox reactions within the mitochondria, MitoRP.123 MitoRP consists of a TEMPO (2,2,6,6-tetramethyl-piperidin-1-yl)oxyl moiety coupled to the monocationic fluorophore coumarin 343 (Figure 1.36). It was reported that MitoRP is able to assess redox changes within Complex I of the mitochondria reversibly. However, an increase in ROS production increases the fluorescence intensity of MitoRP, unlike a decrease in fluorescence intensity within TER.

The difference in fluorescence response in relation to ROS production between MitoRP and TER could be the fact that MitoRP is a tetramethyl-based system but TER is a tetrathyrl-based system. As described earlier, methyl-based system has a higher reduction rate compared to that of ethyl-based system. In this regard, the whole oxidation and reduction process occurring for both MitoRP and TER may be of a different mechanism. In addition to that, the experiments carried out by Hirosawa et al. (2012) were with isolated mitochondria and not intact viable cells as in the case of TER.
Despite the ability of TER to assess redox status within biological systems, it is vital that the PFN probes do not affect the normal physiological functioning of the system. The cytotoxic effect of TER in cells is therefore crucial. An understanding on the cytotoxic effect of TER provides a greater understanding on the applicability of the PFN probe. The commonly used assay for assessing the cytotoxicity of compounds, MTT or (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out to assess the cytotoxicity effect of TER.

**Experimental**

hTRET immortalized human fibroblast cells were cultured in 96-well plate at a concentration of $2 \times 10^4$ cells per mL over a period of 24 hours. The cells were then incubated with 1 µM of compounds namely, TER, MT-TER and MitoSox. DMSO was used in place of the compounds as blank.

Cells were incubated with the compounds for 45 minutes, washed and replaced with 100 µL of fresh phenol red free DMEM supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin (final concentration 1% v/v).

A 12 mM stock solution of MTT was prepared by adding 1 mL of 1xPBS to a 5 mg vial of MTT (commercially available). 10 µL of the 12 mM MTT stock solution was added to each well within the 96-well plate. A negative control was prepared by adding of 10 µL of the 12 mM MTT stock solution to 100 µL media.

The mixtures were incubated at 37°C for 4 hours. After which, 85 µL of the solution was removed and 50 µL of DMSO was added to each well. The mixtures were thoroughly mixed and incubated for a further 10 minutes at 37°C.

The mixtures within the 96-well plate were subsequently mixed to form a homogenous solution and absorbance within each well was obtained at 540 nm using the BioTek Synergy plate reader.
Results and discussion

Figure 4.3.10: MTT cell proliferation assay upon incubating cells with different compounds [tetraethylrhodamine nitroxide, TER; its non-radical alkylated derivative, methyl trap tetraethylrhodamine (MT-TER) and MitoSox] for 45 mins. Cells were left to grow over a period of 1, 12, 24, 48 and 72 hours. Results were normalized to DMSO blank as percentage change in absorbance intensity of sample, $F_S$ relative to DMSO blank, $F_B$. (GraphPad prism was used to compare times within each treatment using a two-way repeat measure ANOVA with Tukey’s mulptile comparisons test used for pairwise comparisons. The standard errors to conduct pairwise comparisons between treatments were calculated manually, with the t-statistic used for the comparisons. P values of > 0.05 were obtained between different treatments. Hence, results were considered non-significant between treatments).

Under all the treatments described in Figure 4.3.10, the percentage change in absorbance intensity of sample, $F_S$ relative to DMSO blank, $F_B$ were similar. Figure 4.3.10 hence indicated that TER, MT-TER as well as MitoSox were not significantly toxic to cells for periods up to 3 days and do not affect normal functioning of the mitochondria. Results therefore enable TER to be used over extensive time period to assess biological redox status.

In summary, experiments described demonstrated that TER has a slow reduction rate. It was also shown that the probe has good pH stability at biologically relevant pH (6-
7). In addition, microscopy indicated that TER localizes significantly into the mitochondria. The potential of the probe to assess the redox changes within the mitochondria of hTERT immortalized human fibroblast cells was demonstrated using flow cytometry.

Despite the demonstrated value of TER as a mitochondrially targeted probe, the lack of complete localization of TER into the mitochondria potentially remains an issue. As aforementioned, due to the occurrence of ETC within the mitochondria, the mitochondria have a net negative charge across the mitochondrial membrane. The net negative charge across the mitochondrial membrane increases the uptake of cationic group into the mitochondria.\textsuperscript{136,137} Subsequent effort was hence carried out to increase the overall positive charge of TER, with the aim to increase uptake of TER within the mitochondria.
CHAPTER 5
Improved Cellular Response of PFNs by Esterification

Previous studies by Hardy et al. (2007),94 Dikalov et al.,96,97,98 Cocheme and Murphy et al. (2011),104 Hirosawa et al. (2012),125 as well as Trnka and Murphy (2008),163,166 reported that cationic probes show an increased localization within the mitochondria. Having the objective to increase the localization of TER into the mitochondria, an attempt was made to increase the overall positive charge of TER. This was carried out through esterification of the carboxylic group within the rhodamine moiety of TER, giving rise to the PFN, methyl ester tetraethylrhodamine nitroxide (ME-TER) with increase positive charge (Figure 5.1). Synthesis was carried out by Dr. Benjamin Chalmers based on synthesis developed by Morrow.126

Figure 5.1: Comparing the chemical structures between A) tetraethylrhodamine nitroxide (TER) and B) methyl ester tetraethylrhodamine nitroxide (ME-TER).

Compared to TER, ME-TER has an overall positive charge (Figure 5.1). Theoretically, ME-TER should show increased mitochondrial localization compared to TER. This was confirmed by repeating the previously described mitochondrial localization experiment using ME-TER as the PFN. Details of the experiment are described below.
5.1 Localization of methyl ester tetraethylrhodamine nitroxide (ME-TER)

Experimental

Similar cell preparation and experimental procedures as detailed in Chapter 2 for assessing the localization of PFN probes within cells were carried out. However, hTERT human fibroblast cells were incubated with 1 µM of ME-TER instead of TER.

Cells within IBIDI slides were incubated with 1 µM of ME-TER by replacing previous medium within each well with 2 mL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)]. The cells were incubated for 45 minutes at 37°C under an atmosphere of 5% CO₂ in air. Care was taken to avoid any detachment of cells from the slide.

Upon incubation, the cells were washed once with 2 mL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)] and were subsequently co-stained with 100 nM of MitoTracker Green and 50 nM of LysoTracker Blue in 2 mL of phenol red free medium [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)]. The cells were incubated for 30 minutes at 37°C under an atmosphere of 5% CO₂ in air.

The cells were subsequently washed once with 2 mL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)] and a new batch of 2 mL phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)] was added to each well prior imaging.

The localization of ME-TER, MitoTracker Green and LysoTracker Blue were imaged under an Applied Precision Deltavision microscope. An excitation at wavelength 515-569 nm and emission detection at wavelength 549-639 nm was applied in the case of ME-TER, while in the case of MitoTracker Green, an excitation at wavelength 447-503 nm and emission detection at wavelength 487-559 nm was applied. In the case of LysoTracker Blue, an excitation wavelength of 372-408 nm and an emission
wavelength of 417-453 nm were applied. Imaging conditions were maintained at 37°C under an atmosphere of 5% CO₂ in air in phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin (final concentration 1% v/v)].

Results and discussion

![Image](image)

**Figure 5.1.1:** Localization of 1 μM methyl ester tetraethylrhodamine (ME-TER) within hTERT immortalised human fibroblast cells. Cells were co-stained with MitoTracker Green (100 nM) and LysoTracker Blue (50 nM). (Triplicate life cell images were taken, only one image is being displayed).

Based on Figure 5.1.1, it can be observed that incubating hTERT cells with 1 μM of ME-TER cause clumping of the mitochondria into spherical structures instead of remaining as a filamentous network as observed previously in the case of 1 μM TER. Such observation could possibly indicate the disruption of the mitochondrial membrane which could possibly be due to an increased uptake of ME-TER into the mitochondria, owing to the increased positive charge of ME-TER. The increased uptake in turn could have possibly perturbed the ETC within the mitochondria by disrupting the transfer of electrons along the mitochondrial membrane. The disruption in electron transfer along the mitochondrial membrane results in loss of membrane potential, observed as the rounding up of the mitochondrial filamentous network and subsequently apoptosis or cell death.
The experiment was hence repeated using lower concentrations of ME-TER, in the nanomolar (nM) range and localization of ME-TER imaged accordingly under the Deltavision microscope. The localization of ME-TER was compared with that of TER.

hTERT immortalized human fibroblast cells were incubated separately with 5 nM TER and ME-TER along with their respective non-radical alkylated derivatives. The localizations of the TER and ME-TER as well as their respective non-radical alkylated derivatives within the mitochondria were subsequently compared.

![Image of localization comparison](image)

**Figure 5.1.2:** Comparing localization of 5 nM of tetraethylrhodamine nitroxide (TER) with 5 nM methyl ester tetraethylrhodamine nitoxide (ME-TER), along with the non-radical alkylated derivatives of both nitroxide probes, within hTERT immortalized human fibroblast cells. (Triplicate life cell images were taken, only one image is being displayed).

Observations made based on Figure 5.1.2 shows that at 5 nM concentration, the fluorescence intensities given off by TER and its non-radical alkylated derivative were
too weak to be observed. ME-TER and its non-radical alkylated derivative on the other hand, showed significant signal. Overlay image between MitoTracker Green and ME-TER (Figure 5.1.3) confirmed significant increase of ME-TER localization within the mitochondria.
Figure 5.1.3: A) Comparing the localization of 1 µM of tetraethylrhodamine (TER) and 5 nM of methyl ester tetraethylrhodamine nitroxide (ME-TER) within hTERT immortalized human fibroblast cells. B) Colocalization plot between ME-TER (5 nM) and MitoTracker Green (100 nM) within mitochondria. (Triplicate life cell images were taken, only one image is being displayed).

Based on Figure 5.1.3, the localization of ME-TER within the mitochondria has a coefficient of correlation of 0.9217. The coefficient correlation is much higher than that of TER with MitoTracker Green (0.7269) as described in Figure 3.3.4. The increase ME-TER / MitoTracker Green coefficient correlation confirmed an increased localization of ME-TER into the mitochondria compared to TER. The increased mitochondrial localization of ME-TER is evident through Figure 5.1.3A where the endosomal localization of TER is absent in the case of ME-TER. Compared to TER which only displayed approximately 73% colocalization with MitoTracker Green (Figure 3.3.4B), ME-TER displayed approximately 90% colocalization within the mitochondria (Figure 5.1.3B) as shown by the coefficient of correlation.

The increased localization of ME-TER within the mitochondria therefore renders ME-TER possibly a better probe for assessing mitochondrial redox status relative to TER. Only a small quantity of ME-TER is required for assessing mitochondrial redox status.

In an attempt to determine if esterification of TER giving rise to ME-TER affects the ability of ME-TER to assess mitochondrial redox status, the previously described flow cytometry redox experiment was repeated using ME-TER as probe. ME-TER treated hTERT immortalized human fibroblast cells were treated with ROT / AMC and the subsequent change in fluorescence intensity of ME-TER within cells were monitored via flow cytometry techniques.

5.2 Methyl ester tetraethylrhodamine nitroxide (ME-TER) as mitochondrial redox probe

Similar cell preparation and experimental procedures as detailed on page 129 for assessing the capability of TER to monitor redox changes within the mitochondria as
was carried out. However, instead of TER, cells were incubated with ME-TER. Details of the experimental procedures are as follows.

**Experimental**

Cells cultured within 6-well plates were subjected to different treatment as detailed below.

i) Rotenone (ROT) treatment

The cells were initially incubated with ME-TER (5 nM) or MitoSox (1 µM) for 45 minutes at 37°C, 5% CO₂, in 1 mL of DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v) and phenol red]. The medium containing ME-TER was subsequently removed by pipette and a fresh batch of DMEM containing varying concentrations of ROT (0, 0.01, 0.1, 1, 10 and 100 µM) in a final volume of 1 mL, was added to each of the 6 well respectively. The cells were incubated with ROT for 15 minutes at 37°C under an atmosphere of 5% CO₂ in air.

ii) Antimycin (AMC) treatment

Similar experimental procedures to that of ROT treatment were carried out in the case of AMC treatment. Upon treatment with ME-TER, the cells were incubated with varying concentrations of AMC (0, 0.01, 0.1, 1, 10 and 100 µM) instead of ROT, in a final volume of 1 mL of DMEM for 15 minutes.

Blanks were carried out by replacing ROT or AMC with DMSO in a final volume of 1 mL of DMEM, followed by 15 minutes incubation at 37°C under an atmosphere of 5% CO₂ in air.

Upon treatment, the cells were washed with 1 mL of 10% v/v versene, treated with a stock solution of trypsin (0.5 mL of 0.5% v/v trypsin) and centrifuged (speed- 1200xg, time- 4 mins and temperature- 37°C). Supernatant was removed and cells resuspended in 1 mL 1xPBS. Fluorescence readings for approximately 10000 cells were subsequently obtained via the BD LSRFortessa flow cytometer.
ME-TER was excited around wavelength 556 nm and emission collected at wavelength 575 nm, while MitoSox was excited around wavelength 510 nm and emission collected at wavelength 580 nm.

Results and discussion

![Bar graph showing the percentage reduction in fluorescence intensity of treated hTRET immortalized human fibroblast cells, F_t, compared to blank, F_c. The graph shows data for treatments with varying concentrations of rotenone (ROT) and antimycin (AMC) with error bars indicating standard deviation.](image)

Figure 5.2.1: Percentage reduction in fluorescence intensity of treated hTRET immortalized human fibroblast cells, F_t [cells incubated with 5 nM methyl ester tetraethylrhodamine nitroxide (ME-TER) for 45 minutes, followed by treatment with varying concentrations of rotenone (ROT) or antimycin (AMC)] for 15 minutes relative to blank, F_c [cells incubated with 5 nM ME-TER for 45 minutes, followed by treatment with DMSO for 15 minutes]. (Each data point are mean values. Data were analysed using Kruskal Wallis test with pos hoc testing using the Dunn’s multiple comparison test. Relative to blank, all treatment showed a P value < 0.05, results are therefore significantly different to blank).

As indicated in Figure 5.2.1, ME-TER showed similar results to the parent TER (Figure 4.3.2A). As described in Chapter 4 (Figure 4.3.3), due to the reducing environment within the mitochondria, the localized ME-TER nitroxides are reduced to hydroxylamines. The reduction give rise to an initial increase in fluorescence intensity upon the uptake of ME-TER nitroxides into the mitochondria. Subsequent
treatment with ROT or AMC, increases ROS production within the mitochondria.\textsuperscript{105,162} An increase in ROS production within the mitochondria oxidises the hydroxylamines to nitroxides, resulting in a drop in ME-TER fluorescence intensity (Figure 5.2.1). Increasing the level of ROT and AMC present, increases ROS production within the mitochondria of ME-TER treated cells. Such an increase in ROS production in turn decreases the fluorescence intensity of ME-TER present in the cells.

Analysis made from observation based on Figure 5.2.1, concluded that modification made on the parent TER structure to give the ester ME-TER still provides a fluorescence response that is sensitive to mitochondrial redox changes.

In an attempt to confirm if ME-TER fluorescence changes observed with ROT and AMC treated ME-TER incubated cells were due to changes in redox status within cells, the experiment above was repeated with the non-radical alkylated derivative of ME-TER or also known as methyl trap methyl ester tetraethylrhodamine, MT-ME-TER (Figure 5.2.2). MT-ME-TER served as a control to examine the role of radical reduction compared to probe retention in governing the fluorescence response detected by flow cytometry.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{structures.png}
\caption{Comparing chemical structures between A) methyl ester tetraethylrhodamine nitroxide (ME-TER) and its B) non-radical alkylated derivative or methyl trap methyl ester tetraethylrhodamine (MT-ME-TER).}
\end{figure}

Figure 5.2.2: Comparing chemical structures between A) methyl ester tetraethylrhodamine nitroxide (ME-TER) and its B) non-radical alkylated derivative or methyl trap methyl ester tetraethylrhodamine (MT-ME-TER).
hTERT immortalized human fibroblast cells were incubated with MT-ME-TER instead of ME-TER and subjected to different ROT or AMC treatment as detailed below.

**Experimental**

hTERT immortalized human fibroblast cells were seeded at an initial concentration of approximately 5x10^4 cells per mL in 6-well plates as described on page 124. Within different wells, the cells were treated differently as follows.

i) Rotenone (ROT) treatment

The cells were initially incubated with MT-ME-TER (5 nM) for 45 minutes at 37°C, 5% CO₂, in 1 mL of DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v) and phenol red]. The medium containing ME-TER was subsequently removed by pipette and a fresh batch of DMEM containing varying concentrations of ROT (0, 0.01, 0.1, 1, 10 and 100 µM) in a final volume of 1 mL was added to each of the 6 well respectively. The cells were incubated with ROT for 15 minutes at 37°C under an atmosphere of 5% CO₂ in air.

ii) Antimycin (AMC) treatment

Similar experimental procedures to that of ROT treatment were carried out in the case of AMC treatment. Upon treatment with ME-TER, the cells were incubated with varying concentrations of AMC (0, 0.01, 0.1, 1, 10 and 100 µM) instead of ROT, in a final volume of 1 mL of DMEM for 15 minutes.

Blanks were carried out by replacing ROT or AMC with DMSO in a final volume of 1 mL of DMEM, followed by 15 minutes incubation at 37°C under an atmosphere of 5% CO₂ in air.

Upon treatment, the cells were washed with 1 mL of 10% v/v versene, treated with a stock solution of trypsin (0.5 mL of 0.5% v/v trypsin) and centrifuged (speed- 1200xg, time- 4 mins and temperature- 37°C). Supernatant was removed and cells resuspended in 1 mL 1xPBS. Fluorescence readings for approximately 10000 cells were
subsequently obtained via the BD LSRFortessa flow cytometer. MT-ME-TER was excited around wavelength 556 nm and emission collected at wavelength 575 nm.

Results and discussion

Figure 5.2.3: Percentage reduction in fluorescence intensity of treated hTRET immortalized human fibroblast cells, $F_s$ [cells incubated with 5 nM methyl trap methyl ester tetraethylrhodamine nitroxide (MT-ME-TER) for 45 minutes, followed by treatment with varying concentrations of rotenone (ROT) or antimycin (AMC)] for 15 minutes relative to blank, $F_c$ [cells incubated with 5 nM MT-ME-TER for 45 minutes, followed by treatment with DMSO for 15 minutes]. (Each data point are mean values. Data were analysed using Kruskal Wallis test with pos hoc testing using the Dunn’s multiple comparison test. Relative to blank, all treatment showed $P$ values > 0.05, results are therefore not significantly different to blank).

Based on Figure 5.2.3, there was no significant reduction in fluorescence intensity of MT-ME-TER upon treating the cells with increasing level of ROT. A similar trend was observed in the case of AMC treatment of concentration 0.01 to 10 µM. The analysis made based on Figure 5.2.3 hence confirmed that the drop in ME-TER fluorescence intensity previously observed in cells treated with ROT and AMC (Figure 5.2.1) was due to changes in mitochondrial redox status and not leakage of the probe out of the mitochondria and the cell.
However, upon treating cells with AMC concentration of 100 µM, there was a significant drop in MT-ME-TER fluorescence intensity (Figure 5.2.3) upon running a One-way ANOVA with pos hoc testing using the Dunnett’s multiple comparison test (P value 0.0009). This suggests that at AMC concentration of 100 µM, the drop in fluorescence intensity of ME-TER (Figure 5.2.1) was possibly due to leakage of the probe out of the cell.

A positive control experiment based on CCCP treatment to disrupt mitochondrial membranes within cells incubated with MT-ME-TER was subsequently carried out. A similar drop in MT-ME-TER fluorescence intensity upon 100 µM AMC treatment compare to 100 µM CCCP treatment would possibly confirm the leakage of the probe MT-ME-TER out of the cell. CCCP treatment experimental procedures carried out were described below.

**Experimental**

**CCCP treatment**

hTERT immortalized human fibroblast cells were incubated with MT-ME-TER for 45 minutes as previously described in the case of ME-TER. The cells were washed with fetal calf serum (final concentration 12% v/v), penicillin streptomycin (final concentration 1% v/v). Subsequently, the cells were treated with CCCP (100 µM) prepared in phenol red free media added with fetal calf serum (final concentration 12% v/v), penicillin streptomycin (final concentration 1% v/v) for 15 minutes. Incubation conditions were maintained at 37°C under an atmosphere of 5% CO2 in air.

Upon treatment, cells were washed with 1 mL of 10% v/v versene, treated with a stock solution of trypsin (adding 0.5 ml of 0.5% v/v trypsin) and centrifuged (speed- 1200 rpm, time- 4 mins, temperature- 37°C). Supernatant was removed and cells resuspended in 1 mL 1xPBS. Fluorescence readings for approximately 10000 cells were subsequently obtained via the BD LSRFortessa flow cytometer. MT-ME-TER was excited around wavelength 556 nm and emission collected at wavelength 575 nm.
Results and discussion

Figure 5.2.4: Percentage reduction in fluorescence intensity of treated hTRET immortalized human fibroblast cells, $F_s$ [cells incubated with non-radical alkylated derivative of methyl ester tetraethylrhodamine nitroxide (MT-ME-TER) for 45 minutes, followed by treatment with varying concentrations of rotenone (ROT) or antimycin (AMC) or carbonyl cyanide m-chlorophenyl hydrazone (CCCP)] for 15 minutes relative to blank, $F_c$ [cells incubated with 5 nM MT-ME-TER for 45 minutes, followed by treatment with DMSO for 15 minutes]. (Each data point are mean values. Data were analysed using Kruskal Wallis test with pos hoc testing using the Dunn’s multiple comparison test. Relative to blank, CCCP and AMC 100 µM treatment showed P values < 0.05, results are therefore significantly different to blank).

A decrease in MT-ME-TER fluorescence intensity was observed at 100 µM CCCP treatment (Figure 5.2.4). Compared to 100 µM AMC, it is apparent that a greater decrease in MT-ME-TER was observed in the case of 100 µM CCCP treatment. Nevertheless, a decrease in MT-ME-TER fluorescence intensity was observed in both cases, 100 µM AMC and CCCP treatment. The decrease in MT-ME-TER fluorescence intensity in both 100 µM AMC and CCCP treatment could possibly confirm the leakage of MT-ME-TER out of the mitochondria and subsequently out of the cell. The leakage of MT-ME-TER out of the mitochondria and cell was possibly due to the
disruption of the mitochondrial membrane potential under both 100 μM AMC and CCCP treatments.

In an attempt to confirm the possibility that the leakage of MT-ME-TER out of the cell under both 100 μM AMC and CCCP treatment is due to disruption of the mitochondrial membrane, imaging studies were carried out. The condition of MT-ME-TER incubated cells upon treatment with varying concentrations of ROT, AMC and CCCP were imaged based on the following experimental procedures.

**Experimental**
Cells were cultured within IBIDI slides as described on page 97 and were subjected to different treatment as follows.

i) Incubation with MT-ME-TER
5 nM of MT-ME-TER was incubated with hTERT cells for 45 minutes in 200 μL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v)] at 37°C under an atmosphere of 5% CO₂ in air. The cells were subsequently washed once with 200 μL of phenol red free DMEM [added with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v)].

ii) Incubation with mitochondrial specific probe, MitoTracker Green
Upon incubation with MT-ME-TER, the cells were subsequently incubated with 100 nM of MitoTracker Green probe in 200 μL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v)] for 30 minutes at 37°C under an atmosphere of 5% CO₂ in air. After which, the cells were washed once with phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v)] and subjected to ROT or AMC or CCCP treatment.

iii) ROT / AMC / CCCP treatments
The previously treated with MT-ME-TER and MitoTracker Green cells were subsequently treated with ROT or AMC (1, 10 and 100 μM) or CCCP (100 μM) in
200 µL phenol red free DMEM [added with fetal calf serum (final concentration 12% v/v), penicillin streptomycin (final concentration 1% v/v)] for 15 minutes at 37°C under an atmosphere of 5% CO₂ in air.

Blanks were carried out by replacing ROT or AMC or CCCP treatment with DMSO. Upon ROT / AMC / CCCP treatments, cells were washed once with phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin (final concentration 1% v/v)]. The localization of MT-ME-TER and MitoTracker Green was imaged under an Applied Precision Deltavision microscope (excited at wavelength 515-569 nm and emission detected at wavelength 549-639 nm in the case of MT-ME-TER and excited at wavelength 447-503 nm and emission detected at wavelength 487-559 nm in the case of MitoTracker Green). Imaging conditions were maintained at 37°C and 5% CO₂ in phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin (final concentration 1% v/v)].
Results and discussion

A
Figure 5.2.5: Comparing localization of non-radical alkylated derivative of ME-TER, methyl trap methyl ester tetraethylrhodamine (MT-ME-TER) within hTRET immortalized human fibroblast cells upon treatment with varying concentrations of A) rotenone, ROT (1, 10 and 100 µM) and B) antimycin, AMC (1, 10 and 100 µM) as well as carbonyl cyanide m-chlorophenyl hydrazone, CCCP (100 µM). (Triplicate life cell images were taken, only one image is being displayed).

It can be observed from Figure 5.2.5, that upon treatment with 100 µM of AMC and CCCP, there was a lost of the filamentous network within the cells. The loss in filamentous network was absent in the case of ROT (1, 10, 100 µM) and AMC (1, 10 µM) treatments. Such loss of the filamentous network as aforementioned (Figure 5.1.1) is possibly an indication of disruption towards the mitochondrial membrane potential.

The disruption of the mitochondrial membrane potential possibly leads to leakage of MT-ME-TER out of the mitochondria and the cell which can be observed by lower fluorescence intensity among the MT-ME-TER incubated cells treated with 100 µM AMC and CCCP. Such a decrease of fluorescence intensity among MT-ME-TER incubated cells with 100 µM of AMC and CCCP was prominent through Figure 5.2.5 upon comparing the fluorescence intensity of MT-ME-TER incubated cells treated with 100 µM of AMC and CCCP relative to the other ROT (1, 10, 100 µM) and AMC (1, 10 µM) treatments. In addition, cells treated MT-ME-TER and 100 µM CCCP resulted in lower MT-ME-TER fluorescence intensity compared to that of 100 µM AMC treatment, agreeing with previous flow cytometry results (Figure 5.2.4).

Upon analysing the images in Figure 5.2.5, it can therefore be concluded that 100 µM AMC and CCCP treatments could possibly results in the disruption of mitochondrial membrane potential. The disruption of the mitochondrial membrane leads to the leakage of PFN probes out of the mitochondria and subsequently out of the cell. A high concentration of AMC (in this case 100 µM) therefore could possibly lead to disruption of mitochondrial membrane potential in ME-TER treated cells. Despite of it
all, ME-TER still has the capacity to serve as a potential probe to monitor redox changes at lower ROT and AMC concentrations.

Upon confirming the redox sensing capability of ME-TER, cytotoxicity of ME-TER and MT-ME-TER remained a crucial factor in determining the potential use of ME-TER and MT-ME-TER in biological system. An ideal redox sensing probe does not affect the normal physiological functioning of the cells over prolong incubation period. The cytotoxicity level of ME-TER and MT-ME-TER was therefore assessed.

**5.3 Cytotoxicity of Methyl Ester Tetraethylrhodamine (ME-TER)**

In assessing the cytotoxicity of ME-TER and MT-ME-TER, the previously described MTT assay assessment (Figure 4.3.10) was repeated for ME-TER and MT-ME-TER treated cells. In addition, the effect of ME-TER within cells was imaged over a period of more than 24 hours using the spinning disk fluorescence microscope, so as to assess the potential use of ME-TER to monitor changes in mitochondrial redox status within cells over extended time periods.

Conventional fluorescence microscopy involves exciting the sample under observation with a broad stream of light. The fluorescence given off by the sample, both within and out of focal plane is subsequently collected and imaged. In the case of confocal fluorescence microscopy, a pinhole is used to block out light which is out of focus. This improves the resolution of image.

A commonly used confocal fluorescence microscopic technique is the confocal laser scanning microscope. Such a laser scanning microscope uses a laser of distinct wavelength governed by the nature of the sample to rapidly scan the sample at several different points. The light returning from each point is quantified by a photomultiplier tube and a digital image is reconstructed.\(^{167}\)

Although the confocal laser scanning microscope is able to provide a fast response, the microscope however suffers from a trade off between image resolution and imaging speed. In order to obtain a significantly good resolution in a short period of time, relatively intense laser power has to be used. Such intense power can result in photobleaching and even phototoxicity in live cell imaging.\(^{168}\)
Another technology that has the potential to overcome this limitation is a spinning disk instrument where the microscope is equipped with multiple pin holes. A spinning disk containing rapidly rotating pinhole disk enables thousands of light points to scan the sample simultaneously and can achieve frame rates up to 1000 frames per second. This enables the use of a lower intensity laser power and multiple point detection simultaneously, giving high resolution images in a short period of time.\textsuperscript{168}

Due to its sensitivity and low phototoxicity, spinning disk microscopic techniques along with MTT assay were carried out to assess the cytotoxicity level of ME-TER. The experimental procedures were detailed below.

**Experimental**

i) MTT assay

Similar MTT assay experimental procedures as described for assessing the cytotoxicity of TER (page 146) were repeated for assessing the cytotoxicity level of ME-TER and MT-ME-TER.

hTRET immortalized human fibroblast cells were cultured in 96-well plate at a concentration of $2 \times 10^4$ cells per mL over a period of 24 hours. Upon incubation, the cells were incubated with 25 nM of compounds namely, ME-TER and MT-ME-TER. DMSO blank was used in place of the compounds as blank.

Cells were incubated with the compounds for 45 minutes, washed and replaced with 100 µL of fresh phenol red free DMEM added with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin (final concentration 1% v/v).

A 12 mM stock solution of MTT was prepared by adding 1 mL of 1xPBS to a 5 mg vial of MTT (commercially available). 10 µL of the 12 mM MTT stock solution was added to each well within the 96-well plate. A negative control was prepared by adding of 10 µL of the 12 mM MTT stock solution to 100 µL media.

The mixtures were incubated at 37°C for 4 hours. After which, 85 µL of the solution was removed and 50 µL of DMSO was added to each well. The mixtures were thoroughly mixed and incubated for a further 10 minutes at 37°C.
The mixtures within the 96-well plate were subsequently mixed to form a homogenous solution and absorbance within each well was obtained at 540 nm using the BioTek Synergy plate reader.

ii) Imaging using spinning disk fluorescence microscopy

4x10^5 cells per mL of hTERT immortalized human fibroblast cells were cultured in IBIDI slides overnight in DMEM media [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin (final concentration 1% v/v)]. The cells were subsequently incubated with 25 nM of ME-TER (prepared in 2 mL of phenol red free DMEM media supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin (final concentration 1% v/v) and localization of probe was imaged under the CV1000 spinning disk microscope every 3 minutes for over 24 hours. Imaging conditions were maintained at 37°C and 5% CO₂. The probe was excited at wavelength 561 nm and emission detected at wavelength 582 nm.

Results and discussion

![Graph A](image)

![Graph B](image)
Figure 5.3.1: A) hTERT immortalized human fibroblast cells were incubated with varying compounds (25 nM) for varying time point. Cells were then washed and cytotoxicity of compounds on cells were determined via MTT assay. Results were expressed as percentage absorbance change of sample, F relative to blank, F_b (DMSO treatment). (Each data point are mean values of replicated experiment. Data were analysed using Kruskal Wallis test with post hoc testing using the Dunn’s multiple comparison test. Relative to blank, all treatment showed P values > 0.05, results are therefore not significantly different to blank). B) hTERT immortalized human fibroblast cells were incubated with 25nM of methyl ester tetraethylrhodamine nitroxide (ME-TER) over 24 hours. Cells were imaged every 3 minutes for over 24 hours. Image of cells before and after treatment period was shown. (Triplicate life cell images were taken, only one image is being displayed).

Based on Figure 5.3.1A, both ME-TER and MT-ME-TER have similar change in absorbance relative to the non-treated and DMSO control up to 3 days (72 hours) of incubation with the probes. The similar change in absorbance relative to the non-treated and DMSO control indicates that both ME-TER and MT-ME-TER have limited impact on the normal mitochondrial function in the cells studied. Both ME-TER and MT-ME-TER hence have low cytotoxic effect towards the treated cells.

The low toxicity effect of ME-TER towards cells was further confirmed through images of cell incubated with ME-TER observed in Figure 6.3.1B. Cells incubated with ME-TER for more than 24 hour incubation, undergone normal mitosis or cell division and increased in cell number. ME-TER therefore, has the potential to be used as a mitochondrial redox monitoring probe over extended time period.

Although the coupling of an extra methyl group at the nitroxide radical centre of MT-ME-TER, renders it non-reactive, MT-ME-TER could possibly serve as a probe to stain mitochondria as it has its fluorescence fully switched on, in addition to showing good mitochondrial localization (Figure 5.2.5) and low cytotoxicity within cells (Figure 5.3.1A).

Having established the potential use of ME-TER as a mitochondrial redox probe due to its increase mitochondrial localization and low cytoxicity within cells, studies were
carried out to determine the capability of ME-TER to compare differential redox status within normal and Ataxia Telangiectasia, A-T diseased cells, which as previously described suffers from oxidative stress.

5.4 Comparing redox status between normal and Ataxia Telangiectasia, A-T diseased cells

The first step in determining the capability of ME-TER to assess the redox status within normal and A-T diseased cells, was to determine if ME-TER localizes within the mitochondria of both normal and A-T diseased cells. Imaging experiments as described in Figure 5.1.3 were carried out. Normal and A-T diseased fibroblast cells were incubated with 10 nM ME-TER and MT-ME-TER. Details of the experimental procedures are described below.

A) Localization of ME-TER within normal and A-T diseased cells

Experimental

Primary (non-immortalized) normal human fibroblast and A-T diseased human fibroblast cells were cultured overnight in IBIDI slides in DMEM media [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v) and phenol red] at an initial concentration of $1.5 \times 10^5$ cells per mL.

The cells were subsequently incubated with 10 nM of ME-TER and MT-ME-TER by replacing previous medium within each well with 2 mL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)]. The cells were incubated for 45 minutes at $37^\circ$C under an atmosphere of 5% CO$_2$ in air and care was taken to avoid any detachment of cells from the slide.

Upon incubation, the cells were washed once with 2 mL of phenol red free DMEM (supplemented with 12% fetal calf serum and 1% penicillin streptomycin antibiotics) and were subsequently co-stained with 100 nM of MitoTracker Green in 2 mL of phenol red free medium [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)]. The cells were incubated for 30 minutes at $37^\circ$C under an atmosphere of 5% CO$_2$ in air.
The cells were subsequently washed once with 2 mL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)] and a new batch of 2 mL phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)] was added to each well prior imaging.

The localization of ME-TER, MT-ME-TER and MitoTracker Green were imaged under an Applied Precision Deltavision microscope. An excitation at wavelength 515-569 nm and emission detection at wavelength 549-639 nm was applied in the case of ME-TER and MT-ME-TER, while in the case of MitoTracker Green, an excitation at wavelength 447-503 nm and emission detection at wavelength 487-559 nm was applied. Imaging of LysoTracker Blue was based on an excitation wavelength of 372-408 nm and an emission wavelength of 417-453 nm. Imaging conditions were maintained at 37°C under an atmosphere of 5% CO₂ in air in phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin (final concentration 1% v/v)].
Results and discussion

A

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
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<th>MitoTracker Green</th>
<th>Overlay</th>
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B

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C

![Graph](image17.png)

Pearson Coefficient of
Correlation 0.8602

![Graph](image18.png)

Pearson Coefficient of
Correlation 0.8702
Figure 5.4.1: Localization of tetraethylrhodamine nitroxide (ME-TER) (10 nM, 45 minutes incubation) and MitoTracker Green (100 nM, 30 minutes incubation) within A) normal and B) Ataxia Telangiectasia, A-T diseased human fibroblast cells. C) Correlation plots of mitochondrial localization of ME-TER relative to MitoTracker Green in normal human fibroblast cell line (left) and Ataxia Telangiectasia, A-T diseased human fibroblast cell line (right) were shown. (Triplicate life cell images were taken, only one image is being displayed).

Figure 5.4.1A and B shows that both ME-TER and MT-ME-TER showed significant mitochondrial localization within normal and A-T diseased cells. Colocalization plots between ME-TER and MitoTracker, displayed a correlation coefficients of 0.8902 and 0.8767 for the localization of ME-TER within the mitochondria of normal and A-T diseased cells, respectively. This correlates with previous results on the localization of ME-TER and MT-ME-TER within the mitochondria of hTERT immortalized cells (Figure 5.1.3 and Figure 5.2.5).

Having established significant uptake of ME-TER and MT-ME-TER into the mitochondria of normal and A-T diseased cells, subsequent flow cytometry experiments were carried out to assess the capability of ME-TER to compare differential redox status within normal and Ataxia Telangiectasia, A-T diseased cells.

B) Assessing redox status of normal and A-T diseased cells with ME-TER via flow cytometry techniques

Experimental

Primary (non-immortalized) normal and A-T diseased human fibroblast cells were grown in cell culture flasks and treated with a stock solution of trypsin (adding 0.5 mL of 0.5% v/v trypsin) and centrifuged (speed- 1200xg, time- 4 mins and temperature-37°C) in 10 mL FALCON tube. Cells were subsequently count to a concentration of 2 x 10^5 cells per mL in a final volume of 0.3 mL. The cells were incubated with varying concentrations of compounds for varying periods of time as listed below, in a final volume of 2 mL of DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v) and phenol red].
i) 10 nM ME-TER and MT-ME-TER for 45 minutes  
ii) 5 µM MitoSox for 45 minutes  
iii) 100 nM MitoTracker Green for 30 minutes  
iv) 20 nM TMRM for 30 minutes

Upon incubation cells were centrifuged (1200 rpm, 4 minutes at 37°C). The supernatant was removed and cells resuspended in 1 mL of 1xPBS in the case of ME-TER, MT-ME-TER, MitoSox and MitoTracker Green treatment, but 1 mL of 20 nM TMRM (prepared in 1xPBS) in the case TMRM treatment. Fluorescence readings were subsequently obtained for 5000 cells via flow cytometry (BD LSRFortessa).

Results and discussion
Figure 5.4.2: Figures showing percentage changes in fluorescence intensity of sample, Fs (compounds) relative to control, Fc (DMSO) upon treatment with A) 45 minutes incubation with 10 nM ME-TER; B) 45 minutes incubation with 5µM MitoSox; C) 30 minutes incubation with 10 nM of MT-ME-TER; D) 30 minutes incubation with 20 nM TMRM probe. Results were normalized to mitochondrial density as measured by 30 minutes incubation with Mitotracker Green (100 nM). (Result were significantly different between normal and A-T diseased cells with P values < 0.1 under different treatment conditions, as determined by T-test. Cells were taken from a minimum of 3 different patients).

Subsequent flow cytometry studies based on analysis from Figure 5.4.2 affirmed the capability of ME-TER to compare the redox status between normal and A-T diseased cells. MitoSox serves as a positive control, confirming that A-T diseased cells have a higher fluorescence intensity signifying a higher oxidative stress level (higher ROS level) compared to that of normal cells (Figure 5.4.2B). Similar result was portrayed by ME-TER where the oxidative stressed A-T diseased cells have lower fluorescence intensity compared to that of normal cells (Figure 5.4.2A). These results align with our previous observation where ROT / AMC stressed ME-TER incubated hTERT cells portrayed decreasing fluorescence intensity with increasing ROT / AMC concentration (Figure 5.2.1).

Further studies with MT-ME-TER and TMRM, confirmed that the difference in ME-TER fluorescence intensity between normal and A-T diseased cells reflects a difference in oxidative status between both cell lines and not due to difference in uptake of probe into cells. Figure 5.4.2D (treatment with commercially available TMRM to assess mitochondrial membrane potential) shows that A-T diseased cells (higher fluorescence response) have higher mitochondrial membrane potential compared to normal cells (lower fluorescence response). The higher membrane potential should therefore lead to an increased uptake of ME-TER and MT-ME-TER into the mitochondria of the A-T diseased cells relative to normal cells. The increased uptake therefore should lead to a higher ME-TER fluorescence response within A-T diseased cells relative to normal cells. Such response was prominent in the case of MT-ME-TER treatment (Figure 5.4.2C) but missing from ME-TER treatment (Figure
5.4.2A). Instead of a higher fluorescence response, ME-TER treated A-T diseased cells displayed a lower fluorescence response relative to ME-TER treated normal cells (Figure 5.4.2A). Such reduction hence confirmed that the difference in ME-TER fluorescence intensity was due to difference in redox status between both normal and A-T diseased cells, where A-T diseased cells are under oxidative stress.

Results obtained coincide with previously reported findings from other research groups. Studies carried out by Reichenbach et al. reported of an increase in lipid H$_2$O$_2$ content in 33 A-T patients compared to normal patients. In addition to that, they have also analysed the content of 8-oxodeoxyguanosine (8-OHdG) formed from the addition of OH• to the C-8 position of guanine. A-T patients were found having increased 8-OHdG levels, indicating an increase in OH• radicals within A-T diseased patients.151

A more recent experiment carried out by Sharma et al. also coincided with the findings shown on Figure 5.4.2. They treated normal and A-T fibroblast cells with H$_2$O$_2$ and subsequently measured the oxidative stress level via the use of MitoSox. A-T cells were seen to exhibit a higher fluorescence compared to normal cells.153

Results hence confirmed the capability of ME-TER to assess redox status within immortalized, normal as well as A-T diseased human fibroblast cells. However, an effective redox probe should have the potential to be used over a wide range of cell types. As all the studies carried out thus far were based mainly on human fibroblast cells, the capability of ME-TER to assess redox status within other cell lines was subsequently assessed.

**5.5 Versatility of ME-TER in other cell lines**

The versatility of a redox probe to monitor the redox status of a variety of cell lines is desirable. This enables the redox probe to be used to compare the redox status within different cell lines, which in turn provides a better understanding of the biochemical response of oxidative damage over a variety of cell lines.

Studies on the redox sensing capability of ME-TER carried out thus far were based mainly on human fibroblast cells. In an attempt to assess the versatility of ME-TER
within other cell lines, four different cell lines ranging from normal to diseased states were incubated with ME-TER. The mitochondrial localization of ME-TER within the different cell lines was assessed. The cell lines used were:

i) Healthy normal cell- immortalized retinal cells (mouse origin), cerebellum cell (DAOY, human origin)

ii) Diseased cells- cervical cancer cells (HeLa, human origin), adenocarcinomic alveolar basal epithelial cells (A549, human origin)

Experimental

The above mentioned cells were cultured overnight in IBIDI slides in DMEM media [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v) and phenol red] at an initial concentration of 1.5x10^5 cells per mL. The cells were cultured in 2 mL of DMEM, supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin antibiotics (final concentration 1% v/v) and phenol red within each well.

The cells were subsequently incubated with 10 nM of ME-TER and MT-ME-TER by replacing previous medium within each well with 2 mL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)]. The cells were incubated for 45 minutes at 37°C under an atmosphere of 5% CO2 in air and care was taken to avoid any detachment of cells from the slide.

Upon incubation, the cells were washed once with 2 mL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)] and were subsequently co-stained with 100 nM of MitoTracker Green in 2 mL of phenol red free medium [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)]. The cells were incubated for 30 minutes at 37°C under an atmosphere of 5% CO2 in air.

The cells were subsequently washed once with 2 mL of phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)] and a new batch of 2 mL phenol
red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)] was added to each well prior imaging.

The localization of ME-TER and MT-ME-TER as well as MitoTracker Green was imaged under an Applied Precision Deltavision microscope. An excitation at wavelength 515-569 nm and emission detection at wavelength 549-639 nm was applied in the case of MT-TER and MT-ME-TER, while in the case of MitoTracker Green, an excitation at wavelength 447-503 nm and emission detection at wavelength 487-559 nm was applied. Imaging conditions were maintained at 37°C under an atmosphere of 5% CO₂ in air in phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin (final concentration 1% v/v)].

Results and discussion

![Image of Retinal Cells, Nitrooxide, MitoTracker Green, Overlay]

Pearson Coefficient of Correlation: 0.8814

[Graph showing correlation between two variables]
B  **Cerebellum cells (DAOY)**

<table>
<thead>
<tr>
<th>Nitroxide</th>
<th>MitoTracker Green</th>
<th>Overlay</th>
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<tbody>
<tr>
<td>ME-TER</td>
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<tr>
<td>MT-ME-TER</td>
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C  **Cervical cancer Cells (HeLa)**

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<th>MitoTracker Green</th>
<th>Overlay</th>
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<tbody>
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<tr>
<td>MT-ME-TER</td>
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</tbody>
</table>
D. Adenocarcinomic alveolar basal epithelial cells (A549)

<table>
<thead>
<tr>
<th>Nitrooxide</th>
<th>MitoTracker Green</th>
<th>Overlay</th>
</tr>
</thead>
</table>

ME-TER

MT-ME-TER
Figure 5.5.1: Localization of 10 nM methyl ester-tetraethylrhodamine nitroxide (ME-TER) and its non-radical alkylated derivative, methyl trap methyl ester tetraethylrhodamine (MT-ME-TER) along with 100 nM MitoTracker Green in A) immortalized retinal cells; B) DAOY, cerebellum cell; C) HeLa, cervical cancer cell; D) A549, adenocarcinomic alveolar basal epithelial cells. Pearson Coefficient of Correlation plots between nitroxide and MitoTracker Green were shown. (Triplicate life cell images were taken, only one image is being displayed).

Figure 5.5.1 showed that ME-TER and MT-ME-TER localizes into the mitochondria of a range of different cell lines, both normal and diseased states as well as from different origins (human and animal). This extends the application of ME-TER as redox probe to a wider range of cell types and cell lines, not limiting only towards human fibroblast cells as previous described.

Due to the fast growth rate of the immortalized mouse retinal cells, along with an attempt to assess the capability of ME-TER to monitor the redox status within animal cell model, the previously described ME-TER redox monitoring flow cytometry experiment (Figure 5.2.1) was repeated by incubating immortalized mouse retinal cells with ME-TER.
Figure 5.5.2: Percentage reduction in fluorescence intensity of treated immortalized mouse retinal cells, $F_s$ [cells incubated with probes for 45 minutes, followed by treatment with varying concentrations of rotenone (ROT) or antimycin (AMC)] for 15 minutes relative to blank, $F_c$ [cells incubated with probes for 45 minutes, followed by treatment with DMSO for 15 minutes]. A) Cells treated with 10 nM ME-TER. B) Cells treated with 10 nM MT-ME-TER. C) Cells treated with 1 µM MitoSox. [Each data point are mean values. Data were analysed using Kruskal Wallis test with pos hoc testing using the Dunn’s multiple comparison test where $P < 0.05$ means results are significantly different to blank. Results in both Figures A and C showed a $P$ value < 0.05 (significantly different to blank), while Figure B showed a $P$ value > 0.05 (non-significantly different to blank)].

Figure 5.5.2A shows that ME-TER responded to ROT / AMC-stimulated ROS production in a dose respond manner over a ROT and AMC concentration range of 0.01 to 100 µM. Similar trend was observed in the case of MitoSox treatment (Figure 5.5.2C). As aforementioned, MitoSox reacts with ROS giving rise to hydroxyl triphenylphosphonium ethidium. Hydroxyl triphenylphosphonium ethidium fluoresce with an emission wavelength of 580 nm. An increase in MitoSox fluorescence was therefore used as a control to detect possible increase in ROS concentration. Under the ROT and AMC treatments described in Figure 5.5.2, increasing the concentration of ROT and AMC increases fluorescence of MitoSox. The increasing
MitoSox fluorescence hence indicates an increase in ROS production under those ROT and AMC treatment conditions.

The drop in ME-TER fluorescence intensity (Figure 5.5.2A) was due to the changes in ROS production with increasing ROT and AMC concentrations. ME-TER nitroxides are reduced to hydroxylamine upon uptake into the mitochondria of the retinal cells. The reduction of ME-TER occur as a result of the reducing environment within the mitochondria (Figure 4.3.3).\textsuperscript{163} The reduction give rise to an initial increase in ME-TER fluorescence intensity within the mitochondria. Subsequent dose response treatment with ROT or AMC, increases ROS production within the mitochondria.\textsuperscript{105,162} The increasing ROS oxidises the hydroxylamines to nitroxides, resulting in a drop in ME-TER fluorescence intensity (Figure 5.5.2A).

Interestingly, no significant decrease in fluorescence intensity of was observed between ROT treatment concentration of 10 µM and 100 µM. This deviates from reported evidence for rotenone-stimulated O$_2$•$^-$ production in human fibroblast cells.\textsuperscript{169} Although the reason for such an observation remains unknown, it has been proposed that retinal cells have in place a mechanism which decreases O$_2$•$^-$ production within Complex I. Such mechanism serve to prevent aberrant signalling as O$_2$•$^-$ serves as an intracellular messenger to trigger the initiation of apoptosis or cell death.\textsuperscript{170}

Based on analysis from Figures 5.5.1 and 5.5.2, it can be concluded that ME-TER has the capability to localize into the mitochondria and assess the redox status within a variety of cell types and cell lines. The versatility of ME-TER possibly enables it to be used as a universal redox sensing probe.

In an attempt to further explore the capability of ME-TER, further studies were carried to assess the potential use of ME-TER as a potential probe to monitor the efficacy of antioxidant therapy in reducing oxidative stress.

### 5.6 The use of ME-TER as potential probe to monitor the efficacy of lutein as antioxidant therapy in reducing oxidative stress

Lutein (Figure 4.6.1) has been reported to be an effective antioxidant in reducing oxidative damage. Studies have suggested the ability of lutein to function as a reactive
oxygen species scavenger in the reducting free radicals in eye diseases such as age-related macular degeneration (AMD) and cataract.\textsuperscript{171} Placebo-controlled intervention trials have found that ingestion of lutein containing food or supplements increased macular pigment optical density (which indirectly measures lutein concentration) which possibly helps improve visual function.\textsuperscript{172,173} Lutein consumption was found to be inversely related to eye diseases such as AMD and cataract.\textsuperscript{171}

\begin{center}
\begin{figure}
\includegraphics[width=\textwidth]{lutein.png}
\caption{Chemical structure of lutein.\textsuperscript{171}}
\end{figure}
\end{center}

In assessing the potential use of ME-TER as probe to monitor the efficacy of lutein as antioxidant therapy in reducing oxidative stress, hTERT human fibroblast cells treated with ME-TER were subjected to AMC as well as lutein treatment. Changes in ME-TER fluorescence intensity was subsequently monitored via spinning disk microscopy. Details of experiment are described below.

**Experimental**

4x10\(^5\) cells per mL of hTERT immortalized human fibroblast cells were cultured in IBIDI slides overnight in DMEM media [supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)]. The cells were subsequently incubated with 300 µL of 25 nM ME-TER (prepared from a 10 mM DMSO stock, in 2 mL of phenol red free DMEM media supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v) for 45 minutes. The media was then replaced with a fresh batch of media (300 µL) that does not contain ME-TER [but supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v)]. The ME-TER treated cells were subsequently imaged and the change in fluorescence intensity was imaged every minute for 3 minutes.
During imaging, the cells were then subjected to different treatments as follow. Different compounds were added to the cells within IBIDI slides between imaging intervals.

a) 45 minutes incubation with 25 nM of ME-TER (as described above), followed by a 20 µM AMC treatment (prepared from a 100 mM DMSO stock) in a final volume of 300 µL. In addition to ME-TER, cells were also incubated with 100 nM MitoTracker Green (prepared from a 100 µM DMSO stock, in 2 mL of phenol red free DMEM media supplemented with fetal calf serum (final concentration 12% v/v) and penicillin streptomycin antibiotics (final concentration 1% v/v) for 30 minutes.

b) 45 minutes incubation with 25 nM of ME-TER (as described above), followed by a 2 mM lutein treatment (prepared from a 0.6 M DMSO stock) in a final volume of 300 µL.

c) 45 minutes incubation with 25 nM of ME-TER (as described above), followed by a 20 µM AMC treatment (prepared from a 100 mM DMSO stock) and 2 mM lutein treatment (prepared from a 0.6 M DMSO stock) in a final volume of 300 µL.

d) A blank solution was prepared where upon 45 minutes incubation with 25 nM of ME-TER (as described above), cells were incubated with DMSO in place of AMC and lutein, in a final volume of 300 µL.

Fluorescence intensity of ME-TER treated cells subjected to the different treatment were imaged and compared accordingly. An excitation at wavelength 515-569 nm and emission detection at wavelength 549-639 nm was applied in the case of ME-TER, while in the case of MitoTracker Green, an excitation at wavelength 447-503 nm and emission detection at wavelength 487-559 nm was applied. Imaging conditions were maintained at 37°C under an atmosphere of 5% CO₂ in air in phenol red free DMEM [supplemented with fetal calf serum (final concentration 12% v/v), penicillin streptomycin (final concentration 1% v/v)].
Results and discussion

A

ME-TER + DMSO

ME-TER + AMC

ME-TER + Lutein

ME-TER + AMC + Lutein
Based on Figure 5.6.2, it can be observed that despite the treatment, all ME-TER cells shows similar response. ME-TER incubated cells have increased fluorescence at time point 0 hour. However, upon 6 hours incubation, cells show a reduction in fluorescence intensity. Subsequent incubation up to 13 hours shows an increase in fluorescence intensity. Both AMC and lutein treatment does not affect the cell response.

A possible deduction could be due to the detachment of cells from the culture slide upon treatment during imaging intervals. The detachment of cells from the culture slide results in a lost of focus within the spinning disk microscope. Further incubation period, helps the cells to acclimatize and reattach themselves to the culture dish. This in turn, puts the cells back into focal plane, restoring the focus within the spinning disk and hence increasing fluorescence intensity. A similar observation was prominent in the case of overlay image between ME-TER and MitoTracker Green treated with DMSO (Figure 4.6.2B), confirming such deduction.

As a solution to the above experimental setup, the use of animal model was looked into as an alternative. In collaboration with the Queensland Eye Institute (QEI), the potential use of ME-TER to monitor in vivo oxidative status within an acute ischaemia / reperfusion injury rat model along with antioxidant treatment was assessed by Dr.
Nigel Barnett and Miss Cassie Rayner. This study was published and the following provides a brief discussion with reference to the results and findings reported.

5.7 Assessing redox status within animal model using ME-TER as redox probe

The rat retinal ischemia / repurifusion (I/R) model has been reported to be an established animal model for monitoring retinal responses upon the induction of an ischaemic insult. The I/R model mimics clinical situations such as glaucoma (increase pressure in eye) and retinal vascular occlusion disease.25,174

To investigate the potential application of ME-TER as a redox probe for studying the redox condition within the glaucoma model, Barnett and Rayner administered ME-TER through injection into the vitreous of rat eyes. The rat was subsequently subjected to ischaemia / reperfusion and changes in ME-TER fluorescence intensity within the rat eyes were imaged.
Figure 5.7.1: The change in methyl ester tetaethylrhodamine nitroxide (ME-TER, 2 µM) fluorescence intensity in the retina during reperfusion following an acute ischaemic insult in Sprague-Dawley rats. A) Fluorescent fundus images. B) Quantification of ME-TER fluorescence: Comparing fluorescence intensity between control and ischaemic reperfusion (I/R) treatment. (The data was fitted with a linear model and subsequently ANOVA statistical test was carried out. Relative to control, P values < 0.05 were obtained. Results are therefore significantly different to control). (Figure was taken from Nigel with permission).

The obstruction followed by reflow of blood is known as ischaemia / reperfusion. It is known to result in a burst of ROS generation. Both Figures 5.7.1A and B show a drop in fluorescence intensity of ME-TER upon reperfusion following an acute ischaemic insult. In addition, the intensity reduces with increase reperfusion period (Figure 5.7.1B). The observed results coincide with that observed in cell-based experiments.

Further studies by Rayner et al. (2014) investigated the possible toxic effect of ME-TER on the retina. Immunohistochemistry analysis revealed that ME-TER treated
retina has similar morphology to that of the non-treated control retina. This confirmed that ME-TER does not result in or has minimal toxic effect in animal retina models. Such observation agrees with previous ME-TER imaging and MTT toxicity results (Figure 5.7.1).

Subsequently studies by Rayner et al. (2014)$^{175}$ also shows that the ROS-induced change in ME-TER probe was ameliorated with lutein treatment. It was observed that with upon inducing reperfusion, the fluorescent intensity declined during the first 60 minutes. Such decline in fluorescence intensity however, was significantly less in lutein treated retina. No decline in fluorescence response was observed in the case of control (non-ischaemic) and lutein (non-ischaemic) treated retinas.

The capability of ME-TER to detect the changes in ME-TER fluorescence intensity within lutein treated ischaemic retina, suggest the potential of ME-TER to serve as a tool for testing antioxidant therapies in reducing retinal oxidative damage.

It can therefore be concluded that ME-TER serve as a verstatile redox probe. The application of ME-TER ranges from cell based model of varying cell lines and cell conditions (normal or diseased) to animal based model.

In an attempt to further explore the capability of ME-TER, further studies were subsequently carried to assess the potential use of ME-TER as a potential probe to monitor the efficacy of antioxidant therapy in reducing oxidative stress.
CONCLUSION & FUTURE WORK
CHAPTER 6
Conclusion & Future Work

Studies within this project focus on assessing the capabilities of fluorescein and rhodamine-based profluorescent nitroxides (PFN) to monitor mitochondrial redox state. In achieving this, several factors play an important role, namely the reduction rate of the PFN, the influence of changing pH conditions on the fluorescence stability of the PFN, the intracellular localization of the PFN as well as the applicability of the PFN within biological systems.

Findings from comparing the reduction rate between tetraethylfluorescein (TEF) and tetramethylfluorescein (TMF) shows that ethyl-based probe (TEF) provides a better representation of biological redox status within intracellular organelle relative to methyl-based probe (TMF). This is due to the slower reduction rate of TEF compared to TMF. Such slower reduction rate prevents the probe from undergoing reaction prior reaching the mitochondria. The assessment of redox status provided is therefore, a better reflection of the oxidative condition within the mitochondria.

A possible reason for the decreased reduction rate of TEF compared to its methyl counterpart, TMF, is thought to be due to the increased steric bulk around the nitroxide radical centre within TEF. The increased steric bulk is contributed by the ethyl groups around the radical centre within TEF. Such an increase in steric bulk slows down the rate at which the radical centre within TEF is being reacted upon, hence decreasing the reduction rate. In addition, the ethyl groups within TEF also contribute to an increase in the electron donating effect around the nitroxide centre of TEF. Such increase in the electron donating effect, increases the shielding around the radical centre within TEF. This subsequently slows down the rate of reaction around the radical centre within TEF. Both the increase in steric bulk and the shielding effect around the nitroxide centre within TEF contribute to a decrease in the reduction rate of the PFN.

Subsequent studies concluded that fluorescein-based probes such as the tetramethyl- (TMF) and tetraethyl- (TEF) system suffer from pH instability. Such pH instability
arises due to the nature of the chromophore fluorescein, which exhibits drastic changes in fluorescence intensity with slight changes in pH.

In addition, fluorescein-based PFN shows lysosomal localization instead of mitochondrial localization. These factors limit the potential use of TMF and TEF as redox probes for assessing redox status within the mitochondria.

Follow up studies were hence carried out to incorporate the chromophore rhodamine within the PFN. Rhodamine has been used as an effective probe for staining mitochondria since the 1980’s. Coupling a tetraethylisoiindoline nitroxide with rhodamine has successfully gave rise to an effective redox probe, tetraethylrhodamine nitroxide (TER) for assessing the redox status within the mitochondria. Apart from showing relatively good mitochondrial localization (73% colocalization with MitoTracker Green), TER showed increased pH stability relative to TEF around biologically relevant pH.

Further studies reveal TER exhibits a reversible sensing nature and via flow cytometry along with microscopy imaging, the capability of TER to assess redox status within ROT / AMC oxidative stressed hTERT immortalized human fibroblast cells was confirmed. The use of flow cytometry along with TER presents a sensitive tool for assessing mitochondrial redox status, where an increase in oxidative stress results in a reduction in the fluorescence intensity of TER treated cells. Via MTT assay, it was concluded that TER does not affect the normal functioning of treated cells.

Despite the proven functionality of TER, the lack of complete localization of TER into the mitochondria remains an issue. Subsequent effort was hence carried out to increase the overall positive charge of TER. Esterification of TER was carried out giving rise to a PFN probe, methyl ester tetraethylrhodamine (ME-TER) with increased overall postive charge.

ME-TER showed increased mitochondrial localization (approximately 90% colocalization with MitoTracker Green). Subsequent imaging and flow cytometry experiments confirmed the redox sensing capacity of ME-TER within a wide range of cell lines ranging from human to animal origin as well as normal and diseased cells.
ME-TER has the capacity to differentiate the redox condition within normal and Ataxia Telangiectasia (A-T) diseased cells.

Further studies reveal that ME-TER does not affect normal functioning of cells up to 72 hours incubation. Subsequent spinning disk microscopy techniques reveal that cells undergo normal mitosis and growth in the presence of ME-TER.

The application of ME-TER within rat retinal ischaemia / repurfusion (I/R) model concluded that ME-TER serves as a versatile redox probe. The application of ME-TER ranges from cell based model of varying cell lines and cell conditions (normal or diseased) to animal based model.

Comparing different mitochondrial redox sensing probes, TER and ME-TER has several advantages as follow.

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<tr>
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In conclusion, this study is a proof of concept that ethyl based rhodamine nitoxide system has the capacity to act as an effective mitochondrial redox sensing probe.

Although ME-TER shows great potential as a mitochondrial redox sensing probe, much still remains to be discovered. Among the future works that could be carried out are:

1) Further understanding of the mechanism by which ME-TER assesses redox status within the mitochondria. This can be achieved via the use of different inhibitors
apart from rotenone and antimycin such as buthionine sulfoximine (BSO) which alters intracellular antioxidant and glutathione level.

2) Administration and assessment of the efficacy of potential antioxidant drug treatments apart from lutein, based on the use of ME-TER within both cell-based and animal-based models could also be looked into.

3) The application of ME-TER in other animal-based models apart from the glaucoma rat acute ischaemic / reperfusion (I/R) model also serve as an important further work to contribute to the development of an effective mitochondrial redox probe.

4) The development of different variation of ME-TER could also be explored. An example being the attachment of another non-reactive chromophore into the existing probe. This has the potential of enabling a ratio metric analysis of the intracellular redox status to be carried out.

5) Further experiments to assess the applicability of TER within pure or mitochondrial enriched cell fraction could also be looked into.

6) In addition, colocalization of cells treated with the PFN probes with other mitochondrial markers apart from Mitotracker Green could be carried out to serve as further confirmation of the localization of the probes within mitochondria.

7) MTT studies could also be complemented with other assays such as assessing cellular ATP (adenosine triphosphate) level within cells for a better assessment of mitochondrial redox status.
REFERENCES


REFERENCES


