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Hydrogen Peroxide Sensing for Reproductive Health

Hydrogen Peroxide Sensing for Reproductive Health

by Malcolm Stuart Purdey

THE UNIVERSITY of ADELAIDE

A Thesis submitted for the degree of

Doctor of Philosophy

in the

Faculty of Science

School of Physical Sciences

December 2015

For Ainsley: my patient, beautiful, longsuffering wife.

If we knew what it was we were doing, it would not be called research, would it?

Albert Einstein

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PUBLICATIONS

Journal Papers

These papers comprise full chapters in this thesis:

Purdey, M. S.; Connaughton, H. S.; Whiting, S.; Schartner, E. P.; Monro, T. M.; Thompson, J. G.; Aitken, R. J.; Abell, A. D., Boronate probes for the detection of hydrogen peroxide release from human spermatozoa. *Free Radical Biology and Medicine* **2015,** *81* (0), 69-76.

Purdey, M. S.; Thompson, J. G.; Monro, T. M.; Abell, A. D.; Schartner, E. P., A dual sensor for pH and hydrogen peroxide using polymer-coated optical fibre tips. *Sensors* **2015**, *15* (12), 31904-31913.

Purdey, M. S.; Abell, A. D., New BODIPY-based probes for the detection of hydrogen peroxide. **2015**, *In Preparation.*

These publications represent other work by the author described in this thesis:

Sutton-McDowall, M. L.; Purdey, M.; Brown, H. M.; Abell, A. D.; Mottershead, D. G.; Cetica, P. D.; Dalvit, G. C.; Goldys, E. M.; Gilchrist, R. B.; Gardner, D. K.; Thompson, J. G., Redox and anti-oxidant state within cattle oocytes following in vitro maturation with bone morphogenetic protein 15 and follicle stimulating hormone. *Molecular Reproduction and Development* **2015,** *82* (4), 281-294.

Sutton-McDowall, M. L.; Wu, L.; Purdey, M.; Brown, H. M.; Abell, A. D.; Goldys, E. M.; MacMillan, K. L.; Robker, R. L.; Thompson, J. G., Non-Esterified Fatty Acid-Induced Endoplasmic Reticulum Stress in Cattle Cumulus Oocyte Complexes Alters Cell Metabolism and Developmental Competence. *Biology of Reproduction* **2015**, *Available online,* doi:10.1095/biolreprod.115.131862

Zuber, A.; Purdey, M.; Schartner, E.; Forbes, C.; van der Hoek, B.; Giles, D.; Abell, A.; Ebendorff-Heidepriem, H., Detection of gold nanoparticles with different sizes using absorption and fluorescence based method. *Sensors and Actuators B: Chemical* **2016**, *227*, 117-127.

Schartner, E. P.; Henderson, M. R.; Purdey, M. S., Dhatrak, D.; Monro, T. M.; Gill, P. G.; Callen, D. F., Tumour detection in human tissue samples using a fibre tip pH probe. **2016**, *In preparation*.

Patent

Australian patent application 2015902890 – "*Detection of Gold Nanoparticles*" (Deep Exploration Technologies CRC Limited) – 21 July **2015**

Conference presentations

Conference paper:

Purdey, M. S.; Schartner, E. P.; Sutton-McDowall, M. L.; Ritter, L. J.; Thompson, J. G.; Monro, T. M.; Abell, A. D., Localised hydrogen peroxide sensing for reproductive health. *Proceedings of SPIE* **2015,** *9506*, 950614.

Invited talks:

Purdey, M. S.; Schartner, E. P.; Heng, S.; Zhang, X. Z.; Stubing, D. B.; Monro, T. M.; Abell, A. D.; Functionalisation of Optical Fibres for Biosensing Applications. *ANFF Research Showcase,* Canberra, ACT, Australia, November **2014**.

Purdey, M. S.; Schartner, E. P.; Sutton-McDowall, M. L.; Ritter, L. J.; Monro, T. M.; Thompson, J. G.; Abell, A. D.; A Non-invasive Sensor for Hydrogen Peroxide and pH. *Society for Reproductive Biology,* Adelaide, SA, Australia, August **2015**.

Conference posters:

Purdey, M. S.; Connaughton, H. S.; Whiting, S.; Thompson, J. G.; Aitken, R. J.; Abell, A. D.; Reactive Oxygen Species Detection in Human Spermatozoa with Aryl Boronates. *RACI National Congress,* Adelaide, SA, Australia, December **2014**.

Purdey, M. S.; Schartner, E. P.; Monro, T. M.; Aitken, R. J.; Thompson, J. G.; Abell, A. D.; Hydrogen Peroxide Sensing for Reproductive Health. *249th ACS Nation Congress,* Denver, CO, USA, March **2015**.

ABBREVIATIONS

4HNE	4-Hydroxynonenal
AA	Arachidonic Acid
ACN	Acetonitrile

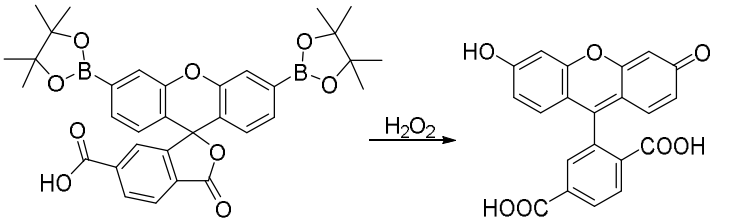
APTES	3-(Aminopropyl)triethoxysilane
BODIPY	Boron Dipyrromethene
$CDCl_3$	Deuterated chloroform
$CHCl_3$	Chloroform
CPF1	Carboxy Peroxyfluor-1
CPF1-NHS	N-Succinimide ester of Carboxy Peroxyfluor-1
DCFH	2',7'-Dichlorohydrofluorescein diacetate
DCM	Dichloromethane
DHE	Dihydroethidium
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DPI	Diphenyl Iodonium
EEPF1	2-Ethoxy(2-Ethoxyethoxy) Peroxyfluor-1
ETC	Electron Transport Chain
FCR2	Flavin Coumarin Redox sensor 2
FRET	Förster Resonance Energy Transfer
H_2O_2	Hydrogen Peroxide
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HNO	Nitroxyl
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
IVF	*In Vitro* Fertilisation
MeOH	Methanol
MitoPY1	Mitochondrial PeroxyYellow-1

MRI	Magnetic Resonance Imaging
MSR	MitoSOX Red
$NaCNBH_3$	Sodium Cyanoborohydride
NbzB	NitrobenzoylBODIPY
NbzF	NitrobenzoylFluorescein
NCR3	NicotinamideCoumarin Redox sensor 3
NEFA	Non-Esterified Fatty Acid
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
NPF1	Naphthoperoxyfluor-1
NpFR1	Naphthalimide Flavin Redox sensor 1
$O_2^{\bullet-}$	Superoxide
^{-}OCl	Hypochlorite
$^{\bullet}OH$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
PB1	PeroxyBODIPY-1
PBS	Phosphate Buffer Solution
PEG	Poly Ethylene Glycol
PET	Photoinduced Electron Transfer
PF1	Peroxyfluor-1
ROS	Reactive Oxygen Species
SE	Standard Error of the mean
SNARF2	Seminaphthorhodofluor-2
TBHP	Tert-Butyl Hydroperoxide

GLOSSARY OF FLUORESCENT PROBES

Carboxyperoxyfluor-1 (**CPF1**)

See **Chapters 2-4**, **6** and **7**

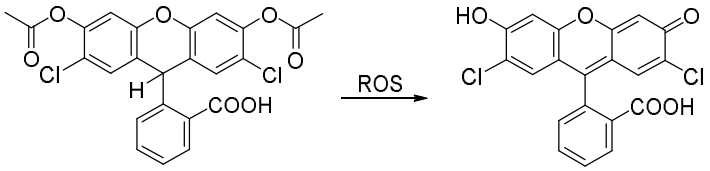


CPF1
Non-Fluorescent

Carboxyfluorescein
Exc. 485nm; Em. 525nm

2',7'-Dichlorohydrofluorescein Diacetate (**DCFH**)

See **Chapters 1** and **2**

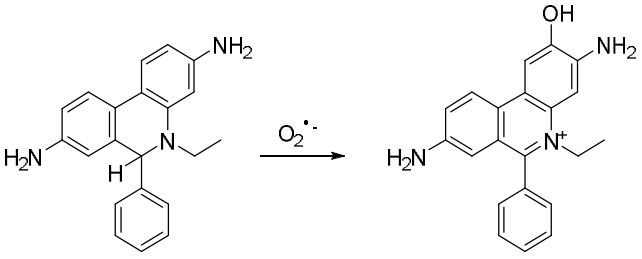


DCFH
Non-Fluorescent
(Acetates removed by intracellular esterases)

Dichlorofluorescein
Exc. 485nm; Em. 525nm

Dihydroethidium (**DHE**)

See **Chapters 1** and **2**

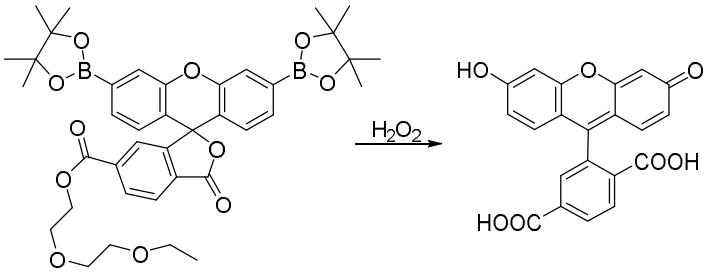


DHE
Non-Fluorescent

2-hydroxyethidium
Exc, 500 nm; Em. 580 nm

2(2-Ethoxyethoxy)ethoxy Peroxyfluor-1 (**EEPF1**)

See **Chapters 2**, **6** and **7**

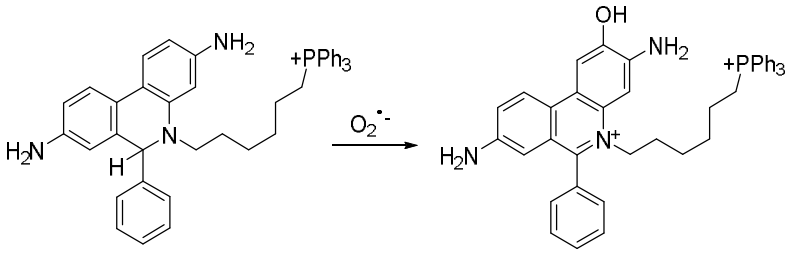


EEPF1
Non-Fluorescent
(PEG likely removed by intracellular esterases)

Carboxyfluorescein
Exc. 485nm; Em. 525nm

MitoSOX red (**MSR**)

See **Chapters 1** and **2**

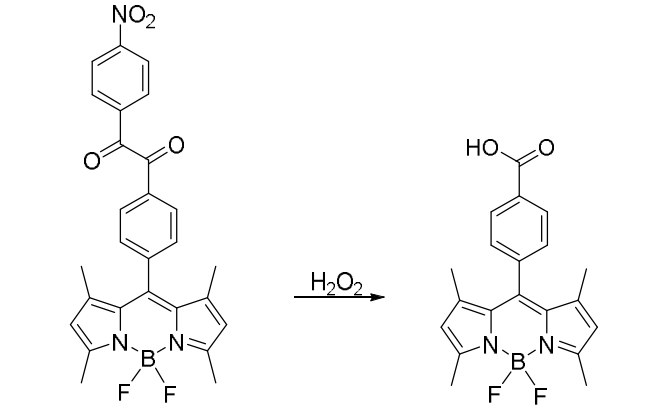


MSR
Non-Fluorescent

2-hydroxy Mitoethidium
Exc, 500 nm; Em. 580 nm

NitrobenzoylBODIPY (**NbzB**)

See **Chapters 5-7**

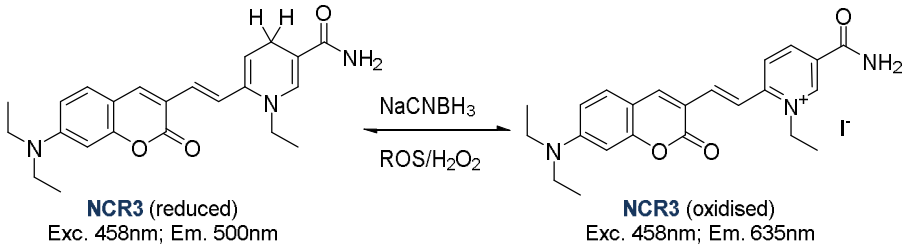


NbzB
Weakly fluorescent
Exc. 490nm; Em. 510nm

20
Exc. 490nm; Em. 510nm

Nicotinamide Coumarin Redox Sensor 3 (**NCR3**)

See **Chapters 6** and **7**

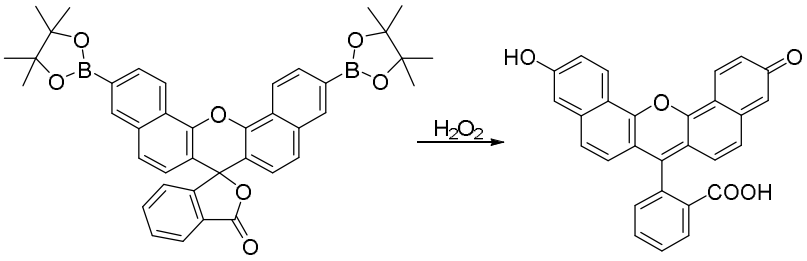


NCR3 (reduced)
Exc. 458nm; Em. 500nm

NCR3 (oxidised)
Exc. 458nm; Em. 635nm

Naphthoperoxyfluor-1 (**NPF1**)

See **Chapters 6** and **7**

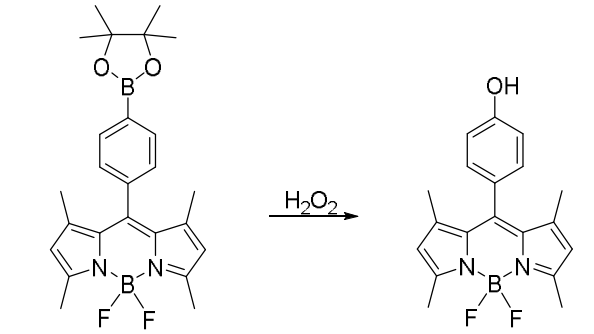


NPF1
Non-Fluorescent

Naphthofluorescein
Exc. 598nm; Em. 650nm

PeroxyBODIPY-1 (**PB1**)

See **Chapters 5-7**

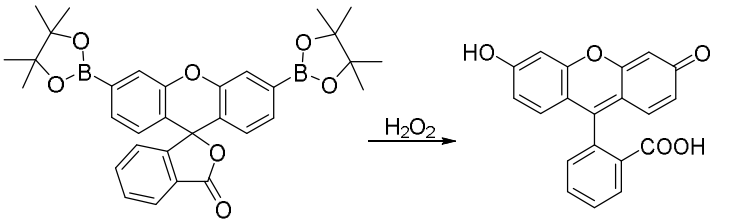


PB1
Exc. 490nm; Em. 510nm

19
Weakly Fluorescent

Peroxyfluor-1 (**PF1**)

See **Chapters 1-7**

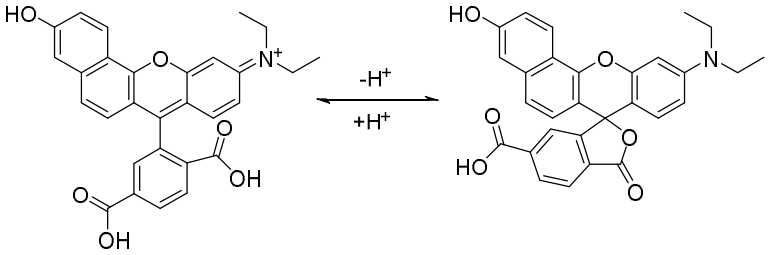


PF1
Non-Fluorescent

Fluorescein
Exc. 490nm; Em. 520nm

Seminaphtharhodafluor-2 (**SNARF2**)

See **Chapters 4** and **7**



SNARF2 (Low pH)
Exc. 530nm; Em. 600 nm

SNARF2 (High pH)
Exc. 530nm; Em. 650 nm

ABSTRACT

The research presented in this thesis details the synthesis, surface functionalisation and photochemical studies of fluorescent probes for the detection of hydrogen peroxide (H_2O_2) in reproductive health.

Chapter 1

H_2O_2 is an important reactive oxygen species (ROS) that is detrimental to the health of spermatozoa and embryos. Fluorescent probes are commonly used for the detection of ROS and here examples with different mechanisms of detection are examined; such as turn-on probes, turn-off probes, Förster resonance energy transfer (FRET)-based and photoinduced electron transfer (PET)-based probes. Specific reference is given to the aryl boronate and benzil classes of probe, which show good selectivity for H_2O_2 over other ROS. The attachment of the fluorescent probe to an optical fibre as a non-invasive sensing platform is discussed. This then allows sensing in a sensitive biological environment, such as an embryo, without exposure to the probe in solution. Fibre tip sensors and microstructured optical fibre-based sensors are discussed for use in such biological environments. Finally, a summary is provided detailing the objectives of this thesis and the chapters in which these are addressed.

Chapter 2

Three aryl boronate probes [peroxyfluor-1 (**PF1**), carboxy peroxyfluor-1 (**CPF1**) and a novel probe 2(2-ethoxyethoxy)ethoxy peroxyfluor-1 (**EEPF1**)] were synthesised for use in the detection of H_2O_2 in human spermatozoa. The activity and selectivity of these probes was then compared to three commonly used commercial probes, 2',7'-dichlorohydrofluorescein diacetate (**DCFH**), dihydroethidium (**DHE**) and MitoSOX red (**MSR**). **PF1** and **EEPF1** were found to be effective at detecting H_2O_2 and peroxynitrite ($ONOO^-$) produced by spermatozoa when stimulated with menadione or 4-hydroxynonenal. Flow cytometry was used to demonstrate that **EEPF1** is more effective at detecting ROS in spermatozoa compared to **DCFH**, **DHE** and **MSR**. Furthermore, **EEPF1** distinguished poorly motile sperm from motile sperm as revealed by an enhanced production of ROS.

Chapter 3

A fibre-tip based probe constructed by encapsulating **CPF1-NHS** in a polyacrylamide matrix is reported for the detection of H_2O_2. This non-invasive platform avoids the need to introduce an organic fluorophore into a sensitive cell such as an embryo as discussed above. A number of derivatives of **PF1** were investigated, with carboxylated fluorophore **CPF1** proving to be the easiest to synthesise and characterise. **CPF1** was functionalised to glass slides

using layer-by-layer deposition of polyelectrolytes. This functionalised surface showed a fluorescent response to H_2O_2 comparable to solution-based measurements. Three surface functionalisation methods were then investigated for attachment to an optical fibre tip, specifically polyelectrolyte deposition, silane monolayer formation, and light-catalysed polymerisation of acrylamide. The most effective method of functionalisation was found to be light-catalysed formation of a polyacrylamide matrix with the **CPF1** embedded. These polyacrylamide fibre tip probes were then guided into microdroplets of bovine *in vitro* fertilisation (IVF) media using a micromanipulator. This was visualised under an optical microscope to detect the controlled release of H_2O_2. This fibre probe is thus compatible with imaging techniques used in IVF research laboratories.

Chapter 4

This chapter presents the development of a single optical fibre tip probe capable of detecting both the concentration of H_2O_2 and the pH of the associated solution. The sensor was constructed by embedding two fluorophores [**CPF1** and seminaphtharhodafluor-2 (**SNARF2**) for H_2O_2 and pH detection respectively] on the tip of an optical fibre using the previous developed polyacrylamide matrix methodology. The functionalised fibre probes reproducibly sensed pH with a resolution of 0.1 pH units. The probe also accurately detected H_2O_2 over a biologically significant concentration range, of 50-100 μM. This study revealed the importance of simultaneous detection of H_2O_2 and pH, where changes in pH were shown to affect the fluorescent response of **CPF1**. This new fibre probe offers potential for non-invasive detection of pH and H_2O_2 in biological environments using a single optical fibre.

Chapter 5

Two new cell-permeable boron-dipyrromethene (**BODIPY**) based fluorescent probes for the detection of H_2O_2 were designed and synthesised. The aryl boronate peroxyBODIPY-1 (**PB1**) gave rise to a decrease in fluorescence on reaction with H_2O_2, while the fluorescence of the benzil-based nitrobenzoylBODIPY (**NbzB**) probe increased on reaction with H_2O_2. The benzil probe **NbzB** exhibited a high degree of selectivity for H_2O_2 over other ROS. The aryl boronate **PB1** showed a greater change in fluorescence on reaction with H_2O_2 compared to **NbzB**, and **PB1** also detected H_2O_2 in bovine oocytes under oxidative stress. These results suggest that aryl boronates (i.e. **PB1**) and benzils (i.e. **NbzB**) have use in biological environments requiring higher sensitivity or selectivity to H_2O_2.

Chapter 6

The research discussed here extends the solution-based and fibre tip experiments to the detection of H_2O_2 in biological environments. Detection of H_2O_2 within cells is often frustrated

by autofluorescence in the green emission region. Contrastingly, the red emission region in biological systems shows a lower autofluorescence background signal. Therefore a red-emitting fluorescent probe for H_2O_2, naphthoperoxyfluor-1 (**NPF1**), was synthesised. However, when incubated with H_2O_2 in cuvette, **NPF1** showed a greater than 20-fold reduced fluorescent response to H_2O_2 compared with **CPF1**. This poor sensitivity suggests that **NPF1** should not be used for the detection of H_2O_2, but rather fluorophores with a greater fluorescent response should be utilised (e.g. **CPF1**). A reversible optical fibre-based sensor for H_2O_2 was then explored by attaching a reversible fluorescent probe for ROS (nicotinamide coumarin redox sensor 3, **NCR3**) to an optical fibre tip. The sensor was constructed using light-catalysed polymerisation to give a polymer matrix on the tip containing **NCR3**. This allowed the fibre tip to be reversibly oxidised by H_2O_2 and reduced by $NaCNBH_3$. The sensor exhibited good reversibility over at least seven cycles of oxidation and reduction, with consistent fluorescent ratios of its maxima at 500 and 635 nm. However, its fluorescence intensity decreased over time, suggesting that **NCR3** leached from the polymer into the buffer solution. This nevertheless represents the first example of a reversible fibre sensor for ROS and is as such an important first step towards a reusable optical fibre probe for H_2O_2.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Signed:

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Malcolm Purdey

Tuesday, 8 December 2015

ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisor, Prof. Andrew Abell. There are few things that can aid a PhD student more than a fantastic supervisor, and I could not have asked for a better supervisor than Andrew. I have learned enormous amounts through the course of this study, and certainly a vast proportion of that is due to Andrew's input. It was always reassuring to know that whenever I was stuck with an issue I could take it to Andrew and there would be a solution.

Thanks to Prof. Tanya Monro for the input into the project and for invaluable knowledge about optical fibres and sensor designs. In this area I would also like to acknowledge Dr. Erik Schartner for his endless assistance with optics, optical fibres and sensing in general. It also helped to have someone constantly there to top me up with fresh cynicism towards any given experiment when I may be running dry. There were a lot of good laughs had, as well as many valuable discussions and timely assistance.

I wish also to thank Prof. John Carver, for initial supervision of this project, and for further support even after his move to ANU in Canberra.

Importantly, I would like to acknowledge Cook Medical Australia, along with the ARC Linkage grant (LP 110200736) that supported this work. The outcomes have been fantastic and it was immensely helpful working so closely with industry.

For those post-docs in the Abell group; particularly Dr. William Tieu, Dr. Sabrina Heng and Dr. Niels Krogsgaard-Larsen, I thank profoundly for listening patiently to any issues I might be having. It would not be a stretch to call each of them unending sources of chemical wisdom, and I am extremely grateful for the number of hours chopped off my PhD studies due to helpful suggestions. A special thanks to Niels for extended periods of time listening to ideas and helping with aesthetics of figures and presentation of this thesis.

I also thank my contemporary and past PhD students, particularly Daniel Stubing, Michelle Zhang, Jacko Feng, Kelly Keeling and Tim Engler for making the lab a fun place to be all the time. I really don't think I would have finished all this if there weren't people to bounce ideas off and to empathise with on the tougher days.

I must thank Assoc. Prof. Jeremy Thompson and Dr. Mel Sutton-McDowall for direction and help with biological studies with embryos. Working with them enabled the whole project to be grounded in applications, as well as them just being fantastic people to work with.

The technical staff at IPAS and the Discipline of Chemistry also deserve a huge thanks. I would particularly like to mention the assistance of Dr. Herbert Foo, who was instrumental in helping with surface functionalisation and analysis.

I would further like to thank Prof. John Aitken, Haley Connaughton and Sara Whiting at the University of Newcastle for assistance with experiments in human spermatozoa.

It would be remiss of me to leave out thanking the superb lab placement students I supervised during my studies: Pang-Chong (Alex) Yun, Yvonne Yusa, Camilia Tan Huici, Alex “Bumpmaster” Jackson and Conner Sangster. Each were exceptional students and were extremely helpful in the lab. I enjoyed teaching them as much as they hopefully enjoyed learning!

I would also like to thank all involved in the formation of the Centre for Nanoscale BioPhotonics. It has been a pleasure to get to know everyone and the level of research taking place is certainly inspiring to students like me.

On a more personal note, there is again nearly too many people to thank! I will start by thanking my parents, Don and Annette Purdey, for all their support during all my studies throughout my entire life. I would especially like to thank Mum for her continued support, help and excellent proof-reading even after Dad passed away mid last year. It is truly inspiring to have parents of such incredible love and wisdom.

I must thank also my Grandad, Dr. J. Howard Bradbury, who really piqued my interest in science by his continued tireless work at the Australian National University for more than 25 years since his retirement. He remains a profound input into my studies, and the only difficulty I now have is continuing on such a legacy of academic excellence and personal integrity.

If I were to name all the other family and friends I would wish to thank and reasons why, I believe it would take at least as many pages as the research presented here! Suffice it to say that I feel blessed to have such amazing brothers, sisters, parents (& in-laws), and friends. I can’t thank you all enough for putting up with me in this amazing yet lengthy journey.

Finally, it is no surprise that I thank my wife Ainsley. This last three and a half years has been full of joy and sorrow, and I can’t thank you enough for the constant support and friendship that you’ve shown me. There is no other way to put it – this PhD would not have been the same without you. I entered this program not yet married, and exited it married to

the best woman ever, complete with a beautiful daughter Elsie who is a constant joy! Thank you so very much, you are simply the best!

I also can’t help but acknowledge God who led me here and continues to prod me with new thoughts and ideas. He is incessantly consistent and perfectly reliable, I never thought I wanted to end up where I am, but He had much better ideas.