AUTOREFERAT - ENGLISH

Development and practical application of fluorescent probes for reliable detection and visualisation of bioinorganic substances in biological systems

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1. NAME

Jacek Łukasz Kolanowski

2. DIPLOMAS, DEGREES CONFERRED IN SPECIFIC AREAS OF SCIENCE OR ARTS, INCLUDING THE NAME OF THE INSTITUTION WHICH CONFERRED THE DEGREE, YEAR OF DEGREE CONFERMENT, TITLE OF THE PHD DISSERTATION

PhD in chemistry (Awarded *Très Honorable*) École Normale Supérieure de Lyon, France
Year awarded: 2013
Advisor: Prof. Jens Hasserodt, Thesis title: "Bispidine-iron(II) complexes as a novel platform for the design of magnetogenic probes"

Master of chemistry (Awarded *Maxima Cum Laude*) Adam Mickiewicz University, Poznań, Poland
Year awarded: 2009
Advisor: Prof. Henryk Koroniak, Thesis title: "Novel synthetic method for preparation of ortho-alkylfluorinated arylphosphonates and their reactivity"

Bachelor's Degree in biotechnology Adam Mickiewicz University, Poznań, Poland
Year awarded: 2007
Advisor: Prof. Przemysław Wojtaszek, Thesis title: "Potential therapeutic application of resveratrol in processes triggered by Sirtuins"

3. INFORMATION ON EMPLOYMENT IN RESEARCH INSTITUTES OR FACULTIES/DEPARTMENTS OR SCHOOL OF ARTS

Director of the Centre for High-Throughput Screening Studies
Institute of Bioorganic Chemistry PAS, Poznan, Poland
Employment contract, 10.2018 – Present

Principal Investigator in the Department of Molecular Probes and Prodrugs
Institute of Bioorganic Chemistry (IBCH), Polish Academy of Sciences (PAS), Poznan, Poland,
Employment contract, 09.2017 – Present

Postdoctoral Research Associate
School of Chemistry, The University of Sydney, Australia
Employment contract, 05.2015 – 05.2017

***Fondation ARC* Postdoctoral Research Fellow**
School of Chemistry, The University of Sydney, Australia
Fellowship stipend, 04.2014 – 05.2015

PhD student
École Normale Supérieure de Lyon, France
Employment contract, 01.2020 – 10.2013

Researcher (MSc)
Jacobs University Bremen, Germany
Stipend, 10.2009 – 12.2009

Internship (LLP Erasmus Programme)
University of Bremen, Germany
Stipend, 10.2007 – 02.2008

4. DESCRIPTION OF ACHIEVEMENTS, SET OUT IN ART. 219 PARA 1 POINT 2 OF THE ACT.

4.A Title of scientific achievement

"Development and practical application of fluorescent probes for reliable detection and visualisation of bioinorganic substances in biological systems"

4.B Publications included in scientific achievement

P1 – P6: Experimental publications		**IF***	**Cit****
P1.	KG Leslie, JL Kolanowski, N Trinh, S Carrara, MD Anscomb, K Yang, CF Hogan, KA Jolliffe*, EJ New*, Nicotinamide-Appended Fluorophores as Fluorescent Redox Sensors, **2020**, *Australian Journal of Chemistry* **73,** 10, 895-902. *My contributions include:* • Optimising the synthesis of NCR1 probe, designing the synthetic route and for the first-time synthesising and successfully purifying the NCR2 probe • Designing, performing, and interpreting the spectroscopic characterisation of those two probes and their performance and response to various analytes.	1,073	2
P2.	JL Kolanowski, LJ Dawson, L Mitchell, Z Lim, ME Graziotto, WK Filipek, TW Hambley, EJ New*, A fluorescent probe for investigating metabolic stability of active transplatin analogues. *Sensors and Actuators B: Chemical* **2018**, 255 (3), 2721-2724 *My contributions include:* • Conceptually conceiving, designing, performing and interpreting the key experiments and theses of the manuscript, including in particular spectroscopic characterisation, validation of probes' performance and response as well as selectivity. • Designing, carrying out and interpreting unique experiments to better test the robustness of the response of the probe to potential biological interferents.	5,667	10

	• Originating the main observations and theses of the manuscript, preparing the presentation of the spectroscopic data, co-writing and co-editing the manuscript. • Co-preparing the response to the reviewers, which was key to successful publication.		
P3.	IJ Carney, JL Kolanowski, Z Lim, B Chekroun, TW Hambley, EJ New*, A ratiometric iron probe enables investigation of iron distribution within tumour spheroids. *Metallomics*, **2018**, 10(4), 553-556 *My contributions include:* • Optimizing the synthesis of the probe. • Compiling experimental data. • Contributing to planning the manuscript and jointly formulating the main theses of the manuscript. • Editing the manuscript and preparing the response to reviewers.	4,069	7
P4.	C Shen, JL Kolanowski, CM-N Tran, A Kaur, MC Akerfeldt, MS Rahme, TW Hambley, EJ New*, A ratiometric fluorescent sensor for the mitochondrial copper pool. *Metallomics* **2016**, 8, 915-919 *My contributions include:* • Supervising daily a PhD student in the design and performing of the synthesis. • Performing regular interpretation of the results of the synthetic effort and making decisions on next steps of synthesis and spectroscopic characterisation of all the probes. • Designing imaging experiments, developing robust protocols for imaging data collection and analysis, Interpreting the imaging data, co-writing the manuscript and co-preparation of the response to reviewers.	3,54	22
P5.	M Hu, KE Schulze, R Ghildyal, DC Henstridge, JL Kolanowski, EJ New, Y Hong, AC Hsu, PM Hansbro, PAB Wark, MA Bogoyevitch, DA Jans*, Respiratory Syncytial Virus co-opts host mitochondrial function to favour infectious virus production. *eLife*, **2019**, 8, e42448 *My contributions include:* • Advising on the selection of the right tools to reliably study ROS in mitochondria • Selection and helping in the implementation of data acquisition and data analysis protocol to ensure the robustness of the measurements of oxidative stress in mitochondria.	7,551	20
P6.	KL Chong, BA Chalmers, JK Cullen, A Kaur, JL Kolanowski, BJ Morrow, K Fairfull-Smith, MJ Lavin, NL Barnett, EJ New, MP Murphy, SE Bottle*, Pro-fluorescent mitochondria-targeted real-time responsive redox probes synthesised from carboxy isoindoline nitroxides: Sensitive probes of mitochondrial redox status in cells. *Free Radical Biology & Medicine* **2018**, 128, 97-110.	6,02	10

My contributions include:

- Designing and jointly performing, processing (with the group leader) and leading the analysis and interpretation of the confocal imaging and Fluorescence Lifetime Imaging experiments in a way that maximised the reliability of the results.

P7 – P9: Original reviews			
P7.	JL Kolanowski, A Kaur, EJ New*, Selective and reversible approaches towards imaging redox signaling using small molecule probes. *Antioxidants & Redox Signalling* **2016**, April, 713-730. *My contributions include:* • Conceiving the main concept and formulating the main theses of the work as well as a novel way of representing and organising the data to analyse the topic from previously unexplored angle. • Collecting, analysing, and interpreting the majority of literature sources, preparing the majority of figures and co-writing and co-editing the manuscript.	7,093	14
P8.	A Kaur, JL Kolanowski, EJ New*, Reversible fluorescent probes of biological redox state. *Angewandte Chemie International Edition in English* **2016**, 55, 1602-1613. *My contributions include:* • Analysing and interpreting the majority of sources used in this manuscript and synthesising several major theses of this review., including, among others, those regarding the criteria for design of such tools and correlating structure with fluorescence response mechanisms • Designing and preparing all figures and co-written and co-edited the manuscript.	11,709	93
P9.	JL Kolanowski[+], F Liu[+], EJ New*, Fluorescent probes for the simultaneous detection of multiple analytes in biology. *Chemical Society Reviews* **2018**, 47, 195-208. *My contributions include:* • Analysing and interpreting part of the sources used in this manuscript and synthesising several theses of this review • Co-designing selected figures, co-writing and co-editing the manuscript (with corresponding author)	40,182	215

*from year preceeding publication year

** without self-citations (Web of Science, 20.12.2022)

4.C Description of scientific aim of the above-mentioned series of articles and the achieved results together with the discussion of their application

The scientific aim of my research was to improve, in three different dimensions, the reliability of detection of biologically important and particularly difficult to detect bioinorganic analytes (i.e., detectable substances) like transitional metal ions and reactive oxygen and nitrogen species, with the use of fluorescent small molecule tools:

- **Design:** Introducing novel design strategies and recommendations to inform a future generation of more reliable probes with a better understanding of the structure-performance relationship, and providing instructions to validate them
- **Probes development:** Developing probes with uniquely high levels of specificity and reliability for bioinorganic analytes based on a variety of strategies (structure-selectivity relationship as well as ratiometricity and fluorescence lifetime as more reliable parameters for detection of interaction of a probe with an analyte).
- **Methodology and probes application:** Developing methodologies and workflows based on experiment and literature to enable scientists equipped with probes to reliably acquire and extract data about elusive bioinorganic analytes in biological models

Background & Context

Fluorescent probes are one of the most popular tools used to better understand physiological and pathological processes. Studying such processes requires extremely reliable probes that do not disturb these processes and allow observations of analytes with high spatial and temporal resolution. Fluorescent probes work by detecting and visualising (bio)chemical substances (analytes), often in real time in live cells through a selective reaction or recognition event with an analyte that results in a detectable change in fluorescent properties (e.g., emission intensity).

In the last decades, fluorescent probes have enjoyed a dramatic upsurge in interest, but over 90% of reported probes have never been applied in biological systems beyond proof-of-concept experiments.[1] This is primarily due to insufficient characterisation and limited reliability of detection with these tools in a biological environment.

The biochemical targets I have focussed on in this work were reactive oxygen and/or nitrogen species (RONS) and transition metal ions – two categories of bioinorganic substances of diagnostic and therapeutic significance, which play critical yet poorly understood biological roles, e.g. in cancer, neurodegeneration and others. This is due to several challenges:

- Different forms of RONS change dynamically between each other even down to the nanosecond scale making it difficult to design specific and selective probes for their visualization. Similarly, transition metal ion complexes readily exchange composition, structure, and oxidative state
- Generated signals arising from existing probes can often be ambiguous
- The role or function of RONS and metal ions depend on their local biochemical environment, that changes in time and spatially on subcellular scale

Much of my career has been dedicated to addressing these challenges from multiple perspectives, from the design and validation steps through to data analysis for more reliable biological studies and introducing new generations of probes. **Therefore, the primary contribution of my research has**

been expanding and improving the reliability, design, and use of fluorescent tools for molecular imaging. This scientific achievement has been described in terms of activities under the following 4 themes:

1. Providing original theoretical findings regarding design and criteria of probes to support and improve the reliability of generating, validating, and applying fluorescent tools for bioinorganic analytes (Based on P7 and P8)
2. Understanding and harnessing the structure-response relationship of probes, sensing groups and analytes to develop probes for bioinorganic analytes (P1 and P2)
3. Employing my reported strategies to discover, actively develop and apply probes for RONS and labile metal pools with more reliable response independent of probe concentration (P3-P6)
4. Founding and establishing a new field of research surrounding the development and use of dual-analyte probes (P9).

4.C.1 Developing new criteria and approaches for the design and validation of fluorescent probes for bioinorganic analytes in biological models

Fluorescent probes are typically composed of a fluorophore (signalling moiety) and an analyte-sensitive moiety, which can be a part of a fluorophore itself, attached directly or via a spacer (Figure 1). For some probes, additional elements (second fluorophore, quencher or targeting group) are attached. A distinct advantage of fluorescent responsive probes versus tags (always emitting the same signal) is their ability to modulate fluorescent properties in response to interaction with an analyte, generating a more unambiguous signal that is easier to interpret.

Figure 1 Modular structure of a fluorescent probe

Bioinorganic compounds found in organisms can be elusive and difficult to detect, especially due to the dependence of their activity on their concentration and speciation. Therefore, interrogation of their activities at the cellular level requires probes that are capable of:

1. **High specificity** (Figure 2, left) to address the diversity of forms of such analytes, and each having diverse biological roles
2. **Reversibility of response** (Figure 2, right) to tackle the challenge of bioinorganic analytes quickly switching between these forms

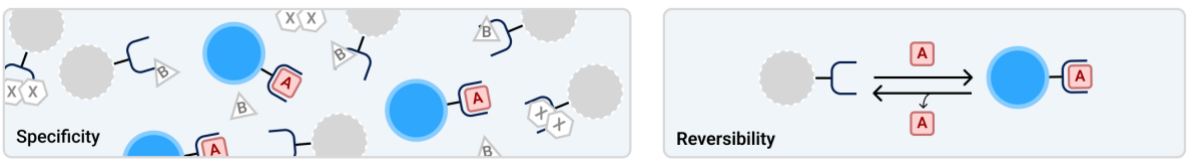

Figure 2 Specificity of fluorescent probes vs Reversibility of analyte-sensing interaction

Review P7 contains a discussion on the difficulty of developing fluorescent probes and tools to tackle these challenges simultaneously (specificity vs time-variable, reversible response), on the example of probes for RONS (with conclusions transferrable to probes for any targets). It also introduces a new method of predicting specificity of a fluorescent tool by analysing the link between chemical reactivity (mechanisms) of the responsive group and chemical character of analyte as a substrate of these reactions. It also draws links between the above-mentioned chemical nature of the responsive group before and after interaction with analyte, and its position on the probe and subsequent relationship to the fluorescence mechanism of the signalling moiety (fluorescent scaffold). Review P8 in turn focuses on how to achieve reversibility, identifies gaps in validation protocols and provides solutions to increase the reliability of this validation processes (subchapter 4.C.1.3). As the articles are based on deep analysis of reported data leading to original conclusions and findings, they reflect the results of novel, theoretical research that constitute a significant input and expand the potential of this research field.

4.C.1.1 Establishing comprehensive list of universal criteria for design, selection, and validation of fluorescent probes for chemical analytes in cells

To design and develop more optimal probes suited to application in biology, I have defined an improved, comprehensive list of criteria for designing fluorescent probes, which includes:

1. **Fluorescent properties**, in particular **(i)** selecting the ideal excitation / emissions wavelengths, **(ii)** brightness, which is a product of the **(iia)** efficiency of absorption of light (characterised by extinction coefficient - ε), and **(iib)** efficiency of emitting the absorbed energy as light (fluorescence quantum yield - Φ or QY),
2. **Modes of interaction with an analyte** (covalent or non-covalent, reversible, or irreversible), which requires appropriate responsive group and should be selected based on the analyte's properties and the information we wish to obtain during imaging experiments,
3. **Fluorescent response upon interaction with analytes,** characterised by the type and magnitude of change in florescence properties caused by the analyte: **(i)** intensity-based response with is characterised by increase or decrease in signal intensity and **(ii)** ratiometric type response which means a change in the ratio of signal intensity at two or more wavelengths,
4. **Specificity of interaction with the analyte** of interest against other forms of this analyte, similar analytes as well as stability (inertness) towards other chemical and physical elements of the environment (e.g., proteins, pH but also polarity or viscosity),
5. **Sensitivity,** which is a consequence of the **(i)** strength/probability of interaction between the analyte and the probe, **(ii)** the resulting analyte-induced type and magnitude of change in fluorescent properties and **(iii)** noise (background signal and non-specific interactions),
6. **Behaviour of the probe in the cellular environment** including localisation and local concentration of the probe in time which is a consequence of uptake, retention, and stability / metabolism, and
7. **Effect of the probe on cellular environment** which for visualisation and monitoring should be eliminated, minimised or possible to account for and includes (**i)** probes' but also **(ii)** experimental (e.g. light-induced) toxicity, which can cause both acute and long-term sub-lethal effects as well as more subtle changes in cellular processes, metabolism and overall state.

4.C.1.2 Correlating structure with responsiveness of fluorescent probes for RONS for improved selectivity

Understanding the relationship between structure and specificity of response is seemingly an obvious key to improving reliability of fluorescent probes. Most existing reviews classify fluorescent probes for RONS according to type of fluorophores used or the analyte they aim to detect, which has not yet provided clear understanding of how to achieve *specificity of response*. Our work (P7) has re-framed this question by studying RONS through the lens of the possible chemical reactions they can undergo and structures of potential responsive motifs which are susceptible to these reactions. On this basis, we uniquely highlighted the most probable non-specific interactions of existing probes and developed a strategy to assist scientists in future identify and avoid potential interference.

After in-depth analysis of the results from previously reported probes, I discovered that **the best predictor of probe specificity is the compatibility of an analyte's mechanism of reaction and the chemical reactivity of the probe's responsive group.** I defined four classes of chemical transformations used in probes for RONS. Those groups of probes differ from each other but share, within each group, the advantages and challenges in their specificity, identified gaps and proposed improvements in their experimental validation:

1. Radical oxidations - highly sensitive but they suffer from non-specific reactivity with other radicals (e.g. H_2O_2, $O_2^{\bullet-}$, $^{\bullet}OH$, $^{\bullet}OtBu$, $ONOO^{\bullet-}$) and side-reactivity, as well as self-propelling re-generation of radicals. Examples include dichlorofluorescein (DCFH) and dihydroethidine (DHE) and nitroxyl-radical-based probes.[2–5]
2. Oxidative nucleophilic transformations – based on tuning electrophilic group on the fluorophore, they are susceptible to interreference from other nucleophilic RONS (H_2O_2, $ONOO^-$ and $O_2^{\bullet-}$) and other nucleophiles (e.g., glutathione, lysines etc). Examples include boronate-based probes.[6–9]
3. Electrophilic non-radical oxidations – based on RONS acting as an electrophile (e.g. $^{\bullet}OCl$ with propensity to react with S-electrons, NO with N-electrons and peroxynitrite with aromatic rings). They usually exhibit high specificity over other RONS but can frequently generate radicals and are often insufficiently evaluated against other biologically relevant electrophiles and radical species.[10–14]
4. Metal-centred reactions - opens possibilities of using a wider range of types of parameters to distinguish the analytes (reactivity, geometry, charge and redox potential – similar to protein-based redox sensors); only very few examples reported.[15,16]

Most probes focussed on selectivity are irreversibly modified by the RONS analyte. The few examples of both specific *and* reversible probes for RONS are largely inspired by redox enzyme active sites. They use selenium as the reactive moiety, which by judicious substitution pattern can be rendered specific toward oxidation by $ONOO^-$, H_2O_2 or $^{\bullet}OCl$ and reduced by glutathione or other thiols enabling a redox cycling-dependent fluorescent response. However, their validation protocols are lacking when determining the specificity *in cellulo*. Therefore, we have proposed that while creating single probes which are both reliably specific and reversible is highly challenging, the simultaneous use of one specific and one reversible probe with complementary fluorescent properties (e.g., non-overlapping emission wavelengths) can be a solution. Alternatively, we suggest for the first time tethering two probes (reversible and specific) together to ensure co-localisation and metabolic stability - a concept we have further developed and described in another manuscript (P9) which is discussed at the end of this description.

In this work (P7) I have demonstrated the importance of understanding the chemical determinants of reactivity of responsive group and the analytes to ensure specificity. This enables determining classes of compounds which could be most probable interferents in biological models and therefore reliably validate the probe.

4.C.1.3 Establishing design and validation requirements for reversible fluorescent probes for bioinorganic analytes

As mentioned above, to monitor changes more reliably in the levels and localisation of bioinorganic analytes over time, fluorescent probes that respond reversibly to an analyte's presence or absence are strongly preferred. This can be achieved through a non-covalent interaction of the analyte with the probe, or a reaction that breaks / forms covalent bonds, with an assumption that it does not split the probe into separate fragments –Figure 3.

In the case of probes that detect transitional metal ions, the former app roach is typically used. This is due to unique characteristics of metal ions, in particular their high but diverse charge density of and their intrinsically varied preference for certain binding geometries and types of ligands. This allowed for the development of highly specific, multi-dentate binding motifs that operate even in highly competitive aqueous environments. [17,18,19–21]

For RONS, due to their high reactivity and weaker specificity due to lower charge densities, their probes usually take part in a reversible reaction that results in a change in fluorescence. Such interaction, however, leads to consumption of the analyte molecule (RONS) and requires presence of another analyte or intrinsic system with opposite activity (e.g., reduction property) to "revert" the probe to its previous state before it can detect the analyte again. This creates challenges in designing reversible fluorescent probes for RONS and despite a need for these tools, no reviews aiding the understanding the relationship between the structure and reversibility have been reported.

Design considerations: to design reversible probe for RONS we proposed to focus principally on matching the chemical character and reactivity of the responsive group (which needs to be reversibly modified by the analyte) to a mechanism of generating fluorescence change. This in turn requires a deep understanding of how the structure of the responsive group determines the interaction with an analyte and how it influences fluorescence of the fluorophore/ signalling group. To this end we have evaluated reported reversible fluorescent probes for RONS from two perspectives:

1. The mechanism that translates a reversible analyte-responsive group interaction to a change in fluorescent signal (P7):
   a) Analyte reacts directly with a chromophore leading to its destruction / formation (e.g., reaction with fluorescein, rhodamine, flavin or nicotinamide)[22–26] – **Error! Reference source not found.**A
   b) Analyte-induced modulation of energy/electron transfer capacity (e.g.through PeT or ICT mechanism) of the probes' responsive moiety influencing fluorescence (e.g. with nitroxyl radical or quinone-like moieties)[27,28] – **Error! Reference source not found.**B
   c) Analyte-induced changes in spatial proximity between fluorophore and quencher /enhancer (e.g. through creating / destruction of disulfide bridges)[29] – **Error! Reference source not found.**C
2. The chemical structure and character of reactive moieties responsible for reversible reaction with the analyte (P8) (nitroxyl radicals, quinones, dichalcogenides and chalcogenoxides, nicotinamides and flavins), (structure-reactivity)

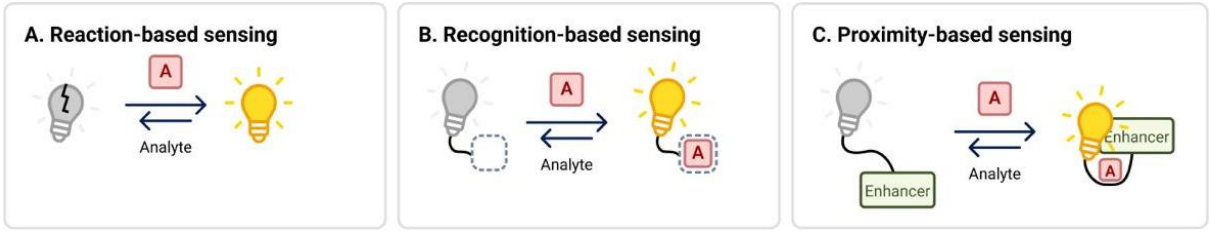

Figure 3 Schematic representation of mechanisms translating reversible probe-analyte interaction into a detectable signal

Validation considerations: While all the reported motifs and mechanisms could be used to design reversible fluorescent probes for RONS, their direct comparison was obscured by discrepancies in probe validation protocols. Many studies lacked evaluation of reversibility, biological sensitivity, or biological compatibility on multiple levels, hindering their reliable application in biology. Therefore, we have provided guidelines for the robust validation of the performance of reversible probes to ensure their reliability in biological experiments. In particular, validating a probe's reversibility requires additional considerations, which include

a) a response time fast enough to monitor rapid changes in concentrations in real-time.
b) reversibility and stability after multiple cycles (low system fatigue).
c) the ability to attune binding affinity and sensitivity to the range of physiologically and pathologically relevant conditions in a particular process we want to monitor (e.g., by measuring those ranges with alternative techniques).
d) development of robust protocols and assays with the use of the probes beyond microscopy (e.g., flow-cytometry, rapid miniaturised multi-well plate reader assays)

4.C.1.4 Summary (P7 and P8)

I have significantly contributed to the theory of designing and validating fluorescent probes for bioinorganic analytes in cells, enabling the development of more reliable tools. In manuscript P7 I have improved the understanding of the relationship between structure, reactivity, and fluorescence response, establishing strict criteria for designing **highly specific** probes for RONS. Then, in P8 I introduced comprehensive prerequisites for the design of **reversible** probes and protocols on how to validate them to maximise their reliability. It is important to note that while the manuscripts presented in this section share characteristics with a scientific review, I conducted theoretical work and meta-analyses to arrive at newfound conclusions that significantly impact the way fluorescent probes are being used to investigate biological questions.

4.C.2 Harnessing structure-reactivity-responsiveness relationship to develop specific probes for bioinorganic analytes

In this section, I will describe my efforts to apply the theoretical findings described in 4.A.2 to investigate how the chemical structure and reactivity of a fluorescent group and responsive moiety can be manipulated to develop highly specific probes in the context of RONS and metal ions.

4.C.2.1 Nicotinamide-based redox probes

Publication P1 explores the relationship between structure and responsiveness, based on the example of the nicotinamide responsive moiety with a coumarin scaffold. My contribution to this work paved the way for the design and development of redox-active nicotinamide-based probe.

Flavin (present in flavin adenine dinucleotide co-factor FAD) and nicotinamide (part of nicotinamide adenine dinucleotide (phosphate) – $NAD(P)^+$ and its reduced form – NAD(P)H –Figure 4) are the two key redox-responsive moieties mediating redox transformations in live cells. They are also intrinsically fluorescent in one of the redox-forms allowing exploration of changes in their fluorescence as a proxy for intracellular redox state.[30–33] While one part of the research team I worked in explored flavin-based fluorescent redox probes, my work focussed on understanding of the relationship between the structure and responsiveness of nicotinamide redox-active moiety (P1) to inform the development of tuneable redox-responsive probes based on nicotinamide for application in biology.

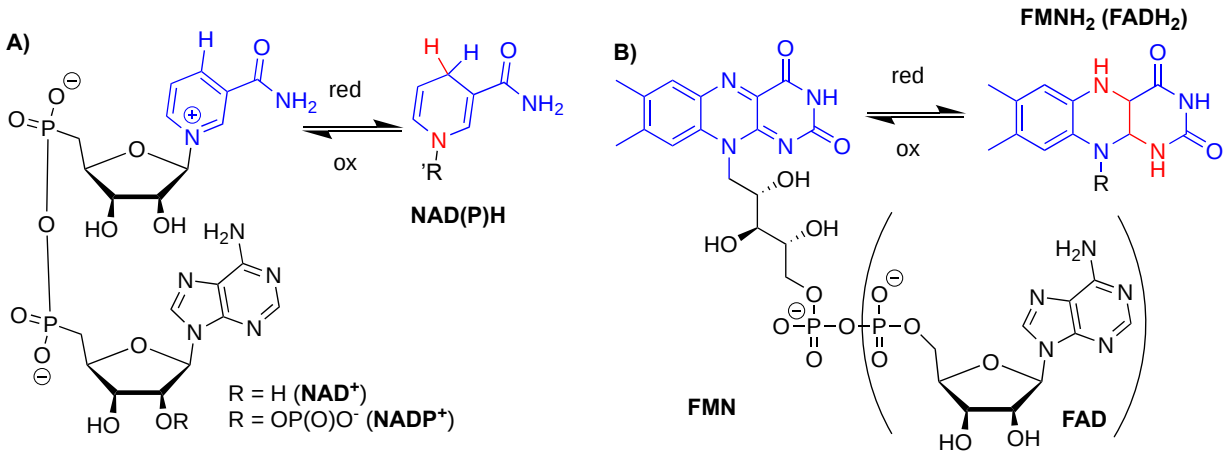

Figure 4 (A) Structures of the two nicotinamide-containing intracellular reversible redox couples (NAD^+/NADH and NADP+/NADPH) (B) Structures of the two flavin-containing intracellular reversible redox couples (FMN/$FMNH_2$ and FAD/$FADH_2$). Blue – redox-active moieties, red – reduced fragments of these moieties.

In order to ensure a distinction of a fluorescent properties of the probe and endogenous nicotinamide, I sought to introduce a nicotinamide motif as a redox responsive element into the coumarin scaffold which would act as a fluorescent reporter. I optimised a Zincke-type reaction to conjugate 7-amino group on coumarin scaffold with *m*-amidopyridine (nicotinamide) and purification of an NCR1 compound (Figure 5) that is based on coumarin 120. I also synthesized and purified for the first time the NCR2 probe based on coumarin 151 to study the influence of the substituents on redox-responsiveness and fluorescent properties of nicotinamide-coumarin based probes. Excitation maxima of NCR1 and NCR2 were blue shifted in comparison to parent coumarins with longer wavelength of excitation maximum in case of NCR2 (374 nm), enabling a non-UV excitation and therefore making it more biocompatible in comparison to NCR1. As both compounds have been isolated in oxidised form, in which nicotinamide should not exhibit any fluorescence, their emission in these conditions might be attributed to the coumarin scaffold. Electrochemical investigation revealed that NCR2 undergoes a single oxidation step of higher amplitude and different redox potential than NCR1, which exhibited a two-step oxidation pattern. Importantly, exposure to the reducing agent (sodium dithionite) in the case of both probes led to the decrease in fluorescence intensity, which could not be reversed upon addition of hydrogen peroxide. A lack of reversibility of

reduction might be explained by a presence of multiple reduction events in cyclic voltammogram of both probes.

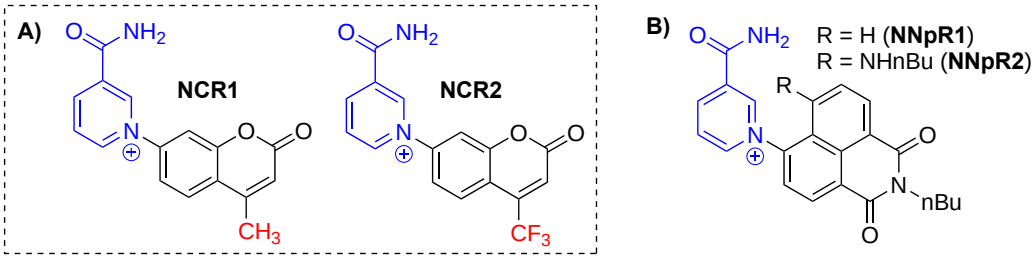

Figure 5 (A) First-generation nicotinamide-coumarin conjugates (NCR1 and NCR2) prepared and characterised by me and (B) second-generation, nicotinamide-naphthalimide conjugates (NNpR1 and NNpR2) designed and developed on the basis of the findings from the first-generation probes. In blue – redox-responsive nicotinamide-moiety, in red – different substituents controlling fluorescent and redox properties of first-generation probes

Differences in redox behaviour and fluorescent properties of NCR1 and NCR2 demonstrate the possibility of tuning properties of nicotinamide-coumarin conjugates by varying an electron-donating / electron-withdrawing character of substituents at position 4 of the coumarin scaffold. Understanding this relationship between the character and relative position of the substituent on fluorescent scaffold with regards to the position of nicotinamide responsive motif informed a design of a more biocompatible nicotinamide-based probe built on napthalimide moiety. This new probe (NNpR1) was then successfully prepared and used in detection of hypoxia in A549 cells. Additionally, a prevalence of fluorescence of the fluorophore scaffold (coumarin or napthalimide) over fluorescence of nicotinamide moiety in these probes bodes well for future more reliable distinction between a probe and endogenous intracellular nicotinamide-derived fluorescence.[35]

4.C.2.2 Development of fluorescent probe for detection of one form of platinum-based metal complexes with therapeutic potential

Therapeutic platinum-based (Pt-based) complexes like cisplatin and transplatin are examples of important yet challenging bioinorganic analytes to monitor. Due to the variable influences of geometry and ligand composition on the bioactivity of Pt complexes (Figure 6), monitoring the specific forms of Pt-based drug candidates can help us understand their biological fate, mechanism of action and potential off-target interactions they undergo, which in turn opens up a pathway to predict their therapeutic potential and design better, more efficient and side effects-free anti-cancer and even other types of drugs.

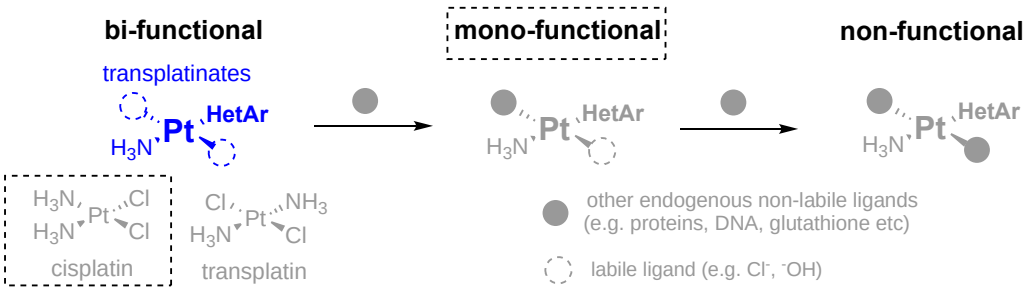

Figure 6 Schematic representation of platinum(II)-based complexes and their metabolic forms (monofunctional and non-funtional). In blue – type and form of platinum complexes detectable by the probe developed within a scope of

my habilitation application. In dashed rectangles – forms of Platinum complexes detectable by the probes developed elsewhere. HetAr – heteroaryl substituents, Pt – Pt(II) ion.

In P2, we aimed to develop highly reliable Pt-sensitive fluorescent probes through achieving extremely high specificity. Designing specific probes for different forms of platinum complexes remains a significant challenge; in fact, only two such probes were reported before the publication of our work (Figure 7 A - Rho-DDTC[36] for cisplatin and derivatives, FDCPt1[37] for platinum(II) complexes with a single labile ligand,– Figure 3 middle) and since then only two variants of Rho-DDTC probes but also for cisplatin, have been developed by the same research group.[38,39]

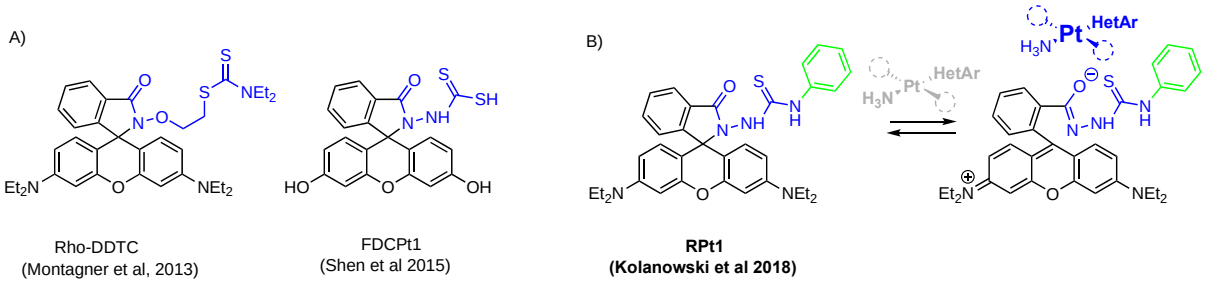

Figure 7 Structures of specific fluorescent probes for platinum (II) metal complexes A) previously reported cisplatin sensitive Rho-DDTC[36] and monofunctional platinum(II) complexes sensitive FDCPt1[37] B) probe developed by myself and co-workers (manuscript xx) together with a mechanism of the response of the probe to platinum analyte. Responsive motifs specific to platinum complexes in blue. In green a specific motif incurring selectivity towards heteroaryl-substituted transplatin analogues.

In chapter 4.C.1.1 I demonstrated the importance of understanding how particular structural elements of responsive moieties influence their susceptibility to certain type of interactions and how this relates to specificity. As seen by the analysis of the structures of reported probes, Pt(II) complex binding motifs are characterised by the abundance of so-called "soft" ligands, in particular sulphur atoms and constrained geometry. By focussing on this approach in the context of non-covalent interactions, we were able to judiciously introduce a hydrophobic aryl substituent to the responsive motif of FDCPt1 and graft it to a rhodamine scaffold for better responsiveness that led to the development of RPt1 (Figure 7 B).

Screening of the fluorescent response of RPt1 against a range of Pt(II) complexes revealed that the probe had a remarkably specific response to transplatin analogues bearing heteroaromatic moieties (PytrPt, QtrPt and AzaIntrPt - Figure 8A and B), leading to an increase in fluorescence. This is a consequence of shifting the equilibrium towards an open, fluorescent form (right structure in Figure 7B) upon binding. The response occurs at physiological pH in aqueous solution, indicating the binding is strong enough to withstand intracellular environments. Interestingly, despite great similarity of the responsive motif on RPt1 probe to that of FDCPt1 (Figure 7A in blue), we observed no response from monofunctional platinum complexes. This specificity may stem from the presence of a phenyl substituent in the vicinity of the sulphur atom, blocking non-specific binding by increasing steric hindrance and contribution of stabilising pi-pi stacking between the phenyl group and heteroaromatic ligand of the Pt complex. Additional screening against a broad range of analytes revealed the probe is sensitive to mercury and silver ions, but as they are not endogenously found in biological environments, this should not interfere with its use.

4.C.2.3 Development of RPt1-based fluorescent assay for evaluation of metabolic stability of aryl-transplatin therapeutics

With RPt1 in hand, I developed a rapid and robust *in vitro* assay to estimate the metabolic stability of aryl transplatin complexes. To this end, I preincubated pyridine-transplatin and quinoline-transplatin complexes with glutathione (GSH) at physiological pH at 1:1 ratio (50 μM) and subsequently added RPt1. A decrease in fluorescence intensity in comparison to time zero (without preincubation of Pt complex with GSH) indicates a lower level of the bifunctional form of the transplatin complex which correlates to sequestration of the Pt-complex by glutathione. In addition, quinoline transplatin showed lower resistance to glutathione vs pyridine-transplatin. Furthermore, the effect is not only dependent on the time of preincubation but also on the concentration of glutathione with 2 mM concentration of glutathione almost completely sequestering PytrPt complex at 50 μM within 1 h.

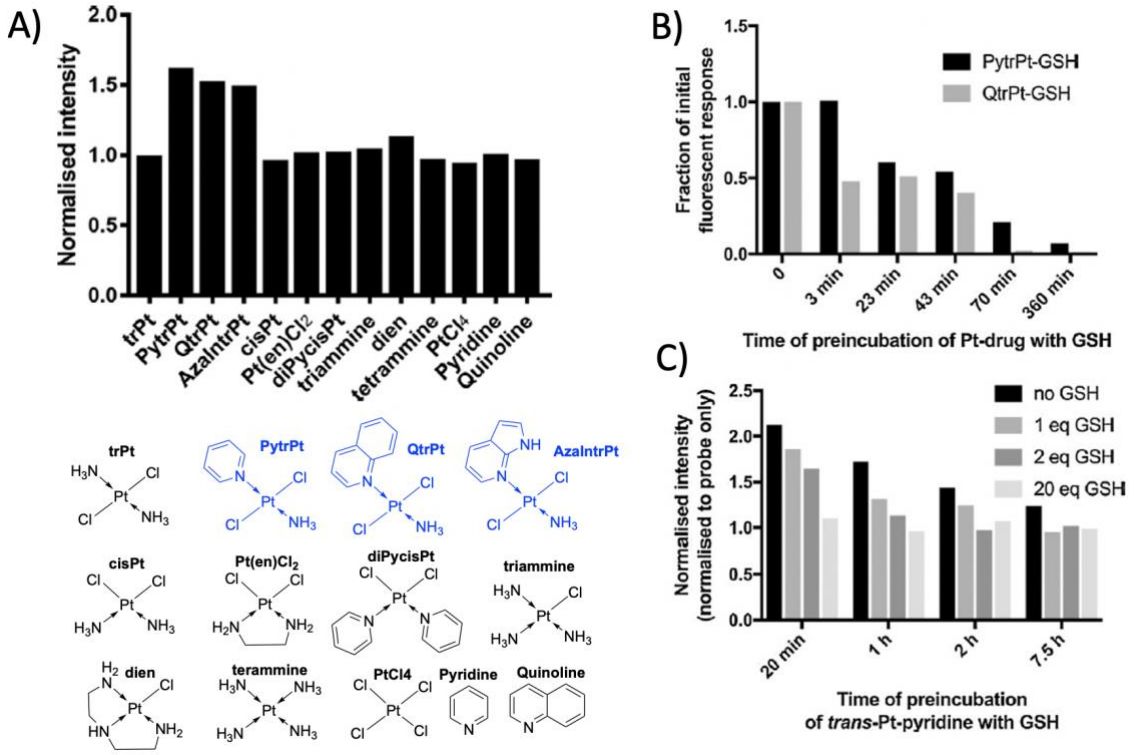

Figure 8 Summary of fluorescent response of RPt1 (50 μM) to the presence of platinum complexes (normalised integrated fluorescent intensity of emission at 582–586 nm for λ_{exc}= 540 nm (in A), 555 nm (in B and C) in HEPES buffer (20 mM, pH 7.4, 1% DMF). A) normalised fluorescence intensity in the presence of 1 eq of platinum complexes and their structures (in blue – platinum complexes generating response). B) RPt1 fluorescence upon exposure to trans-Pt-Pyridine (PytrPt, black) and trans-Pt-Quinoline (QtrPt, grey) before (0 min) and after preincubation of Pt complexes with 1 eq of glutathione, C) RPt1 fluorescence upon exposure to trans-Pt-pyridine preincubated with 0, 1, 2 and 20 eq of glutathione (from black to light grey respectively) for the time indicated. Adapted from P2.

4.C.2.4 Summary (P1 and P2)

The previous section described by contributions towards addressing the design and validation of fluorescent probes, and investigation of the structure-reactivity-specificity relationship of responsive groups and structure-responsiveness dependence to develop more specific probes. In P1 I have demonstrated how an understanding of structure-reactivity-specificity and structure-responsiveness relationship can inform the design of more reliable probes for redox state. Then, in P2 I have

developed a highly specific RPt1 probe and RPt1-based fluorescent assay compatible with microwell plate readers which can be applied to the rapid and reliable assessment of the intracellular stability of aryl-transplatin complexes with therapeutic or biological potential.

4.C.3 Design and use of reliable probes for bioinorganic analytes in biological context

This section describes my work developing and applying fluorescent tools to detect relevant bioinorganic analytes in cellular models with improved reliability. My efforts focussed on both developing highly specific fluorescent probes and optimizing data collection and analysis processes to maximize the reliability of the response in biological studies.

4.C.3.1 Development of ratiometric fluorescent probes for the detection of labile transitional metal ion pools

4.C.3.1.1 A ratiometric response increases reliability of detection

As mentioned in subchapter 4.C.1, analytes can induce different modes of response in probes. The resulting change may be an increase or decrease in emission intensity (for intensity-based probes) or a change in emission colour (a ratiometric probe) (Figure 9). While most reported probes are intensity-based, they suffer due to the dependence of fluorescent intensity not only on analyte concentration but also on concentration, localisation, and metabolism of the probes themselves. Ratiometric probes allow for measurement of the presence of the analyte on the basis of changes in the ratio of intensities at two different wavelengths. The ratio is independent of probe concentration, distribution, and instrument-based variations. Ratiometric probes are therefore significantly more reliable and preferred when using complex biological models, but their development is more challenging.

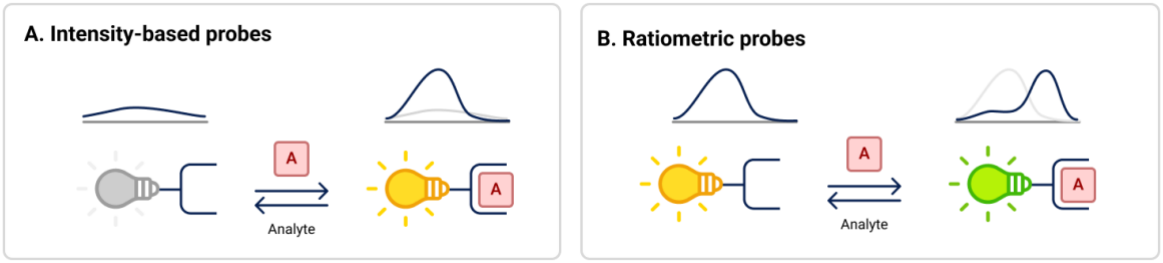

Figure 9 Scheme of A. Intensity-based probes (turn on or turn off) and B. Ratiometric probes

Ratiometric probes can be constructed in multiple ways, including using a fluorescent reporter that natively responds with a change in wavelength of maximum intensity or by linking two fluorescent molecules – one intensity-based reporter with one that emits a stable signal regardless of environment/analyte. In my work I have explored the second strategy to design and develop reliable probes to detect selected labile metal pools in biology.

It is important to note that while metal ions exist in two main pools within cells (bound to proteins and labile), we believe it is the labile pool (dynamically binding and dissociating) that is critical to signalling processes, maintaining metal homeostasis, and providing metal ions to enzymes. While methods exist to measure the *total* metal pool (labile + bound), e.g., ICP-MS, they commonly require destruction of the sample. Therefore, the fluorescent ratiometric probes discussed in this section are

aimed at targeting the labile metal pool, which we also believe is of greater significance to understanding of pathological processes.

Below are examples of my efforts to explore ratiometricity to improve the detection of labile pools of the two most biologically abundant redox-active transition metal ions: iron and copper. We turned to reversible probes to enable studying labile iron pools (LIP) and labile copper pools (LCP) with spatial and temporal resolution. Iron and copper play a very diverse range of functions in cells and are arguably some of the most challenging analytes to detect which further strengthens the importance of my contributions.

4.C.3.1.2 Development of a ratiometric variant of Calcein-AM probe for labile Fe(II) pools in complex biological models (P3)

The labile iron pool (LIP) in the form of Fe(II) is tightly controlled, as it can participate in Fenton chemistry generating reactive oxygen species. LIPs are therefore a diagnostic parameter of iron homeostasis, and can indicate or cause variable pathologies including neurodegeneration,[40] inflammation,[41] cardiovascular disease,[42] and cancer.[43,44] Availability of iron is also associated with the growth and spread of microorganisms and therefore its sequestration is a therapeutic approach to treat infections.[45,46]

Calcein-AM was the first broadly used turn-off intensity-based fluorescent probe for the LIP in cells, operating by reversible chelation of the metal ion leading to decrease in fluorescence intensity of the fluorescein signalling group. One of the key issues in using Calcein-AM is that loss of fluorescence is indistinguishable from low concentrations or decomposition of the probe. This is clearly visible on Figure 10 representing a tumour spheroid stained with Calcein-AM, in which it is impossible to determine if the lack of signal in the internal spheroid is caused by lack of probe penetration, or high amounts of Fe(II).

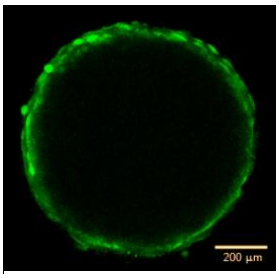

Figure 10 Cross-section image of DLD-1 cell spheroid exposed to Calcein-AM. Adapted from P3.

To overcome its drawbacks, we developed a more reliable, ratiometric probe for Fe(II) based on the Calcein-AM scaffold. We attached a metal-insensitive aminocoumarin to the fluorescein core of Calcein-AM to originate FlCFe1', which responds to the presence of Fe(II) with changes in intensity at two wavelengths. The resulting spectra can be deconvoluted (Figure 11) to show that the emission at 405 nm (from aminocoumarin) is unchanged in the presence of Fe(II), meaning the calculated ratio of two emission peaks can be used to reliably study Fe(II).

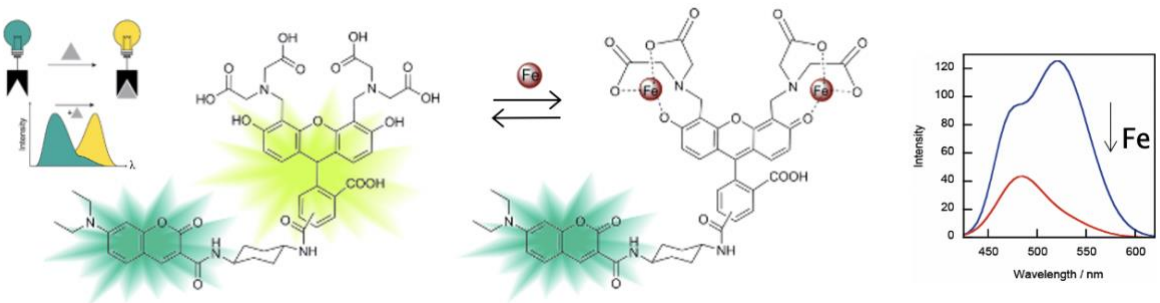

Figure 11 Structure and schematic of the ratiometric fluorescence response of the Calcein-AM-based FlCFe1' probe and the change in fluorescence emission of the probe in the presence of iron (right panel).

For cellular applications, we prepared an acetoxymethyl ester variant of FlCFe1', FlCFe1 which is analogous to Calcein-AM. Treatment of DLD-1 colorectal adenocarcinoma cell line with FlCFe1and subsequent exposure of the cells to Fe(II) salt led to an increase in the ratio of blue (425-480 nm, coumarin emission) to green (520 – 600 nm, calcein and Fe(II)-quenched emission) fluorescence in microscopy, in line with previous observations in buffer. Exposure of cells to iron chelator, in turn, leads to a decrease in blue-to-green ratio in comparison to cells exposed only to FlCFe1 probe with all differences being statistically significant. This clearly demonstrates the reliability of using FlCFe1 probe for studying labile Fe(II) pools in cellular models.

We also demonstrated that FlCFe1 is better suited to studying solid tumour spheroids, allowing evaluation of iron chelation therapeutics. To compare its performance to Calcein-AM, DLD-1 spheroids were treated with FlCFe1 (Figure 12). We found the intensity of fluorescence in each channel decreased towards the centre of the spheroid, but the ratio of blue-to-yellow light can be reliably measured deep into the cross-section of the spheroid even at reduced intensity. We have shown that the concentration of the probe decreases towards the centre of the spheroids, but the concentration of labile Fe(II) is the highest at approx. 50 – 100 μM from the spheroid surface – information which could not be inferred from using the Calcein-AM probe alone.

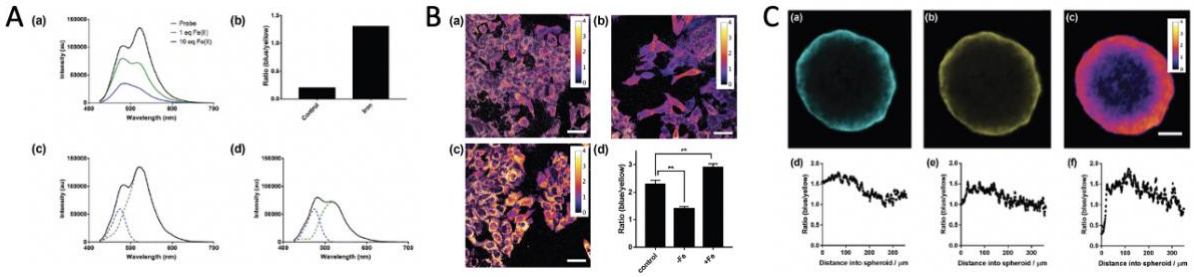

*Figure 12 A. (a) Emission spectra of FlCFe1' with 1 and 10 equivalents of Fe(ii) (λex = 405 nm). (b) Ratios of 490 nm to 530 nm emission in the absence and presence of Fe(ii) (λex = 405 nm). (c) Deconvolution of the FlCFe1' emission spectrum without Fe(ii). (d) Deconvolution of the FlCFe1' emission spectrum with 1 equivalent of Fe(ii). B. FlCFe1 can report on exogenous changes in iron levels. Confocal ratio images (425–480 nm/520–600 nm, λex = 405 nm) of DLD-1 cells treated with FlCFe1 (10 μM, 2 h) followed by 30 min treatment with (a) vehicle control, (b) salicylaldehyde isonicotinoyl hydrazone (SIH; 10 μM) or (c) ferrous ammonium sulfate (FAS; 100 μM). Scale bars represent 20 μm. (d) Changes in the blue/yellow ratio under different conditions. Error bars represent standard deviations of 5 replicates, **p < 0.01. C. Iron chelation agents only alter iron levels at the edge of the spheroid. Confocal microscopy images: (a) blue channel (425–480 nm), (b) yellow channel (520–600 nm) and (c) blue/yellow ratio images of DLD-1 spheroids (106 cells) incubated with FlCFe1 (10 μM, 24 h). Ratio intensity plots of spheroids subsequently treated for 2 h with (d) vehicle control, (e) deferiprone (100 μM) and (f) deferasirox (100 μM). The scale bar represents 200 μm. Adapted from P3.*

With such tool in hand, we then developed an assay to study the efficiency of iron chelators with potential clinical applications. It showed, as expected, that these compounds are effective in removing iron from the outer layers of the cells but cannot efficiently do so for deeper layers. The deeper layers correspond to areas of the solid tumours that exhibit hypoxia and are responsible both for drug resistance as well as metastatic reversal of the cancer after chemotherapy. This fluorescent assay can now be used, therefore, to test new iron chelators as potential therapeutics.

Summary (P3)

This manuscript (P3) describes the development of a novel fluorescent probe for Fe(II) with far improved reliability and cellular penetration than existing tools. I was able to harness the power of ratiometricity to apply this probe to study the effect of hypoxia in solid 3D tumour models and designed assays to study the effect of iron chelators with this probe.

4.C.3.1.3 Development of a ratiometric probe for detection of changes in labile Cu(I) pools in mitochondria (P4)

Like iron, copper is a redox active metal occurring in Cu(II) form when enzyme-bound and as Cu(I) in the labile metal pool. Copper, in addition to its role in enzymatic active sites, is involved in signalling and impacts cell migration, tissue invasion and metastasis. Disturbances in copper homeostasis have been repeatedly suggested as a mechanism of action of anti-cancer drugs, most prominently cisplatin. Most reported fluorescent probes for labile Cu(I) pools (LCPs) are turn-off intensity based and no ratiometric probes for studying the LCP in subcellular organelle were available.

In this work we developed a highly selective, reversible and ratiometric probe for Cu(I) called InCCu1 (Figure 13A), which can reliably detect changes in labile Cu(I) pools in mitochondria. Its thioether motif has binding affinities matching endogenous labile chelators, enabling it to compete for Cu(I) in cells but allowing reversibility of this process. InCCu1 was constructed on a hemicyanine fluorescent scaffold that exhibits biologically compatible fluorescent properties (orange emissions, significant Stokes shifts and high quantum yields). As in the case of the FlCFe1 probe, we also attached aminocoumarin through an alkyl linker to introduce a non-reactive, independent fluorescence band and transform it into a ratiometric probe.

Single-wavelength excitation of InCCu1 results in an emission spectrum with two distinct fluorescent maxima at approximately 480 nm and 600 nm. Addition of Cu(I) in aqueous solution buffered at pH 7.4 led to a decrease of fluorescence emission at 600 nm indicating Cu(I) binding, without an influence on the 480 nm. This allows for measuring a ratio of blue to orange fluorescence which was correlated to the Cu(I) concentration (Figure 13). I have also demonstrated that Cu(I) induced response is unaffected by a simultaneous presence of other metal ions, and that fluorescence is independent of pH, confirming the probe's specificity.

We have further showed that InCCu1 can be reliably used for monitoring of changes in the labile Cu(I) pools in mitochondria. We confirmed co-localisation of the probe with MitoTracker Deep Red (a mitochondrial stain) in colorectal adenocarcinoma cell line DLD-1. This is likely a result of the lipophilic cationic nature of the hemicyanine fluorophore, which promotes accumulation of this compound across the negative membrane potential of the mitochondria. Subsequently, we showed in fluorescence microscopy experiment that fluorescent ratiometric signal from InCCu1 in live cells changes upon exposure to Cu(I). By comparison, the ratio of fluorescence of control *InC* probe which lacks a copper-binding motif was unaffected by copper treatment.

One of my major contributions here included extensive optimisation and subsequent application of a robust and detailed experimental protocol for sample preparation, data acquisition and analysis. I particularly focussed on developing strategies to mitigate common pitfalls and avoid sources of artifacts. This was a critical achievement to avoid misinterpreting artefacts as real biological response, allowing quantitative data to be compared across samples. This original protocol allowed me to reliably correlate a modest fluorescent response of InCCu1 with exposure to increasing copper

concentration to demonstrate statistical significance. This protocol was intended to be applicable to virtually any fluorescent small molecule probes for bioinorganic and related analytes and as such provides a new, original, and powerful tool to ensure the reliability of experiment. The advantage of this new protocol is that it addresses potential sources of misinterpretation, data unreliability and artefacts at multiple stages: from sample preparation and probe selection, through microscopy hardware settings configuration and collection of images, laser excitation through to the final data analysis process. As a result, I further published these methodologies and techniques elsewhere[47] so that it can be readily applied by a wide range of potential users (researchers) who may not be specialised in the field of fluorescent probes.

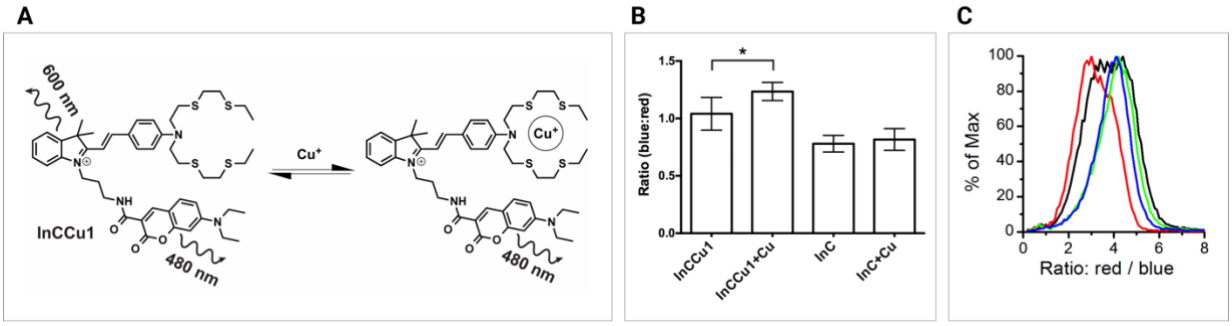

*Figure 13 A) Structure of indolinium-coumarin-copper sensor 1 (**InCCu1**) in equilibrium with Cu(I). B) **InCCu1** reveals a significant change in copper levels while InC does not. Cells were treated either with copper sulfate overnight (100 µM) or nothing, followed by **InCCu1** or InC (0.1 µM, 15 min) prior to imaging on a confocal microscope. Mean fluorescence ratio was calculated from ratios of intensities of blue channel (λ_{em} = 425-525 nm) over red channel (λ_{em} = 570-670 nm). Error bars represent standard error of mean for quintuplicate measurements. C) Flow cytometry with **InCCu1** reveals that cisplatin treatment compromises mitochondrial copper uptake. Histograms show ratio of red emission 585(15) / blue emission 450(50), excitation at 405 nm for DLD1 cells treated with **InCCu1** (0.2 µM, 15 min) following treatment with: PBS vehicle control - black, and copper sulfate (100 µM, 2 h) - red, or cisplatin treatment (10 µM, 16 h) followed by PBS vehicle (2 h) - green, cisplatin (10 µM, 16 h) followed by copper sulfate (100 µM, 2 h) – blue. Figure adapted from P4.*

To demonstrate its practical utility, we wished to use InCCu1 to interrogate the influence of Cisplatin on labile copper pools in mitochondria. As mentioned in 4.C.3., Cisplatin and its analogues are broadly used chemotherapeutics in cancer treatment. They have been initially believed to act through an induction of the DNA damage in fast-dividing cancer cells, but it has been shown that in some cases as little as 1% of cisplatin in cells accumulates in the nucleus with the rest being involved in other, largely unknown pathways. This observation may contribute to understanding the low-selectivity of cisplatin-based chemotherapeutics and major side-effects of its use in clinical practice. A large effort has been made to understand the true fate of platinum-based complexes in cells and possibly identify other mechanisms of action and could allow for the development of a more selective, safe, and efficient generation of anticancer therapeutic.

We wished to test the hypothesis that cisplatin interferes with the copper ion transport and homeostasis. In fact, cisplatin has been shown to interact with several copper transport proteins, but this is poorly understood due to a lack of suitable tools to monitor labile copper pools in different compartments of the cell. We used InCCu1 to reveal that mitochondrial labile Cu(I) pool is unaffected by copper loading in cisplatin pre-treated cells, while in untreated ones, the exposure of the cells to copper leads to increase in the LCP. We were able again to capture subtle disturbance in ratios of fluorescence intensities with statistical significance. The fact that cisplatin compromises uptake of

copper to mitochondria has been further confirmed also by flow cytometry using the same probe, increasing the reliability of this conclusion, and demonstrating the compatibility of this probe with diverse experimental techniques. As mentioned in subchapter 4.C.1, development of protocols for the use of the probes beyond microscopy is important to enable broader use of fluorescent probes in biology.

Summary (P4)

The detection of the labile metal pool is challenging due to the dynamic interaction kinetics of molecular carriers and binding groups found natively in the cell. Thanks to our probe design that includes a competitive binding motif along with a ratiometric fluorescent reporter, and development of protocols and data collection and analysis methods, I was able to show significant changes in mitochondrial Cu(I) levels in treated cells and apply this to study the effects of platinum therapeutic agents on mitochondrial Cu(I) uptake.

4.C.3.2 Selection and application of reliable probes for RONS

4.C.3.2.1 Deciphering of the role of mitochondrial RONS generation upon RSV infection with ratiometric tools (P5)

The achievements in subchapter 4.C.3. and subchapter 4.C.1 combined reveal new robust protocols, design strategies and criteria to improve the development and application of fluorescent probes to detect redox active molecules in live cells. In this section I have applied this knowledge to studying the effects of RSV viral infection of human cells on host mitochondrial reactive oxygen species generation using redox-sensitive fluorescent probes with extremely high reliability of results.

Conducted in collaboration with prof. David A Jans from Monash University (Melbourne, Australia), the main objective of this work was to shed the light on the pathogenesis of respiratory syncytial virus (RSV), which remained largely unknown despite a larger death toll of RSV infections than influenza. Within this work our collaborators demonstrated that RSV induces redistribution of mitochondria in the infected cells through a dynein/microtubule dependent mechanism and showed that RSV gradually reduces maximal and ATP-associated respiratory activity of host mitochondria with the effect increasing with a duration of the infection. As mitochondrial membrane potential was affected (decreased) upon RSV infection, a further investigation of RONS generation in mitochondria was difficult as vast majority of available probes could not be reliably used in this context.

Based on my extensive practical experience and literature knowledge (summarised partially in a separate review published elsewhere[48]), I was able to identify the use of a mitochondrially targeted flavin-rhodamine redox probe (FRR2)[49] as suitable for this application. As RSV infection diminished the mitochondrial potential of the host cells, this would also affect an uptake and subsequently a local concentration of virtually any mitochondria-targeted fluorescent probe. FRR2 is a reversible and excitation-ratiometric probe, which means a ratio between emission intensity in red channel (approx. 580 nm maximum) changes when excited by blue (488 nm) vs green (515 nm) light (**Error! Reference source not found.**). This ratio correlates with an increase in the level of RONS and is independent on the probe's concentration. This critical advantage is further supported by an extensive validation of the probe against a wide range of analytes showing that it is not affected by environmental changes or analytes other than RONS, and it can be reversibly oxidised and reduced upon several cycles with minimal-to-no fatigue[49]. These parameters, as described in subchapter 4.C.1 adhere to most criteria for a robust and reliable probe.

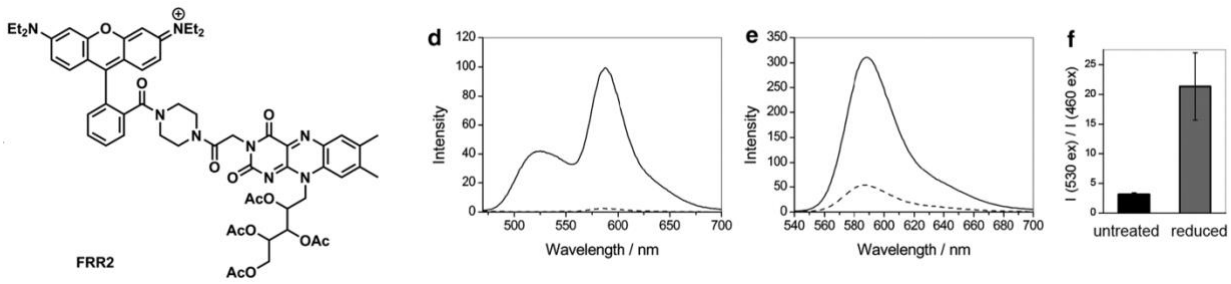

Figure 14 Left: Structure of FRR2 (Flavin-rhodamine redox sensor 2). Right: Fluorescence response of ***FRR2*** *to reduction. Fluorescence emission spectra of* ***FRR2*** *(10 μM) in oxidized (*black line*) and reduced (*dashed line*) forms upon excitation at 460 nm* ***(d)*** *and 530 nm* ***(e)****. Probes were reduced using 200 equivalents of $Na_2S_2O_4$. The ratio of the emission of FRR2* ***(f)*** *at 580 nm upon excitation at 530* versus *460 nm in oxidized (*black*) and reduced (*gray*) forms. All data were acquired in 100 m*M *HEPES buffer, pH 7.4. Error bars represent standard deviation (*n = *3). Figure is adapted from Kaur et al 2016* [49] .

Following the selection of the probe, I developed a protocol for data collection and analysis by implementing the workflow described in section 4.C.1 The intensity ratio image was obtained by individual pixel division of the collected images at two excitation wavelengths. Segmentation of ROIs was performed on a MitoTracker DeepRed image by employing an automated auto-threshold Otsu algorithm followed by a removal of regions smaller than 1000 pixels (smaller than expected size of mitochondria). Such segmented regions were determined to quantify the ratios in the images.

Application of this methodology allowed us to demonstrate that RSV infection increases ROS level in mitochondria of the host cell already after 8 h and significantly after 18 h and that this response is similar to the exposure of the host cells to the rotenone – an inducer of mitochondrial oxidative stress (Figure 14). Interestingly, it has also shown that the addition of MitoQ, which is an inhibitor of the microtubule reorganisation, prevents this increase in RSV-induced mitochondrial oxidative stress (Figure 14B). This observation made possible by judicious choice and robust application of reliable fluorescent redox sensor and appropriate protocols was critical to confirm the main thesis of the manuscript that RSV uses host's mitochondrial oxidative stress for promotion of the infection. It also showed that treatments blocking ROS generation protect against RSV infection in A549 model cell line as well as in patient derived bronchial epithelial cells and *in vivo* in mouse model.

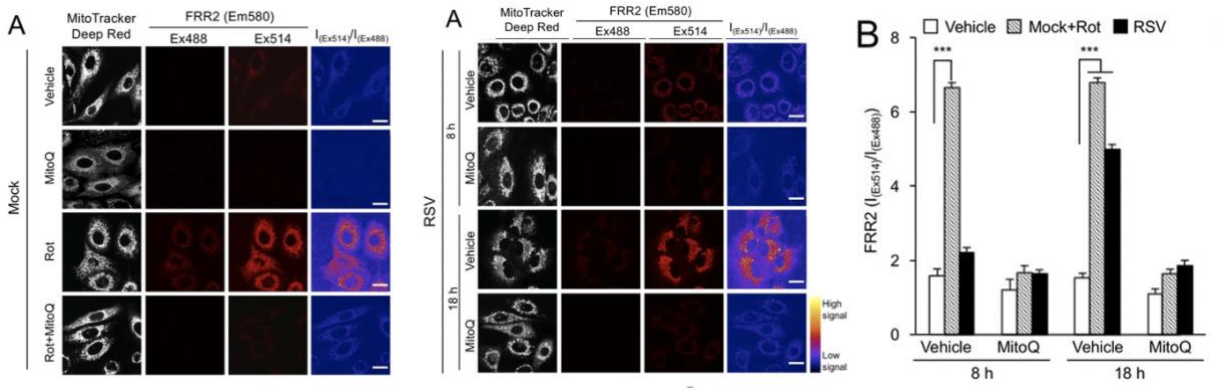

Figure 15 A549 cells mock-infected (panel A left) or RSV-infected (panel A middle) for 8 or18 h p.i., treated as indicated for final 2 hs prior to staining with mitochondria-specific ROS FRR2 probe (red colour on images; 2 μM, 15 min prior to imaging – emission collected for separate excitation at 488 nm and 514 nm and ratio of emission at two different excitations calculated – blue-red-yellow panels)) and Mitotracker Deep Red (white; 100 nM, 15 min): the mitochondrial complex I inhibitor rotenone (Rot, 0.5 μM), the mitochondria-specific ROS scavenger mitoquinone

*mesylate (MitoQ, 1 µM), NCZ (17 µM) or DMSO as a vehicle; in the case of dual Rot/MitoQ addition (Rot + MitoQ), Rot was added 4 hr before staining. Results represent the mean ± SEM for n = 3 independent experiments, where each experiment analysed 25–30 cells per sample, ***p<0.001. Adapted from P5.*

Summary (P5)

The experiments described above shed new light on the pathogenesis of RSV infection and open a way to new therapies against RSV infection. The reliable observation of the changes in mitochondrial ROS generation in this model with affected mitochondrial potential was very difficult to achieve but played key role in the success of this work. I ensured it by a careful selection of the ratiometric and thoroughly validated probe FRR2 meeting criteria from section 4.C.1.1 and application of a robust workflow described in chapter 4.C.1.3 on using fluorescent probes in cells.

4.C.3.2.2 Improving the reliability of sensing RONS with fluorescent lifetime imaging using a nitroxide-based probe (P6)

Fluorescence lifetime is the time between the absorption of the quant of energy and its emission. It is an attractive parameter for analytical purposes as it is sensitive to the environment of the emitter but is independent of the fluorophore's concentration, and unaffected by absorption/emission efficiency. Fluorescence lifetime is increasingly utilised in Fluorescence Lifetime Imaging Microscopy (FLIM), which primarily measures endogenous fluorescence as the number of probes validated and developed for this technique is limited.

As discussed in 4.C.1, nitroxyl radicals are redox sensitive responsive moieties explored in the design of both irreversible and reversible fluorescent probes for RONS. Their reactivity can be fine-tuned through structural modifications of this unusually stable organic radical and can serve for development of RONS-sensitive probes. One example has been developed by the group of Prof. Steven Bottle from the Queensland University of Technology by judicious incorporation of the radical moiety into a Rhodamine scaffold (5b in Figure 16). The probe has been shown to localise in mitochondria and its intensity-based fluorescent response to RONS has been confirmed *in vitro* as well as in hTERT immortalised human fibroblasts treated with rotenone, which induces increased oxidative stress in mitochondria and therefore led to a lower fluorescence intensity. However, as with all intensity-based probes, it was unclear whether an observed change was truly due to the redox response of the probe or a change in its concentration.

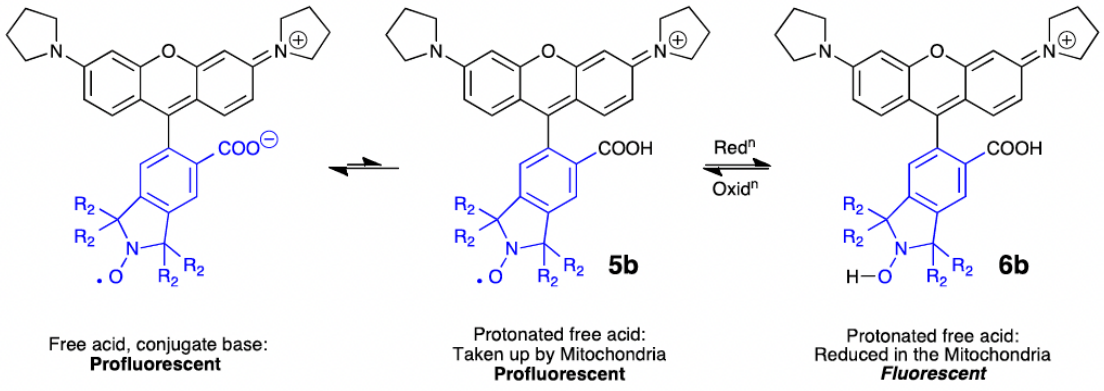

Figure 16 Protonation and redox equilibria of 5b – profluorescent probe for redox state in mitochondria. Deprotonated form of 5b, most probable to occur in cytoplasm, where reduction is not efficient (left structure), and its protonated

form (probably uptaken to mitochondria due to a positive charge) – middle as well as reduced form of 5b (fluorescent, most probably dominant in mitochondria) – right panel (6b). Adapted from P6.

To address this problem, I proposed, designed and performed validation experiments of the redox responsiveness of the probe in cells using FLIM. Fluorescence lifetime of the rhodamine emitter is expected to lengthen when reducing a fluorescence-quenching nitroxyl radical to a hydroxyl amine. This change should be independent of the probe concentration and correlate with the presence or absence of the radical and as such act as reliable proxy for the redox state of the environment. Confocal microscopy experiments on 5b-loaded DLD-1 cells resulted in decrease of fluorescence upon treatment with H_2O_2 as well as two inhibitors, rotenone and antimycin, disrupting mitochondrial redox balance and leading to oxidative stress (Figure 17). To confirm that the effect is truly related to environment-induced shifting of redox equilibrium of the probe towards nitroxyl radical and not just local change in probes concentration, I performed FLIM experiment in the same conditions, Fluorescence lifetime data fitted to bi-exponential decay yielded two lifetime components of 1.3 ns and 2.9 ns, corresponding, respectively to pro-fluorescent radical form 5b and fluorescent hydroxylamine form 6b (Figure 16). Cells exposed to oxidising conditions exhibited decreased average fluorescence lifetime, corresponding to an increased occurrence of shorter lifetime component assigned to radical, oxidised form 5b (Figure 17 B-F). This reliably confirms true sensitivity of the probe to the redox environment of the cell and provides a more reliable way of monitoring it in biological models.

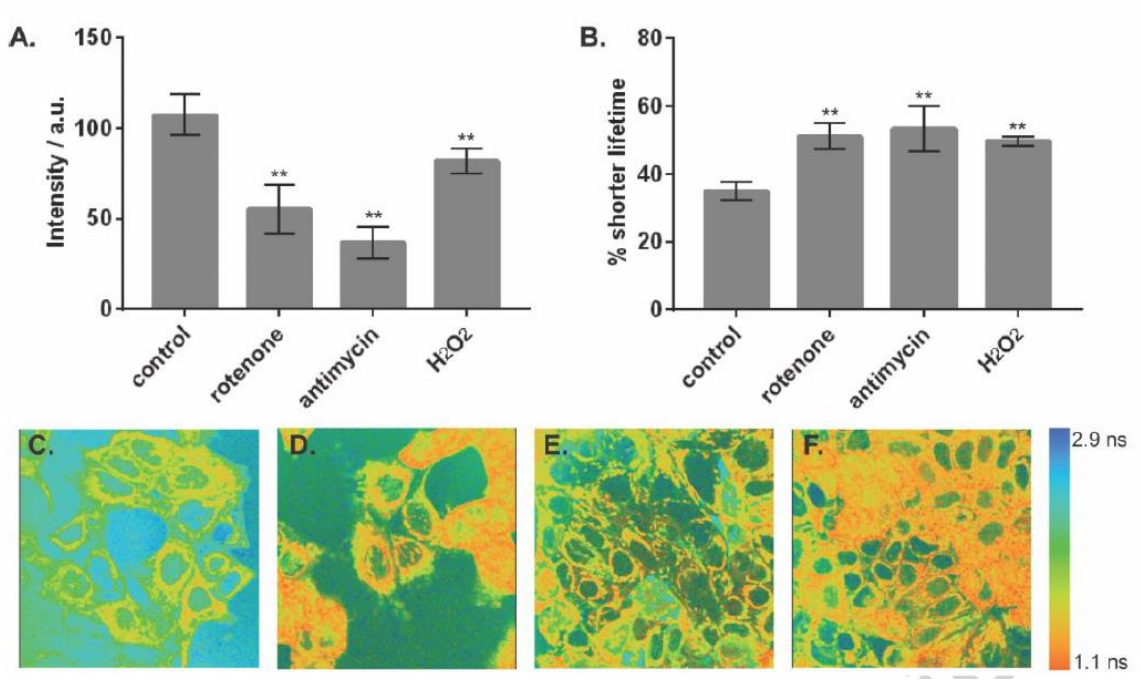

*Figure 17 Fluorescence lifetime imaging microscopy enables ratiometric measurement of the proportions of pro-fluorescent radicals and non-radical forms. DLD-1 cells treated with **5b** (100 nM, 15 min) followed by vehicle control, rotenone (10 µM, 15 min), antimycin (10 µM, 15 min) or H_2O_2 (50 µM, 15 min). Panel **A.** Fluorescence intensities from confocal microscope images of cells, 488 nm excitation, 550-650 nm emission. Panel **B**. % of shorter (1.3 ns) lifetime from fluorescence lifetime imaging, 820 nm two-photon excitation, 550-650 nm emission. Panels **C-F**. Fluorescence lifetime images showing average lifetimes for **C:** Control, **D:** rotenone, **E:** antimycin and **F:** H_2O_2 n=3, data reported as mean ± st. dev. Adapted from P6.*

Summary (P6)

These experiments confirmed that 5b can be used as a reliable fluorescent probe for monitoring of changes in mitochondrial redox state by monitoring of changes of its fluorescence lifetimes. It also demonstrated how by applying FLIM protocols one can eliminate ambiguity in interpretation of the response of intensity-based probes making this approach available for application with a wide range of responsive tools.

4.C.3.3 Summary (P3 – P6)

I have developed and applied protocols for more reliable detection of analytes in complex cellular models with the use of a signal that is independent on probe concentration, overcoming one of the biggest drawbacks of most fluorescent tools.

To introduce improved fluorescent tools to the field, I have developed two ratiometric probes for labile metal ion pools (Fe(II), Cu(I)) with improved reliability of detection in complex biological models (3D tumour spheroids and subtle changes in mitochondria). I was able to apply these tools to study multiple biological questions relevant to the physiology, progression, and treatment of cancer.

Then, in the context of detecting RONS, I worked to address the selection of the right tools and robust application and data analysis protocols to reliably study RONS in cells. By leading the experimental design and probe selection, I was able to use this technique to overcome the tough challenges posed by measuring oxidative stress in mitochondria affected by viral infection (due to the resulting change in redox potential and impact on probe accumulation). I also explored fluorescence lifetime imaging, a new avenue to reliably measure oxidative stress with intensity-based tools.

4.C.4 Introduction and advancement of dual-analyte probe design for biological applications

The achievements described in 4.C.1-4.C.3 focus on the reliability and development of probes detecting *singular* targets. The functions of these analytes (RONS and metal ion species), however, are known to change depending on their biological context and surroundings. For example, H_2O_2 is a relatively stable molecule which can exist in the cytoplasm for long periods, but in the presence of Fe(II) or Cu(I) can undergo Fenton-type transformations into the short-lived hydroxyl radical. This reaction is affected by viscosity and pH, which is estimated to vary dramatically in cells in the range of 4.5 to 9. Furthermore, elevated levels of RONS, Cu(I) and Fe(II) have all been implicated in the onset of neurodegeneration and cancer, which can in turn be targeted to develop therapeutic programs (based on antioxidants and metal chelation).[44,50–55] Therefore, the ability to measure multiple parameters simultaneously with spatial and temporal resolution would provide scientists a way to uncover the biological meaning of their interactions with each other and their environment. To this end, I aimed to unlock a new dimension of biological imaging by introducing the field of multi-analyte sensing probes.

In the seminal work discussed below (P9) I have introduced the first systematic foundation for the development, validation and use of dual-analyte fluorescent probes in biological models. Unlike the above-mentioned probes which detect the presence of only single analytes, dual-analyte probes generate a distinct signal when two analytes are present simultaneously. The ability to detect both targets with one probe provides powerful information about the relative relationship between the

analytes by ensuring the proximity of their occurrence and removing issues of variable localisation, concentration and metabolic stability of two separate single-analyte probes.

The concept of a multi-analyte sensor originates from the field of molecular logic gates. Many such compounds have been reported, but only a handful of isolated examples were tested and used in biology and have not yet been applied with sincere effort to cellular studies. In this work, I have summarised the few successful examples and, what is critical, established prerequisites specific for multiple-analyte probes, including a consideration of a particular type of analyte response required to ensure reliability in biology. These need to be considered in addition to all other criteria of ideal fluorescent probes discussed in chapter 4.C.1 (including biocompatibility, stability and operability in aqueous conditions – something most existing molecular logic gates are not compatible with). My list of original prerequisites and considerations, specific for dual-analyte probes, includes:

1. Type of logic gate that ensures reliable specificity of response

The co-existence of the two analytes needs to be distinguishable from the presence of none or one of either analyte, therefore these probes need to meet the criteria of an "AND" logic gate (signal from the presence of both analytes is different from all other situations – it is specific, Figure 18A). While a negative AND (NAND) logic gate could also be used, it is less preferred because it exhibits a turn-off type of response, which cannot be reliably discerned from lack of probe.

2. From specificity to selectivity

Within the "AND" type of response, three different situations arise (Figure 18B). In all three, signal arising from both analytes binding simultaneously is always distinct from the rest (specific). However, variant (iii) allows for not only a specific detection of simultaneous presence of two analytes but also selective detection of each of the 4 combinations (none, analyte A, analyte B or both analytes) by generating a different signal in each case.

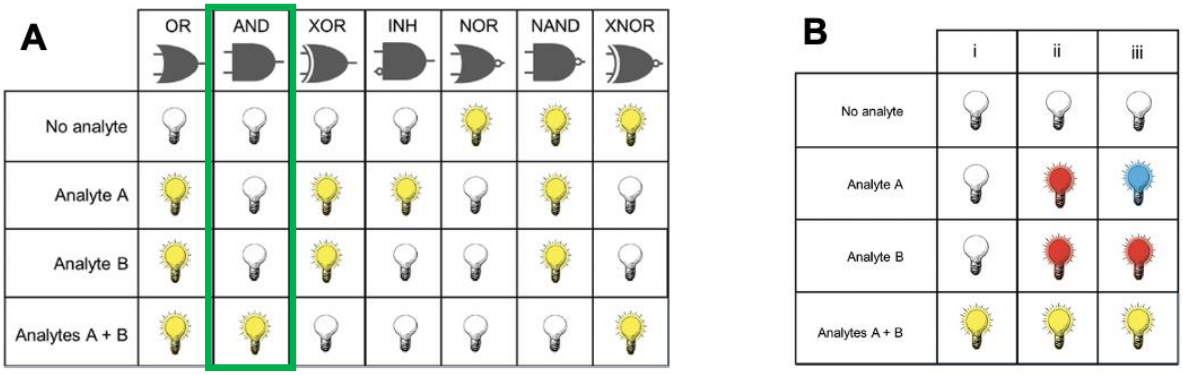

Figure 18 Truth tables A) for two input logic gates, showing the combination of inputs (analytes A and B) for which there is a measurable response in the output. XOR = exclusive OR, INH = inhibit, NAND = negative AND, XNOR = exclusive NOR; B) for sensors in which the response to analytes A + B can be distinguished from the presence of no analytes, or of only analyte A or only analyte B. Adapted from P9.

3. Ratiometric response is required for reliability

As in the case of single analyte probes, if the response of the dual-analyte probe to the analytes is based solely on a change in fluorescence intensity, it suffers from the same issue where a high concentration of probe is indistinguishable from a positive response. Therefore, to ensure a true and

reliable detection, a ratiometric response in the presence of two analytes is even more important that in the case of the single-analyte probes.

4. Reversibility and sequence of response

To detect two analytes, two recognition events need to occur (one for analyte A and one for analyte B). Each can have a reversible or non-reversible character, leading to three possible combinations: both reversible, both irreversible and one reversible one irreversible. In addition, each recognition motif and each recognition event could occur independently or sequentially (e.g., reaction with analyte A allows for analyte B to react). This design could open a new way to study the sequence of appearance of the analytes in biology, which cannot be accessed by available tools.

Taking all the above into consideration, many permutations of designs are possible with each suitable for different types of biological interrogations.

Summary (P9)

In P9 I formulated fundamental theoretical background that initiated in earnest a new field of fluorescent probe research and design based on dual-analyte sensing. With the ability to introduce a completely new dimension of data, dual analyte probes could deepen and even transform our understanding of the function of bioinorganic and other analytes in biology. I have described also why these probes need to exhibit at minimum an AND type, specific response to the presence of two analytes simultaneously and be compatible (specific, stable, membrane permeable and non-toxic) with biological environments. Following the publication of this work, reports of such probes increased exponentially with tens of new dual-analyte probes with demonstrated use in biology reported recently. Over the last four years this work has consistently held a title of "Highly cited paper" in the Web of Science database with over 200 citations, demonstrating the significance and impact of this work.

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4.D Summary of the most important achievements of the candidate in the presented publications

My cumulative knowledge and research experience allowed me to contribute:

- Original theoretical findings to support the design and validation of next generation fluorescent probes,
- Active development and application of fluorescent probes to answer challenging biological questions in cellular models, and
- A burgeoning new field of research based on dual-analyte probes,

which together have made a significant impact on the field of molecular imaging using fluorescence-based tools for bioinorganic analytes and improved the reliability of experiments in which they are applied. The key achievements are summarized as follows:

- **Providing new design criteria and methodologies to improve the reliability of generating, validating, and using fluorescent probes for studying bioinorganic analytes in cells**
  - Identifying new original relationship between the structure and the selectivity/reversibility of response of fluorescent probes to bioinorganic analytes
  - Setting up criteria for ideal fluorescent probe for biological application and outlining required validation workflows to ensure reliability of detection
  - Systematising and developing the concept of dual-analyte fluorescent probes for biological applications in live cells as a means to improve reliability of studying relationships between different parameters
- **Harnessing chemistry for the development and validation of reliable and robust practical fluorescent tools (probes, assays, and protocols) to study bioinorganic analytes**
  - Robust protocol development for applying fluorescent probes to detect bioinorganic analytes in cells
  - Development of a specific probe for trans-aryl platinum(II) complexes with therapeutic potential and related robust assay to evaluate their metabolic stability in vitro
  - Development of a ratiometric version of calcein-AM probe for more reliable detection of labile iron(II) pools and a related assay to test the efficacy of iron chelation therapy in solid tumours
  - Development of the first ratiometric probe for monitoring of labile copper pools in organelle (mitochondria)
  - Using fluorescence lifetime imaging for more reliable (independent on probe's concentration) detection of RONS in cells with a use of intensity-based probes
  - Applying probe and protocol for reliable RONS detection in mitochondria independent on membrane potential
- **Acquiring new understanding of biological processes related to bioinorganic analytes through the use of these tools**
  - Demonstrating that glutathione can sequester transplatin complexes and that their stability to glutathione may correlate with their cellular activity
  - Discovering an existence of a zone of higher concentration of labile iron(II) pools in 3D models of solid tumours (spheroids)
  - Showing significant difference among clinically approved chelators in the efficiency of iron chelation in solid tumour models (spheroids)
  - Uncovering cisplatin inhibition of copper uptake to mitochondria
  - Demonstrating that nitroxyl radicals can reversibly report on redox status of the cells in mitochondria
  - Proving that respiratory syncytial virus uses changes in RONS generation in mitochondria for promoting infection
- **Burgeoning new field of the development and application of dual-analyte probes in biological research**
  - Formulating key foundations, challenges, directions of development and opportunities associated with dual-analyte probes in biological research and drawing attention to these

tools in biological context (over 200 citations in 4 years, uninterrupted "Highly cited paper" label in Web of Science database)
    - Inspiring tens of research teams from around the world to work on dual-analyte probes which led to a creation of tens of new tools of this type (>5 time increase in the number of such tools compatible with applications in biology since publication of my work)

4.E Discussion of possible applications of the achieved results

The achieved results presented above demonstrate significant contributions to the reliable design, development, and use of fluorescent probes in studying bioinorganic analytes in biological models.

- For chemical biologists, the results provide new strategies and pathways to design novel fluorescent probes with unprecedented reliability, as well as protocols and means to validate them and enable their reliable application in answering biological questions.
- The development of new protocols can help large number of biomedical researchers to reliably apply commercial as well as newly published probes to make their results more trustworthy and accurate.
- Understanding disease – the probes developed as well as their future generations can help in fundamental understanding of pathophysiological processes which are increasingly evidenced to be controlled or mediated by bioinorganic analytes. Differences in bioinorganic profiles of different diseases (e.g. cancer, neurodegeneration, inflammation) and their stages can open up a way to develop bioinorganic analyte-based biomarkers of disease and treatment in the future
- The results can inform the design of improved therapies by
    - development of fluorescent assays to identify bioactive molecules rapidly but also reliably by monitoring if they affect bioinorganic analytes in live cells
    - evaluating the (i) stability of Platinum-based anticancer agents (ii) efficacy of drug candidates (iron chelators) as well as
    - deciphering mechanisms of action of drugs – i.e. whether they go through a disturbance in copper(I), iron(II) or RONS homeostasis (e.g. in high throughput high content imaging assays)
- Opening up and initiating a new area of research into dual analyte probes for biological applications, contributing to at least a 5-fold increase in biologically relevant dual analyte probes in the last 4 years and influencing the research programmes of many groups around the world who have now started work on these.

5. PRESENTATION OF SIGNIFICANT SCIENTIFIC OR ARTISTIC ACTIVITY CARRIED OUT AT MORE THAN ONE UNIVERSITY, SCIENTIFIC OR CULTURAL INSTITUTION, ESPECIALLY AT FOREIGN INSTITUTIONS

While my education (BSc and MSc) was completed at Polish institutions, the remainder of my scientific career took place in foreign institutions up until my appointment at the Institute of Bioorganic Chemistry, Polish Academy of Sciences in September 2017.

5.A Description of scientific activities not included in the scientific achievement 4.C

5.A.1 Research carried out at the School of Chemistry, University of Sydney, Australia (2014-2017)

In addition to the scientific achievements described in section 4C, the main subject of this application, I was also involved in several other research projects during my post-doctoral fellowships at the University of Sydney in the group of Prof. Elizabeth New. The first group of projects relate to similar themes as those mentioned in 4C (development of fluorescent probes for biological targets/parameters of interest). One example is my contribution to the *in vitro* characterisation and proof-of-concept demonstrations of the first carborane-based fluorescent probe to stain cellular lipid droplets (P17 – co-first author). I have also resynthesized and confirmed the reversibility of a fluorescent coumarin-based silver(I) probe which was used to study the stability of silver-based nanoparticles in aqueous media mimicking real-life environment (P15). To further the impact of this work I initiated a collaboration with the group of prof. Wojciech Chrzanowski. My expertise in robust validation and characterisation of fluorescent probes have also been employed to study fluorescent properties of a set of terpyridine-aryl tetrazoles synthesized by the group of prof. Max Massi. My work led to identification of several of these compounds to act as sensors of notoriously difficult discrimination of Zn(II) from Cd(II) (P16). Through my broad knowledge on the design, validation, and use of fluorescent probes for redox state (as described in subsection 4.C1.) I have been able to contribute significantly (as co-first author) to a review manuscript discussing the strategies for targeting of fluorescent probes to sense redox changes in mitochondria (P18). This has also informed my work in reliable characterisation of changes in generation of reactive oxygen species in mitochondria of human host cell upon infection by RSV, which have been reported in manuscript P5 described already in the achievement section.

A second group of projects revolved around the development of metal-based complexes as magnetic resonance-compatible probes for biochemical analytes. In this work my main role was contributing to the design of the tools as well as supervise two PhD students in their experimental efforts, analyse results and help draw conclusions to decide on the next steps. We successfully developed the first magnetogenic cobalt complexes with potential application in the detection of reducing environments in cells (P21) and the first Cobalt-based paraSHIFT sensor for anions in aqueous media (P19). By using knowledge I acquired in the research on nicotinamide as redox-responsive moiety, I contributed to the design and then data analysis of a performance of a redox-responsive MRI contrast agent based on Gd(III) and bearing nicotinamide as a redox-active group (P14). I have also applied my expertise built through my PhD work to contribute to the work of prof. Matthew Shores on the development of iron(II)-based magnetogenic complexes (design of the compound, assisting in data interpretation), which resulted in co-authorship of the manuscript P12.

5.A.2 Research carried out in the Department of Molecular Probes and Pro-drugs, IBCH PAS (2018-2022)

After my postdoctoral experience at the University of Sydney, I moved to the Institute of Bioorganic Chemistry, Polish Academy of Sciences in Poznań and established an independent research agenda as head of the Department of Molecular Probes and Prodrugs. With extensive interdisciplinary collaboration, I have been leading my team to work on several projects.

The main direction of my exploration follows the seminal work on the design and concept of the use of dual-analyte probes in biological applications, as described in achievement (P9). I have secured funding and successfully explored the development of a dual-analyte fluorescent (HOMING funding scheme) but also bioluminescent (SONATA funding scheme) probes for the detection of pairs of bioinorganic analytes and enzymes as well as robust assays to use those tools in practice. We have developed a novel robust, green synthetic method for a formation of amide bonds as robust biocompatible linkers (P11) which we can now apply to the conjugation of different components of the probes together. Furthermore, we successfully developed (manuscripts in preparation):

- **dual-analyte fluorescent probes** for more reliable detection of a) hypoxia (reduction & pH) b) ferroptosis (iron(II) & oxidative stress) and c) susceptibility of human (host) cell towards SARS-CoV-2 infection (two enzymes) – subjects of 4 manuscripts in preparation
- **dual-analyte detection tools based on bioluminescence** for a) hypoxia b) iron(II) and c) reduction capacity of the cells (enzymes) – subjects of 3 manuscripts in preparation.
- **robust multiparametric assays for practical applications**, including a) high-throughput miniaturised assay for identification of inhibitors of RNA viruses infection of human cells b) multiparametric and multidimensional assay for detection of ferroptosis – both subjects of patents in preparation.

Under my supervision and guidance, two post-doctoral fellows I am hosting are recently working on projects in multiparametric detection and imaging. One grant was obtained within the PACIFIC funding scheme of Horizon Europe COFUND, led by dr Mariia Dekaliuk aiming at the development of a signal amplification FRET-based assay for the quantification of multiple proteins in liquid biological samples. Our model targets are three broadly used protein biomarkers: prostate specific antigen (PSA), fetoprotein (AFP) and cancer embryonal antigen (CEA). The second project (SONATINA funding scheme) led by Dr Magdalena Derbis aims at developing a toolbox of assays and imaging methodologies for studying an aggregation and dissolution of polyglutaminated FMR1 protein responsible, among others for Fragile X-associated tremor/ataxia syndrome rare disease. The focus is on both the development of new probes to visualise aggregates but also robust image acquisition and analysis protocols to study the process with unprecedented spatial and temporal resolution (including the use of the most advanced fluorescent imaging technology, MInflux, enabling imaging with unprecedented resolution even < 5 nm).

Another research direction I have opened in my department concerns the development of chemical compounds that covalently attach to proteins *in situ* with improved selectivity. We are exploring the development of such tools for a variety of applications, including:

a) imaging of the local microenvironment of proteins with unprecedented resolution by a combination of chemical probes (OPUS funding scheme) and state-of-the-art unique imaging infrastructure for Minflux fluorescent imaging with resolution down to a single nanometer (single molecule - Polish Roadmap of Research Infrastructure project NEBI)
b) identification of targets and off-targets of drug candidates (EU-OPENSCREEN-DRIVE project within Horizon 2020 INFRADEV funding scheme, in collaboration with biomedical expert, prof. Alwin Krämer from Helmholz DKFZ Institute, Heidelberg, Germany.
c) Development of covalent inhibitors for improved specificity and efficacy (e.g. MINIATURA funding scheme project led my postdoctoral fellow, dr Michał Jakubczyk)

5.A.3 Research carried out at the Centre for High Throughput Screening Studies, IBCH PAS (2021-2022)

In addition to leading my research group, I am also Director of the Centre for High Throughput Screening Studies (CHTSS) at the Institute of Bioorganic Chemistry, Polish Academy of Sciences. Although the project has commenced as an infrastructural endeavour to be completed in 2023, I managed to develop and open the centre for research operations in 2021, two years ahead of schedule. Two main aims of CHTSS are (i) to support research of scientific community in Poland and abroad with expertise and infrastructure in high-throughput discovery and validation of bioactive molecules and (ii) to conduct research into the improvement of assays and methodologies for more reliable and informative identification and characterisation of bioactive molecules. The Centre functions as a founding partner site of pan-European EU-OPENSCREEN European Research Infrastructure (ERIC) of 33 groups / platforms from 10 countries in Europe certified for the highest scientific credibility and reliability. The concept of EU-OPENSCREEN has also been described in a peer-review publication (P13).

In the last two years I contributed to over 20 research projects through experimental design, planning and data interpretation of assay development and/or screening phases. These collaborations are listed in part 5B. Below I describe a selection of three research directions which are being developed long-term and led by my own initiative to strengthen the position of the Centre on international arena.

The first area of my research within the framework of CHTSS was to establish high-throughput protocols and workflows for experimental evaluation of the fundamental properties of small molecules to assess their potential to interfere in a non-specific way with commonly used analytical assays and technologies (so called "bio-profiling"). In particular I have been the main EU-OPENSCREEN-ERIC contributor to the conceptual selection and experimental protocol design for maximum reliability of the set of assays to evaluate the tendency of small molecule compounds to (a) generate reactive oxygen species, (b) inhibit luciferases used in bioluminescence-based assays and (c) bind/interact with metal ions, all of which are the most common sources of artifacts in screening campaigns. We have also won a competitive call to perform ROS-generation (three assays identifying different mechanisms of ROS generation) and luciferase-inhibition (three different luciferases) screenings on the EU-OPENSCREEN-designed diversity set (library) of 100 000 compounds. The results of this work are being gradually uploaded into a dedicated EU-OPENSCREEN-owned and fully open-access European Chemical Biology Database (https://ecbd.eu). In addition, on the basis of this work, we are now also developing algorithms and approaches to reliably identify and flag compounds with potential off-target reactivity, to complement the existing algorithms (like PAINS descriptors) and aid in reliable identification of true and specific bioactive molecules and early-eliminate those which can lead to false positives.

The second area focuses on the development of multiparametric fluorescent imaging-based high-throughput assays for phenotypic evaluation of the activity as well as off-target toxicity of any chemical agents. In particular, we employ a so-called cell-painting assay which is based on staining several elements of cellular environment (nucleus, mitochondria, cytoskeleton, membranes and Golgi apparatus) and comparing changes in staining patterns caused by different treatments versus those caused by control treatments of known mechanisms of action.[1] This allows not only for reliable identification of bioactive molecules but also gives insight into their therapeutic mechanism. Depending on biological models (e.g., cell lines / cell types) used to study the effects of small molecules, a cell painting assay can also give insight into the off-target non-specific and/or toxic

activity of the compounds. As such it is one of the most powerful and reliable ways of identifying true and specific bioactives and early stage elimination of those which in later stages (e.g. clinical) may be revealed as unsafe, avoiding waste of resources that can otherwise be invested in further development and clinical testing of bioactives. Our research in this area focuses on alternative staining protocols (including alternative stains as well as staining different sets of cellular targets) as well as new image analysis algorithms to improve the sensitivity and scope of different mechanisms identifiable by the assay. In addition, we also work in collaboration with various partners to adapt and apply cell-painting assay to confirm safety of hit compounds identified in screening (project led by prof. Anna Kurzyńska-Kokorniak within a Fast Track funding scheme of NCBR) and expanding it to testing of safety of nanomaterials (pillar II project iCARE of Horizon Europe funding).

The third research direction I explored within CHTSS is the development and application of tools to identify binders of 3D RNA structures. While the vast majority of treatments focus on proteins as the most common molecular targets of therapies, many of them are considered undruggable. In addition, increasing numbers of RNA targets are believed to be of importance for disease development, progression, and resistance to treatment. This has widened the search of small molecules to those that could bind and modulate the stability and/or activity of RNA molecules. Recently, we have adapted and optimised a previously published indicator-displacement assay[2] for the detection of binders of RNA loops and successfully utilised it in the identification of bioactive/drug candidates for treatment of influenza and SARS-CoV-2 infection. We are now working in close collaboration with several academic and industrial partners (e.g., as partners in ERC Starting Grant and OPUS funding scheme projects) carrying out research to expand a range of assays for detection of binding to different types of 3D RNA structures and identification of broader range of RNA-targeting small molecules.

5.A.4 Research carried out before obtaining the doctoral degree

École Normale Supérieure de Lyon, France

The main topic of my PhD work was focussed on development of iron(II) based metal complexes which could switch between different magnetic states upon external stimulus for potential applications in sensing. In most complexes iron(II) possesses unpaired d-electrons which make those compounds paramagnetic. In contrast, gadolinium is always paramagnetic (7 unpaired electrons), and therefore used as a common contrast agent in the Magnetic Resonance Imaging, one of the most powerful techniques in medical imaging. However, gadolinum-based responsive contrast agents will always suffer from a residual background signal even before interaction with a target due to their constant paramagnetism. This creates a significant risk of mistaking an increase of the contrast due to an interaction with molecular target (switch from off to on state) with an increase caused by an accumulation of the off but not fully silenced form of the Gadolinium-based probes. Iron(II), on the other hand can be switched between a truly diamagnetic state to a paramagnetic state by a change in its coordination sphere.

We published a review in which I defined for the first-time criteria for truly *off-on* magnetogenic (diamagnetic to paramagnetic) probes, and discussed the step-by-step design of such tools while critically evaluating current attempts to develop such tools (P22). I also prepared and characterised a range of binary diamagnetic, paramagnetic, and spin-cross-over iron(II) complexes based on bispidine ligands (3,7-diazabicyclo[3,3,1]nonane (P23). They included a) only a second example of binary diamagnetic-paramagnetic pair of iron(II) complexes suitable for the development of

magnetogenic probes and b) demonstrated a first spin cross-over behaviour in bispidine complexes. My expertise in this field was acknowledged, among others, through an invitation to advise the leading expert in the field (Prof. Peter Comba – author of over 50 manuscripts on bispidine-metal complexes) on the design and experimental protocol to prepare this new type of bispidine ligands and their complexes. This further demonstrates the originality and excellence of my achievement.

Jacobs University, Bremen, Germany

My first experimental projects were carried out from 2007 to 2009 in the group of Prof. Gerd-Volker Roeschenthaler initially at the University of Bremen and then at Jacobs University Bremen (in total 12 months), focussing on the synthesis and thorough spectroscopic characterisation of fluorinated phosphonates. In comparison to phosphates and phosphopolyesters which are present broadly in nature, phosphonates possess a more stable P-C bond which replaces one of the P-O-C links, making phosphonates often less susceptible to hydrolysis while maintaining the sterical and physical character of phosphates. Introduction of fluorine into organic compounds, often in the place of hydrogen, allows for a dramatic change in chemical properties (due to fluorine's electronegativity and reversed polarisation of C-F bond in comparison to C-H) with only small changes in the steric requirements (fluorine has the closest atomic radius to hydrogen out of non-noble gases). This leads to an increasing use of fluorinated compounds in a wide range of applications, from bioactives (e.g., pharmaceuticals, biocides), to surfactants and ionic liquids to anti-corrosives, paints and more. In my work, I synthesized over 20 fluorinated arylphosphonates (P24) and worked on an industrial collaboration towards development of the new methods of introduction of fluorine into organic compounds.

5.B Collaborations with national and foreign institutions or organizations

5.B.1 International Collaborations

5.B.1.1 Ongoing collaborations

University of Sydney, Australia

- **Prof. Elizabeth J New (since 2014)**
  - I have continued collaboration after moving to IBCH PAS, which included completing multiple research projects that led to several publications (including publications from the list of those contributing to the achievement), student exchange and partnership in one of my research projects (Homing) – completed in 2022 and the development of paper-based fluorescent arrays for detection of metal ions in environmental samples (ongoing)
- **Prof. Wojciech Chrzanowski (since 2015)**
  - I have initiated a collaboration when in the group of Prof. EJ New, organised collaboration meetings which led to long-term collaboration between the two groups on 5 different topics
    - **Resulting publication:** Z Lim, DG Smith, JL Kolanowski, RL Mattison, JC Knowles, S-Y Baek, W Chrzanowski, EJ New, A reversible fluorescent probe for monitoring Ag(I) ions. *Journal of the Royal Society Interface* **2018**, 15, 20180346.

  - In my independent work I have also established joined research agenda on development of chemical tools to study interactions of nanoparticles with cells and their effects on cellular environment
    - **Resulting output:** joint project iCARE in Horizon Europe framework (3 mln EUR, HORIZON-CL4-2022-DIGITAL-EMERGING-01-35, commencement in January 2023, consortium of 7 academic and 5 industrial partners)
- **Prof. Stuart Fraser (since 2015)**
  - I collaborated with Prof. Fraser on a project using various fluorophores (in particular, Nile Blue and new coumarin-carborane probe)to reveal changes in the distribution of previously intangible intracellular lipids in mouse embryonic stem cells.
  - **Resulting publications:**
    - BB Boumelhem, C Pilgrim, VE Zwicker, JL Kolanowski, JH Yeo, KA Jolliffe, EJ New, ML Day, SJ Assinder, ST Fraser*, Intracellular flow cytometric lipid analysis – a multiparametric system to assess distinct lipid classes in live cells. *Journal of Cell Science* **2022**; 135 (5): jcs258322 -> performed seminal experiments demonstrating the utility of Nile Blue in detecting different pool of lipids than Nile Red , contributed to the theoretical explanation of the changes of fluorescence of Nile Blue in presence of different lipids, reviewed manuscript
    - A Wu+, JL Kolanowski+, BB Boumelhem, R Lee, A Kaur, ST Fraser, EJ New, LM Rendina, A New Carborane-Containing Fluorophore as a Stain of Cellular Lipid Droplets. *Chemistry – An Asian Journal* **2017**, 12, 1704-1708. -> My role as a lead on bioanalytical part of the work included spectroscopic characterisation of carborane-coumarin probes, validation of their performance in vitro and in cellulo, data analysis and interpretation, manuscript preparation

Ecole Normale Superieure Lyon, France – Prof. Jens Hasserodt (since 2013)

- I continued my collaboration after completing my PhD
- **Topic:** Spin-switching probe for anions sensing in water
- I originated the idea, performed analytical experiments, and designed the manuscript (in preparation)

University of Birmingham, UK (Previously University of Heidelberg, Germany) – Prof. Dirk-Peter Herten (since 2016)

- I initiated this collaboration which focuses on development of chemical probes and protocols for super-resolution imaging (exchange of experimental material and research ideas)

DKFZ Helmholz Insitute, Heidelberg, Germany – prof. Alwin Krämer (since 2021)

- Collaboration initiated within EU-OPENSCREEN-DRIVE project and aimed at development of molecular probes for identification of cellular targets of griseofulvin analogues (through chemoprotoemic studies)

5.B.1.2 Ongoing collaborations within Centre for High Throughput Screening

EU-OPENSCREEN-ERIC, Berlin, Germany – Dr Wolfgang Fecke (since 2018)

- Collaboration within a framework of the European Research Infrastructure Consortia in which I am a representative of the IBCH PAS partner site:

- **Topics:**
  - Robust and reliable workflows for high-throughput screening
  - bio profiling of chemical compound libraries for identification of potential artifacts in screening campaigns
- **Resulting publication:** P Brennecke, D Rasina, O Aubi, K Herzog, J Landskron, B Cautain, F Vincente, J Quintana, J Mestres, B Stechmann, B Ellinger, J Brea, JL Kolanowski, R Pilarski, M Orzaez, A Pineda-Lucena, L Laraia, F Nami, P Zielenkiewicz, K Paruch, E Hansen, JP von Kries, M Neuenschwander, E Specker, P Bartunek, S Simova, Z Lesnikowski, S Krauss, L Lehtio, U Bilitewski, M Bronstrup, K Tasken, A Jirgenson, H Lickert, MH Clausen, JH Andersen, MJ Vincent, O Genilloud, A Martinez, M Nazare, W Fecke, P Gribbon, EU-OPENSCREEN: A Novel Collaborative Approach to Facilitate Chemical Biology. *SLAS Discovery* **2019**, 24(3), 298-413.

University of Cambridge, UK - Prof. Folma Buss, (since 2021)

- **Topic:** identification of specific inhibitor candidates against microbial myosine proteins
- My contributions involve design, data analysis and supervision in assay adaptation, screening and validation of the hits

Ludwig-Maximilians-Universitaet Munich, Germany - Prof. Claudia Veigel (since 2022)

- **Topic:** identification of specific inhibitors of human myosine as tools to study myosine-mediated cellular processes
- My contributions involve design, data analysis and supervision in assay adaptation, screening and validation of the hits

5.B.1.3 Past collaborations

Colorado State University, USA - Prof. Matthew Shores (Collaborator 2015-2019)

- **Topic:** Controlling spin-state in solution with (bio)chemical stimulus
- I initiated a collaboration with A/Prof. Shores and proposed him the original idea of particular design of complexes, provided advice on synthesis, experimental design, and data analysis.
- **Resulting output:**
  - **Conference poster** presented by collaboration at *249th ACS National Meeting & Exposition*, March 2015, Denver (CO), USA
  - **Publication**: TJ Ozumerzifon, RF Higgins, JP Joyce, JL Kolanowski, AK Rappe, MP Shores, Evidence for Reagent-induced Spin-State Switching in Tripodal Fe(II) Iminopyridine Complexes. Inorganic Chemistry, **2019**, 2(12), 7785.

University of Melbourne, Australia - Prof Marie Bogoyevitch (Collaborator 2015 - 2019)

- **Topic:** Investigation of the redox imbalance in the mitochondria of virus infected cells with the use of fluorescent probes
- I initiated and maintained a collaboration by proposing solutions to the problem of studying ROS generation in mitochondria with affected membrane potential, advised on the protocol for the application of our probe, including experimental design, troubleshooting and data interpretation
- **Resulting publication:** M Hu, KE Schulze, R Ghildyal, DC Henstridge, JL Kolanowski, EJ New, Y Hong, AC Hsu, PM Hansbro, PAB Wark, MA Bogoyevitch, DA Jans*. Respiratory Syncytial Virus co-opts host mitochondrial function to favour infectious virus production. *eLife* **2019**, 8, e42448.

Queensland University of Technology, Australia - Prof. Steven Bottle (Collaborator 2015 - 2018)

- Topic: Nitroxyl radical-based probes for real-time monitoring of redox state in mitochondria
- My contributions to this collaboration were performing, analysing and interpreting the results of fluorescence lifetime experiments (FLIM) in cells with the use of probes provided by Prof. Bottle.
- **Resulting publication:** KL Chong, BA Chalmers, JK Cullen, A Kaur, JL Kolanowski, BJ Morrow, K Fairfull-Smith, MJ Lavin, NL Barnett, EJ New, MP Murphy, SE Bottle, Pro-fluorescent mitochondria-targeted real-time responsive redox probes synthesised from carboxy isoindoline nitroxides: Sensitive probes of mitochondrial redox status in cells. *Free Rad. Biol. & Med.* **2018**, 128, 97-110.

Curtin University, Australia - Prof. Max Massi (Collaborator 2015 - 2017)

- **Topic:** terpyridine functionalised aryl tetrazoles for detection of metal ions
- My contributions included working on the compounds to characterize the metal-sensing properties of the reported ligands and establish array-based assay for a differentiation of Zn from Cd
- **Resulting publication**: PJ Wright, JL Kolanowski, WK Filipek, EG Moore, S Stagni, EJ New, M Massi, Versatility of terpyridine functionalised aryl tetrazoles: photophysical properties, ratiometric sensing of zinc cations and sensitisation of lanthanide luminescence. *European Journal of Inorganic Chemistry* **2017**, 44, 5260-5270.

University of Barcelona, Spain – Prof. Patrick Gamez (Collaborator 2016 - 2018)

- advising on the protocol for the application of our redox probes in studying the ROS generation by metal complexes in vitro, experimental design and troubleshooting

McGill University, Canada – Prof. Gerd Multhaup (Collaborator 2016-2017)

- advise on the protocol for the application of our reversible probe for labile copper pools in mitochondria in measuring the Cu(I) efflux efficiency at various physiological and pathological conditions.

State University of Notre Fluminense Darcy Ribeiro, Rio de Janeiro, Brazil - Prof. Adolfo Horn (Collaborator 2016-2017)

- advising on the synthesis and fluorescent tagging of the transition metal complexes with cytotoxic properties.

5.B.2 National Collaborations

Adam Mickiewicz University in Poznań

- **Dr hab Lucyna Mrówczyńska and Prof. Marek Sikorski (Collaborators since 2016)**
  - Topic: Detection of intracellular reactive oxygen species in flavin-exposed human erythrocytes with fluorescent probes
  - I initiated this collaboration and maintained this project, provided advice on protocols for probe applications including experimental design, troubleshooting, data analysis, and quantitative interpretation of microscopy images
  - **Resulting output:**

  - **Conference Poster:** presented by collaborators at *Oxygenalia 2016,* November 2016, Poznań, Poland
  - **Manuscript (in revision):** M Isińska-Rak, A Golczak, M Gierszewski, Z Anwar, V Cherkas, D Kwiatek, E Sikorska, I Khmielnski, G Brudziński, L Mrówczyńska*, JL Kolanowski*, M Sikorski*, 5-Deazaalloxazine as photosensitizer of singlet oxygen and potential redox-sensitive fluorescent probe.
- **Dr hab. Kinga Kamieniarz-Gdula (since 2022)**
  - My role is an identification of bioactive molecules influencing alternative splicing efficiency (design, supervision and interpretation of data generated by my team in the process)
  - Resulting output: I am a co-investigator (formal partner) on the ERC Starting Grant from Horizon Europe

Institute of Human Genetics PAS, Poland – Prof. Natalia Rozwadowska

- Topic: Caged bioluminogenic substrates for a selective molecular imaging of biochemical parameters in cancer
- I am the lead on the research grant of this title (SONATA) in which prof. Rozwadowska is a consortial partner -> their role is to develop cell-based and animal models of lung cancer to demonstrate the use of bioluminogenic substrates for detection of biochemical parameters by bioluminescence

Poznan University of Technology – dr Dariusz Brzezinski

- Development of AI-assisted algorithms and approaches for an analysis of large amounts of numeric and image-based data

6. PRESENTATION OF TEACHING AND ORGANIZATIONAL ACHIEVEMENTS AS WELL AS ACHIEVEMENTS IN POPULARIZATION OF SCIENCE OR ART.

6.A Didactic achievements

6.A.1 Supervision over students (Scientific supervision over students, doctors in the course of the specialization, or doctoral students as a research supervisor or auxiliary supervisor with a list of doctoral studies

PhD students:

- PhD students working on my research projects under my supervision – IBCH PAS doctoral school
  - Francesca Canyelles I Font (2018 – present)
  - Masroor Khan (2019 – present)
  - Anna Wychowaniec (2019 – present)
- Other PhD students I have supervised:
  - Konrad Pakuła (2019 – defended on 19.12.2022 – positive decision) – as auxillary supervisor, head supervisor: Prof. Michał Jasiński
  - Myint Toe (2022 – present) - as member of PhD Thesis Advisory Committee, head supervisor: Prof. Remigiusz Serwa (CeNT, Warsaw)

- Dr Michael Harris (2016 – 2017, defended in 2019) – as daily lab supervisor (diring postdoc), head supervisor: Prof. Tatjana N Parac-Vogt
- Dr Edward O'Neill (2014 – 2017, defended in 2017) – as daily lab supervisor (diring postdoc), head supervisor: Prof. Elizabeth J New
- Dr Clara Shen (2014 – 2017, defended in 2017) – as daily lab supervisor (diring postdoc), head supervisor: Prof. Elizabeth J New

MSc/BSc/Eng students:

- MSc students under my scientific supervision (working on my projects) – due to a formal policy of the Faculty of Chemistry of the Adam Mickiewicz University in Poznan I could not formally play a role of the "Promoter", even though the work has been done fully in my research group)
  - Anna Wychowaniec (MSc in 2019, University supervisor: Prof. Marek Sikorski)
  - Karolina Konsewicz (MSc in 2020, University supervisor: Prof. Marek Sikorski
  - Katarzyna Wodecka (MSc in 2020, University supervisor: Prof. Marek Sikorski)
  - Wiktoria Nitka (MSc in 2020, University supervisor: Prof. Donata Pluskota-Karwatka)
  - Adrian Rufli (MSc in 2020, University supervisor: Prof. Tomasz Pospieszny)
- Other students who carried out their research internships in my group:
  - Joanna Gosieniecka - High school student (2019) and then student at University College London (2022)
  - Xhorxhina Shauli (2019) - Summer intern, student in the SERP programme at Adam Mickiewicz University in Poznan, Poland
- MSc/BSc/Eng students I have provided daily supervision for in the lab during my PhD:
  - Robert Steinhoff from Technische Universität München, Germany
  - Hanno Kossen from University's College London, United Kingdom

6.A.2 Teaching activities

Lecturer at the Institute of Bioorganic Chemistry PAS – Poznań, Poland

- **Molecular Probes for Biological Applications** (15 h course for PhD candidates, two years: 2020, 2022)

Adam Mickiewicz University – Poznań, Poland

- **Modern Chemical Biology** (Faculty of Chemistry, 15 h course for PhD Candidates, 2019)
- **Advances in Molecular Medicine** (Faculty of Biology, 15 h course for MSc students, three years: 2020, 2021, 2022)

Lecturer at Medical University & Technical University of Poznań – Poznań, Poland

- **Bioorganic Chemistry** (7 h lectures, 2 x 8 h seminars, for undergraduate students, 2020, 2021)

Lecturer at the University of Sydney

- **CHEM1101 – 1st year Chemistry** (20 h lectures, 100 h labs supervision, two years: 2015, 2016)

6.B Organizational activities, national academies, and committees

6.B.1 National Science Academies & European Programs

- **Polish Young Academy (Vice-Chair 2019-2021, Chair 2022-present)**

  - This organisation brings together 35 outstanding young Polish scientists from all fields under 38 years of age at the date of their election and hold a doctoral degree, elected by the members of the Polish Academy of Sciences
  - A few months after my election as a member of AMU PAN, I was elected as one of the 3 deputy chairs of AMU PAN coordinating international cooperation and scientific advisory activities, and since the beginning of 2022 I have been the Chair (coordinating the work of all working groups, leading meetings, and discussions, supporting the selection of topics and activities, etc.).
  - The activities at AMU are of a social and voluntary nature and include activities in science popularisation (e.g. regular events promoting science among students, women, and the general public), science policy, especially in the context of the situation of young scientists (since 2019 I have been involved in the preparation of several expert opinions and statements commenting, among other things, on legislative solutions in the area of state science policy) and science advice (e.g. participation in YASAS and SAPEA - details below)
- **Young Academies Science Advice Structure (Co-founder and President, 2019-2022)**
  - As co-founder and President of the YASAS network, I was involved in building the consortium and developing a common charter for the organisation. I built trust and relationships both inside YASAS and externally with organisations with complementary goals. I led YASAS to join the official scientific advice mechanism for the European Commission under the SAPEA project (Scientific Advice for Policy by European Academies)
  - I currently serve as a Board Member in support of ensuring the continuity of the institutional relationship between YASAS and other partners.
- **SAPEA – Scientific Advice for Policy by European Academies (observer on the Board 2021-2022, Board Member 2022, deputy board member 2022 - present)**
  - An initiative of the Network of European Academies of Sciences as a key scientific component of the official Scientific Advice Mechanism for the European Commission (SAM - https://sapea.info.)
  - I contributed to the development of an official scientific advice mechanism for the European Commission, in the area of optimising the quality, transparency and inclusiveness of expert selection processes as well as the involvement and role of young scientists
  - I was involved in the preparation (notably as leader of one of the work packages) of the funding application for the continuation of the SAPEA project, which was successful and its implementation started in May 2022 (budget €3 million, 5 partners, including YASAS led by me at the time)
  - I am currently the YASAS representative to the project working group (work package 4 - Optimisation of the methodology and operational procedures of the SAPEA's work in the framework of scientific advice to the European Commission) and also an alternate member of the SAPEA Supervisory Board on behalf of YASAS.
- **EU-OPENSCREEN European Research Infrastructure Consortium (ICHB PAN representative, 2018 - present)**
  - Certified European Research Infrastructure Consortium (ESFRI member) with 33 partners from 10 European countries in the area of chemical biology and medicinal chemistry
  - I am one of the members of the Executive Committee of the EU-OPENSCREEN-DRIVE project funded by Horizon 2020 and implemented by the whole consortium

  - I am the representative of ICHB PAN in the Polish POL-OPENSCREEN consortium of the Polish Roadmap for Research Infrastructure (7 partners, 3 of which are also members of EU-OPENSCREEN-ERIC)
- **Previous memberships in other associations**
  - Australian Society of Molecular Imaging (ASMI) - regular meeting attendee (2014-2015)
  - Early-Mid Career Researchers of the Australian Academy of Science - Member of the Forum (one year)
  - Sydney Catalyst Network (2016-2017) - member
  - University of Sydney Chemical Society (2014 - 2017)
  - Society of Biological Inorganic Chemistry (2014-2016) - member, conference participant)

6.B.2 Activity in committees and commissions

- **Member of the Scientific Committees of the Polish Academy of Sciences (2019 - present)**
  - Chemistry Committee
  - Committee on Molecular Biology of the Cell
- **Member of the Advisory Committees of the Director of IBCH PAN (2019 - present)**
  - Committee for the introduction of English as a second language - (I proposed and participated in the preparation of a grant proposal for the introduction of facilities at ICHB PAN for English speakers, for which we obtained funding of PLN 500,000)
  - Commission for commercialisation and business cooperation
- **Member of the editorial committee of the scientific journal *Analysis & Sensing* (Wiley & Sons, 2021 - present)**
  - The journal is an initiative of Chemistry Europe, an association of the chemical societies of European countries
- **Organising committees of scientific conferences:**
  - **Polish Scientific Networks: Climate Change - Science & Society,** University of Wrocław, Poland (28-30.09.2022) **-** member of the organising committee;
  - **Young Science Beyond Borders 2021,** online (16-17.12.2021) - chair of the organising committee, session leader and speaker:
  - **9th International Conference on Biotechnology and Bioengineering 2019**, ICHB PAN, Poznań, Poland (25-28.09.2019) - co-organizer, session chair and speaker

6.B.3 Community expert and peer review activity

- **External consultant for the Polish position on European Funds work programmes under Horizon Europe** (2021 - present), more than a dozen interventions in the following areas:
  - European Research Area (including mechanisms for evaluating scientific excellence, diversification of scientific careers and intersectoral cooperation, and development of research infrastructures)
  - Work programmes for Horizon Europe Pillar II programmes in the field of Health
- **Consultant to the Greater Poland Regional Operational Programme 2021 - 2027**
  - Most of my amendments were accepted (2020-2021) and the programme was adopted by the European Commission in December 2022 as one of the first regional operational programmes in Poland.
- **Reviews of scientific articles -** data from Web of Science (search: "Kolanowski Jacek AND Kolanowski JL") - 78 reviews

- Chemical Communications: 49 (distinguished among the top 5 reviewers of Chemical Communications in 2018 and among the top 1% of reviewers in Chemistry in 2018 according to Web of Science) - IF2021: 6.065, 5-year IF: 5.976
- International Journal of Molecular Sciences: 5 - F2021: 6,208, 5-year IF: 6,682
- Sensors: 5 - F2021: 3.847, 5-year IF: 4.05
- Molecules: 3 - F2021: 4.927, 5-year IF: 5.11
- Chemical Society Reviews: 2 - F2021: 60,615, 5-year IF: 56,283
- Biosensors: 2 - F2021: 5.743, 5-year IF: 5.972
- ChemBioChem: 2 - F2021: 3,468, 5-year IF: 3,167
- Chemistry and Biodiversity: 2 - F2021: 2,745, 5-year IF: 2,581
- Chemosensors: 2 - F2021: 4.229, 5-year IF: 4.211
- Organic and Biomolecular Chemistry: 2 - F2021: 3.89, 5-year IF: 3.464
- Interface Focus: 1 - F2021: 4.661, 5-year IF: 5.338
- Journal of Materials Chemistry B: 1 - F2021: 7,571, 5-year IF: 6,788
- Materials: 1 - F2021: 3.748, 5-year IF: 4.042
- Nature Communications: 1 - F2021: 17,694, 5-year IF: 17,764

6.B.4 Invited talks on science advice and policy

- 01-03.11.2022 - InterAcademy Partnership Triennial Conference, Biosphere 2, Arizona, USA: panelist in session "Science Advice by Young Academies".
- 19-20.09.2022 - Euro-CASE Annual Conference, Brussels, Belgium: speaker at workshop session on Engaging Young Workers and Experts on "YASAS: The Young Academies Science Advice Structure"
- 27-28.04.2022 - Science Advice under Pressure conference, European Commission Science Advice Mechanism (SAM), Brussels, Belgium: co-presenter and moderator of the workshop "Society, Policy and Research under Pressure".
- 13-16.07.2022 - European Science Open Forum 2022, Leiden and hybrid: panellist in the session entitled. 'Innovations at the Science Policy Interface: Closing the Gap for Early Career Researchers' Policy Engagement" -
- 15.06.2022 - conference I workshop Scholars at Risk - Ukraine, International Science Council & ALLEA & Science4Ukraine: panelist
- 10-11.05.2022 - combined meeting of the European National Young Academies (ENYA) and ALLEA (The European Federation of Academies of Sciences and Humanities), Brussels, Belgium:
- address at the ENYA plenary session entitled YASAS: The Young Academies Science Advice Structure
- substantive co-organisation and chairing of the ENYA-ALLEA joint session on "Trust in science".
- 26-27.05.2022 - V4 Academies Forum, Budapest, Hungary - speaker in the session on Young Scientists' Activity in Academies entitled 'The Young Academies Science Advice Structure'. "YASAS: The Young Academies Science Advice Structure".
- 01.07.2020 - European Science Open Forum Life, online: panellist in the session "Brain drain, brain gain, brain circulation" on the mobility of young scientists
- 14.10.2019 - 16th International Conference "Youth in Science - 2019", Minsk, Belarus: speaker in the session on the activities of the Academy of Young Scientists

6.C Achievements in the popularization of science

The below summarises my activities in popularising and disseminating scientific concepts to the public, university, and primary school students.

Speeches and popular science events for students

- **Seminar at the 13th National Symposium of the Scientific Circle of Chemists of Adam Mickiewicz University,** Jeziory, Poland (16.03.2019): "Chemical tools for visualisation of biochemical analytes and their interactions in living cells".
- **Do Science seminars** (a grassroots initiative to meet scientists to discuss research career paths - organised by students and young scientists)
    - 08.10.2018, A. Mickiewicz University in Poznań - inaugural seminar of the Poznań branch of the Do Science initiative
    - 06.04.2018, International Institute of Molecular and Cell Biology, Warsaw - seminar by invitation
- **Popular science lecture in the ARS Chemiae series for students,** Adam Mickiewicz University in Poznań, Poland (11.04.2018): "Molecular probes for studying biochemical analytes and their interactions in biology".
- **Speech at the 16th International Scientific Conference "Youth in Science - 2019",** National Academy of Sciences of Belarus, Minsk, Belarus (14.10.2019): "Polish Young Academy".
- **University open days - *'Chemonstrations'* session** at the School of Chemistry, The University of Sydney, Australia**.** 02.2016 - as lead organiser of a session for first year science students, including a series of demonstrations led by PhD students

Seminars and popular science lectures for adults

- **Lecture and discussion on biomarkers and personalised medicine for music festival participants.** 03.08.2018 - Academy of Fine Arts **-** PolAndRock Festival, Kostrzyn, Poland
- ***PAN* popular science conference *from Wieniawski invites*.** ICHB PAN, Poznań, Poland (13.12.2017) - organiser and speaker: 'With Chemistry through the World'.
- **Open lecture as part of the *Inspiring Science* series**, Ultimo Library, Sydney, Australia (05.2016): "Seeing the invisible: MRI".

Popular science lectures and workshops for students

- Lecture and demonstrations for charity fundraiser, School Complex No. 15, Poznań, Poland (28.02.2018) - pupils in the age group 7-11 years.
- Lecture and demonstrations entitled. "With Chemistry through the World: seeing the invisible", School No. 3, Luboń, Poland (10.01.2018) - students in the age group 12-14.
- Lecture and demonstrations to mark National Science Week, Parramatta West Public School, Sydney, Australia (08.2016) - three meetings for different age groups 6-12 years (over 300 students)

Media activity:

- **Co-author of an article entitled "Voice of the Young"** in the monthly journal Academic Forum (FA 7-8/2022) on the Academy of Young Scientists of PAN: https://miesiecznik.forumakademickie.pl/czasopisma/fa-7-8-2022/glos-mlodych%E2%80%A9/

- **Interview in the programme Eureka, as part of the series People of Science, Jedynka Polskie Radio, 28.12.2020 -** fluorescent probes for drug discovery on COVID-19 (my research project funded by the Homing programme of the Foundation for Polish Science), https://jedynka.polskieradio.pl/artykul/2648789
- **Press article on the portal of the Foundation for Polish Science (fnp.org.pl) concerning my Homing research project on intelligent probes for drug discovery on COVID-19, 16.12.2020 -** the article was used by many portals to prepare press materials on the project, e.g.:
    - Polish Press Agency's Science in Poland portal (17.12.2020: (https://scienceinpoland.pap.pl/aktualnosci/news%2C85399%2Cinteligentne-sondy-do-poszukiwan-lekow-na-covid-19.html)
    - bankier.pl (16.12.2020) https://www.bankier.pl/wiadomosc/Naukowcy-z-Poznania-opracowuja-inteligentne-sondy-do-poszukiwan-lekow-na-Covid-19-8021440.html
    - pulsmedycyny.pl, 15.12.2020 https://pulsmedycyny.pl/polscy-naukowcy-opracowuja-inteligentne-sondy-do-poszukiwan-lekow-na-covid-19-1103297
    - epoznan.pl, 15.12.2020 https://epoznan.pl/news-news-112888-poznanscy_naukowcy_opracowuja_inteligentne_sondy_do_poszukiwan_lekow_na_covid_19
- **Guest of the podcast Faceci w Kitlach, episode no. 7 entitled "How to create a medicine?" (11.2020).**
- **Statements in the international press**
    - Article entitled 'Centralised hiring a barrier to research assessment reform', by Ben Upton, Times Higher Education (21.12.2022)
    - Article entitled 'War in Ukraine poses stark choices for scientists', by Richard A. Stone, Science, 03.03.2022 (vol. 375, issue 6584, pp. 942-943)

7. OTHER INFORMATION

7.A Externally funded projects as an independent researcher

- As principal investigator:
    - Four scientific projects funded by national competitions (managed budget: >3.4 mln PLN)
- As task coordinator:
    - Three scientific projects funded by European Commission (Managed budget: 850 000 EUR)
    - Two national research infrastructure projects (managed budget: 30 mln PLN) – more in section 7.B
- As co-investigator: five scientific projects funded by national competitions
- Collaborative projects funded by partners' own budgets (since 2020)
    - Academic partners (from the UK and three from Germany) – managed budget over 650 000 PLN
    - Industrial partners (from the Netherlands, Germany and three from Poland) – managed budget of 1.5 mln PLN

All details about funded projects and competitions can be found in attachment 5B (Section II.9 and III.5).

7.B Development of state-of-the-art research facility

A significant achievement of my career is establishing and directing the first **High Throughput Screening** core facility at IBCH PAS with self-sustainability goals and an orientation on partnerships. Currently, apart for the high throughput infrastructure and expertise, we extended our capacities also to ultra-superresolution imaging and medicinal chemistry. Thanks to the development of the unique expertise and research infrastructure aimed also at addressing research & development needs and effectively marketing our services, I was able to exceed target KPIs (financial sustainability, average of 200,000 PLN revenue per client and approx. 2,000,000 PLN in revenue in its pilot years with multiple returning users).

1. I obtained and coordinated a **budget of PLN 30 million in research infrastructure funding**. As a task manager for the NEBI project from the Polish Map of Research Infrastructure, I obtained funding and **purchased one of only a few fluorescence microscopes in the world using Minflux technology** with resolution < 5 nm (unique in biomedical research in the last 20 years in Poland, the most exclusive piece of research equipment purchased by Poland in recent years - we were the first in the world to secure funding for its purchase).
2. Within 2 years at the Centre, **I managed to acquire over 20 research projects** to be implemented on our infrastructure (the total budget of the tasks carried out by us in these projects is **over PLN 5 million**)
3. I have also **participated in the preparation of 11 further projects involving experimental tasks for a total amount of approximately PLN 2.6 million**, which will be coordinated by the Centre for High-throughput Screening, and which are currently under evaluation by funding institutions.

7.C Summary of invited lectures and participation at conferences (after obtaining the doctoral degree)

After the award of my PhD I have actively participated in 18 research conferences in Poland, Czechia, Spain, Australia, New Zealand, Hungary and USA, out of which 15 as a speaker in a form of:

- 5 invited lectures
- 6 oral presentations and
- 4 flash presentations

Within these activities, as a young researcher, I have received the following distinctions:

- Best short presentation (2016, GRC - Metals in Medicine, Andover, USA),
- Best young investigator presentation (2015 ASMI Conference, Melbourne, Australia)
- Invitation to conference as one of Australia's 100 most promising young scientists - Theo Murphy Australian Frontiers in Science - Materials for the 21st Century, from Design to Application, Melbourne Australia (09.12.2015)
- Three conference travel grants (2016: GRC Metals in Medicine, Andover, USA; EuroBIC-13, Budapest, Hungary; AsBIC-2016, Auckland, New Zealand).

I have also given 16 invited seminars in Poland, Australia, Kazahkstan, Nepal, France, Spain and Germany.

All details of my seminars and presentations can be found in Attachment 5B (Section II.7).

7.D Participation in specialised training and scientific workshops after completion of the doctoral programme

1. Training and certification in the PRINCE2 management method (online, 2021)
2. AAAS Communicating Science Workshop (online, 2019)
3. S4D4Cs 2nd Networking Meeting "Towards a European Science Diplomacy Roadmap" (Berlin, Germany, 2019)
4. How to successfully apply for ERC grants (ICHB PAN, Poland, 2018)
5. Nailing Grants workshop on effective scientific writing (Charles Perkins Centre, University of Sydney, Australia, 2016)
6. Science Pathways 2016 Workshop: Future Leaders EMCR Forum Meeting (University of New South Wales, Australia, 2016)
7. Image analysis workshop (Charles Perkins Centre, University of Sydney, Australia, 2016)
8. DeltaVision Elite Workshop & High Resolution Microscopy Seminar Training (Westmead, Australia, 2015)
9. Semester course Preparing for a Career in Research & Innovation (University of Sydney, Australia, 2014)
10. Principles and Practice of University Teaching and Learning course (University of Sydney, Australia, 2014)

7.E Awards and scholarships won (after obtaining the doctoral degree)

1. **Scholarship of the Minister of Science and Higher Education for outstanding young scientists,** (2020 - 2024)
2. Postdoctoral Teaching Fellowship**, Faculty of Science, University of Sydney, Australia,** AUD 32,000 (awarded in 2015 and 2016).
3. **One-year** Outgoing Postdoctoral Research Fellowship **from the *Fondation ARC pour la Recherché sur le Cancer*** (France) at The University of Sydney, Australia (2014-2015)
   Three-year PhD scholarship from *Ligue Nationale Contre le Cancer* (France) at ENS Lyon, France (2010-2012).

(Applicant's signature)

8. FULL LIST OF SCIENTIFIC PUBLICATIONS

Ref	Authors	Details	Year	Cit.*	IF**	DOI
Publications constituting main scientific achievement						
P1	KG Leslie, JL Kolanowski, N Trinh, S Carrara, MD Anscomb, K Yang, CF Hogan, KA Jolliffe, EJ New	''Nicotinamide-Appended Fluorophores as Fluorescent Redox Sensors.'' Australian Journal of Chemistry, 73, 895-902	2019	2	1,073	10.1071/CH19398
P2	JL Kolanowski, LJ Dawson, L Mitchell, Z Lim, ME Graziotto, WK Filipek, TW Hambley, EJ New	''A fluorescent probe for investigating metabolic stability of active transplatin analogues'' Sensors and Actuators B: Chemical , 255(3), 2721-2724	2018	10	5,667	10.1016/j.snb.2017.09.084
P3	IJ Carney, JL Kolanowski, Z Lim, B Chekroun, TW Hambley, EJ New	''A ratiometric iron probe enables investigation of iron distribution within tumour spheroids.'' Metallomics, 10(4), 553-556	2018	7	4,069	10.1039/c7mt00297a
P4	C Shen, JL Kolanowski, CM-N Tran, A Kaur, MC Akerfeldt, MS Rahme, TW Hambley, EJ New	''A ratiometric fluorescent sensor for the mitochondrial copper pool.'' Metallomics, 8(9), 915-919	2016	22	3,54	10.1039/c6mt00083e
P5	M Hu, KE Schulze, R Ghildyal, DC Henstridge, JL Kolanowski, EJ New, Y Hong, AC Hsu, PM Hansbro, PAB Wark, MA Bogoyevitch, DA Jans	''Respiratory Syncytial Virus co-opts host mitochondrial function to favour infectious virus production.'' eLife, 8:e42448	2019	20	7,551	10.7554/elife.42448.001
P6	KL Chong, BA Chalmers, JK Cullen, A Kaur, JL Kolanowski, BJ Morrow, K Fairfull-Smith, MJ Lavin, NL Barnett, EJ New, MP Murphy, SE Bottle	''Pro-fluorescent mitochondria-targeted real-time responsive redox probes synthesised from carboxy isoindoline nitroxides: Sensitive probes of mitochondrial redox status in cells.'' Free Radical Biology & Medicine, 128, 97-110	2018	10	6,02	10.1016/j.freeradbiomed.2018.03.008
P7	JL Kolanowski, A Kaur, EJ New	''Selective and reversible approaches towards imaging redox signaling using small molecule probes.'' Antioxidants & Redox Signaling, 24(13), 713-730	2016	14	7,093	10.1089/ars.2015.6588
P8	A Kaur, JL Kolanowski, EJ New	''Reversible fluorescent probes of biological redox state.'' Angewandte Chemie International	2016	93	11,709	10.1002/anie.201506353

		Edition in English , 55(5), 1602-1613				
P9	JL Kolanowski, F Liu, EJ New	''Fluorescent probes for the simultaneous detection of multiple analytes in biology'' Chemical Society Reviews, 47(1), 195-208	2018	215	40,182	10.1039/c7cs00528h
Other publications						
P10	BB Boumelhem, C Pilgrim, VE Zwicker, JL Kolanowski, JH Yeo, KA Jolliffe, EJ New, ML Day, SJ Assinder, ST Fraser	"Intracellular flow cytometric lipid analysis – a multiparametric system to assess distinct lipid classes in live cells." Journal of Cell Science, 135(5), jsc258322	2022	4	5,235	10.1242/jcs.258322
P11	A Zarecki, JL Kolanowski, WT Markiewicz	''Microwave-Assisted Catalytic Method for a Green Synthesis of Amides Directly from Amines and Carboxylic Acids.'' Molecules, 26(8), 1761	2020	12	3,267	10.3390/molecules25081761
P12	TJ Ozumerzifon, RF Higgins, JP Joyce, JL Kolanowski, AK Rappe, MP Shores	''Evidence for Reagent-induced Spin-State Switching in Tripodal Fe(II) Iminopyridine Complexes.'' Inorganic Chemistry, 58(12), 7785-7793	2019	10	4,85	10.1021/acs.inorgchem.9b00340
P13	P Brennecke, D Rasina, O Aubi, K Herzog, J Landskron, B Cautain, F Vincente, J Quintana, J Mestres, B Stechmann, B Ellinger, J Brea, JL Kolanowski, R Pilarski, M Orzaez, A Pineda-Lucena, L Laraia, F Nami, P Zielenkiewicz, K Paruch, E Hansen, JP von Kries, M Neuenschwander, E Specker, P Bartunek, S Simova, Z Lesnikowski, S Krauss, L Lehtio, U Bilitewski, M Bronstrup, K Tasken, A Jirgenson, H Lickert, MH Clausen, JH Andersen, MJ Vincent, O Genilloud, A Martinez, M Nazare, W Fecke, P Gribbon	''EU-OPENSCREEN: A Novel Collaborative Approach to Facilitate Chemical Biology.'' SLAS Discovery, 24(3), 398-413	2019	8	2,058	10.1177/2472555218816276

P14	M Harris, JL Kolanowski, ES O'Neill, C Henoumont, S Laurent, TN Parac-Vogt, EJ New	''Drawing on biology to inspire molecular design: a redox-responsive MRI probe based on Gd(III)-nicotinamide. '' Chemical Communications, 54(92), 12986-12989	2018	6	6.29	10.1039/c8cc07092j
P15	Z Lim, DG Smith, JL Kolanowski, RL Mattison, JC Knowles, S-Y Baek, W Chrzanowski, EJ New,	''A reversible fluorescent probe for monitoring Ag(I) ions. '' Journal of the Royal Society Interface , 15(144), 20180346	2018	7	3,355	10.1098/rsif.2018.0346
P16	PJ Wright, JL Kolanowski, WK Filipek, EG Moore, S Stagni, EJ New, M Massi	''Versatility of Terpyridine-Functionalised Aryl Tetrazoles: Photophysical Properties, Ratiometric Sensing of Zinc Cations and Sensitisation of Lanthanide Luminescence'' European Journal of Inorganic Chemistry, 55(SI), 5260-5270	2017	10	2,444	10.1002/ejic.201700663
P17	A Wu*, JL Kolanowski*, BB Boumelhem, R Lee, A Kaur, ST Fraser, EJ New, LM Rendina	''A New Carborane-Containing Fluorophore as a Stain of Cellular Lipid Droplets.'' Chemistry - An Asian Journal, 12(14), 1704-1708	2017	25	4,083	10.1002/asia.201700423
P18	K Yang*, JL Kolanowski*, EJ New	''Mitochondrially targeted fluorescent redox sensors'' Interface Focus, 7(2), 20160105	2017	19	2,693	10.1098/rsfs.2016.0105
P19	ES O'Neill, JL Kolanowski, PD Bonnitcha, EJ New	''A cobalt(II) complex with unique paraSHIFT responses to anions.'' Chemical Communications, 53(25), 3571-3574	2017	7	6,319	10.1039/c7cc00619e
P20	JL Kolanowski, C Shen, EJ New	''Fluorescent probes for the analysis of labile metals in brain cells. '' White A. (editor) Metals in the Brain. Neuromethods, vol. 124, 51-70. Humana Press, New York, NY	2017	1		10.1007/978-1-4939-6918-0_4
P21	ES O'Neill, JL Kolanowski, GH Yin, KM Broadhouse, SM Grieve, AK Renfrew, PD Bonnitcha, EJ New	''Reversible Magnetogenic Cobalt Complexes. '' RSC Advances, 6(36), 30021-30027	2016	12	3,289	10.1039/c6ra04643f
P22	J Hasserodt, JL Kolanowski, F Touti	''Magnetogenesis in Water Induced by a Chemical Analyte. '' Angewandte Chemie International Edition in English, 53(1), 60-73	2014	31	11,336	10.1002/anie.201305662

P23	JL Kolanowski, E Jeanneau, R Steinhoff, J Hasserodt	''Bispidine Platform Grants Full Control over Magnetic State of Ferrous Chelates in Water. '' Chemistry – A European Journal , 19(27), 8839-8849	2013	11	5,696	10.1002/chem.201300604
P24	SN Tverdomed, J Kolanowski, E Lork, G-V Röschenthaler	''An Effective Synthetic Route to Ortho-Difluoromethyl Arylphosphosphonates: Studies on the Reactivity of Phosphorus- and Fluorine-Containing Functions. '' Tetrahedron, 67(21), 3887-3903	2011	22	3,011	10.1016/j.tet.2011.03.076

* without self-citations (from Web of Science Core Collection database accessed on 20.12.2022)
** IF for year preceding publication year (Web of Science Core Collection database)