Organotellurium Reagents for Multiparametric Interrogation of Dynamic Biology

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Department of Chemistry
University of Toronto

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Abstract

Population-averaged measurements (PAMs) of cells have advanced understanding of innumerable biochemical processes and continue to be routinely used in the laboratory. Despite this success, PAMs are limited in their ability to report on the precise state of important individual members of a population. The recent emergence of mass cytometry (MC) has offered a new solution to this problem: the ability to simultaneously measure a large number of parameters on a single cell. Unlike traditional fluorescence-based flow cytometry, which suffers from spectral overlap of fluorophore reporters, MC allows for simultaneous measurement of a massive number of parameters—limited only by the number of unique heavy isotopes on the periodic table. Since its inception, MC has relied on high molecular weight metal-chelating polymer antibody-conjugates (MCPACs) to quantify meaningful static biomarkers. Although very useful, MCPACs are unable to report on active biochemical processes. In response to this limitation, we have developed a modular, bio-compatible organotellurium “tag” with a mass less than 250 Da that can be incorporated into a variety of activity-based scaffolds. The work presented in this thesis outlines our efforts to develop a MC-compatible activity-based probe using this tag that is able to report on cancer cell oxygenation levels. We demonstrate that this molecule can be used to discriminate single cells based on their exposure to oxygen, both in vitro and in vivo. By synthesizing “isotopologous” variants of this probe (molecules that are structurally identical but differ in mass) and administering them to human pancreatic tumour models in a temporally-spaced dosing regime, we demonstrate that tumour microenvironments experiencing temporal fluctuations in oxygenation can be highlighted reliably at the single cell level for the first time. By coupling this
approach with recently developed imaging mass cytometry technology, we present multiplexed images of specific regions within pancreatic tumours that exhibit fluctuating hypoxia with spatiotemporal resolution.
T.E. Lawrence, eponymously of Arabia, but very much an Englishman, favoured pinching a burning match between his fingers to put it out. When asked by his colleague, William Potter, to reveal his trick – how is it he so effectively extinguished the flame without hurting himself whatsoever – Lawrence just smiled and said, “The trick, Potter, is not minding it hurts.”.

– Peter Weyland, 2023
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The most difficult aspect of completing a doctoral degree is not the coursework, demands of research, or the writing of papers—it is rousing the mental fortitude to see the degree as a whole through to the end. For myself, the strength to do this came from my family and friends. Many people have touched my life during my time at the University of Toronto, and as I prepare to venture towards new horizons, I find myself reflecting on just how valuable each and every one of them are to me. My friends have shown me that it is possible to do well professionally while still finding time to have fun. My family have shown me that supporting the ones you love should always be the priority, even if it means making difficult sacrifices. These lessons will serve me well for the rest of my life. I love you all forever.

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To my parents. As I stare at my computer screen writing this section it becomes clear that there are simply no words to describe the love that I have for both of you. Mom and Dad, you have given me both the world and the desire to take it. I am so proud to be your son. My Father. If every child had you as a role model the world would be a far better place. Above all, you have always demonstrated that keeping one’s word is the most important part of being a successful, responsible, and honourable human being. If I am ever considered half the man you are I would burst with pride. My Mother. Our connection is forever and unbreakable. Your impeccable standards and ability to persevere even in the face of great physical adversity are inspirations. More than anything, you have taught me to be honest and kind, and although I know that sometimes I struggle with the latter, you are always there to help me improve. You complete me.
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List of Abbreviations

2-NI = 2-nitroimidazole
β-ME = beta-mercaptoethanol
ABP = activity-based probe
ACN = acetonitrile
$A_{\text{max}} (\lambda_{\text{max}})$ = wavelength of maximal absorbance
amu = atomic mass units
CDCl$_3$ = deuterated chloroform
cts = counts
Cy5 = cyanine 5
CyTOF = mass cytometer instrument
DAPI = (4', 6-diamidino-2-phenylindole)
DART MS = direct analysis in real time mass spectrometry
DCC = $N,N'$-dicyclohexylcarbodiimide
DCM = dichloromethane
DCU = dicyclourea
DIAD = diisopropyl azodicarboxylate
DME = dimethylethane
DMEM = dulbecco’s modified eagle medium
DMSO = dimethyl sulfoxide
DNA = deoxyribonucleic acid
equiv. = number of equivalents
ESI = electrospray ionization
EtOAc = ethyl acetate
FACS = fluorescence activated cell sorting
FAD = flavin adenine dinucleotide
FADH$_2$ = reduced flavin adenine dinucleotide
FBFC = fluorescence based flow cytometry
FBS = fetal bovine serum
FITC = fluorescein isothiocyanate
H&E = hematoxylin & eosin
HPLC = high performance liquid chromatography
IC₅₀ = inhibitory concentration at 50 % maximal concentration
ICP-MS = inductively coupled plasma mass spectrometry
IdU = 5-iododeoxyuridine
IMC = imaging mass cytometry
K/O = knockout
kDa = kilodalton
LAC = laser ablation chamber
LC-MS = liquid chromatography mass spectrometry
LC/MS/MS = liquid chromatography tandem mass spectrometry
MC = mass cytometry
MCPACs = metal-chelating polymer-antibody conjugates
MS = mass spectrometry
nat-Telox 2 = Telox 2 with tellurium at natural isotopic abundance
¹²²-Telox 2 = Telox 2 enriched in the ¹²²-Te isotope
¹²⁵-Telox 2 = Telox 2 enriched in the ¹²⁵-Te isotope
NHS = N-hydroxysuccinimide
NMR = nuclear magnetic resonance
NOD = non-obese diabetic
O.N. = over night
O/E = overexpressed
OCT = optimal cutting temperature
p-NP = para-nitrophenol
PBS = phosphate buffered saline
Ph = phenyl
Pimo = pimonidazole (1-(2-nitro-1H-imidazol-1-yl)-3-(piperidin-1-yl)propan-2-ol)
POR = p450 oxidoreductase
ppm = parts per million
r.t. = room temperature
rcf = relative centrifugal force
RGB = red / green / blue
RPMMI = Roswell Park Memorial Institute
SCID = severe combined immunodeficiency
t = time
$t_{1/2}$ = half-life
TBAF = tetrabutylammonium fluoride
TEA = triethylamine
THF = tetrahydrofuran
TLC = thin layer chromatography
TOF = time of flight
UV-vis = ultraviolet-visible
WST-1 = water soluble tetrazolium salt 1
WT = wild type
XO = xanthine oxidase
1.1 Single Cell Analysis: Analyzing the Fundamental Units of Biology

Population-averaged measurements of cells have advanced understanding of innumerable biochemical processes and continue to be routinely used in the laboratory. Despite this success, these studies are limited in their ability to report on the state of important individual members of a population, as averaging information across all of the cells in a mixture can lead to loss of data pertaining to specific entities. For example, the communication mechanisms that cells within a tissue employ to maintain healthy functioning of an organism can cause neighbours that appear to be the same to phenotypically diverge from each other.\textsuperscript{1-3} Daughter cells can display significant variation from their parent often as a consequence of differences between tissue microenvironments.\textsuperscript{3} Factors such as exposure to growth factors, abundance of nutrients, and even access to oxygen can all cause subtle to extreme changes in cellular phenotypes, genotypes, and ultimately the gross pathology of a tissue.\textsuperscript{4} In order to understand how single cell heterogeneity influences the health of an organism, techniques that enable interrogation of individual members of a complex population are essential.\textsuperscript{2}

A particularly important example of where cellular heterogeneity is critical to pathology may be found within cancerous tumours. Perpetuation and expansion of a tumour requires interplay between many different cell types—cancer, inflammatory, stromal, vascular, immune, and blood, among others.\textsuperscript{5} Tumour-initiating cells (TICs) obscure themselves behind this screen of genetic and phenotypic diversity, providing a defense against endogenous mechanisms and pharma/radiocological strategies that are used to combat tumour growth.\textsuperscript{2,5} TICs even possess the ability to reestablish a tumour after treatment and can often seed lethal metastases at sites distant from the primary cancer.\textsuperscript{6-8} Clearly, TICs are important members of the tumour ecosystem but have been almost impossible to characterize until relatively recently. This is because reliable tools for deep interrogation of single cells in a mixture simply did not exist. Modern advances in instrumentation, biochemistry, and synthetic chemistry have enabled interrogation of cellular
heterogeneity with great success—specifically allowing for simultaneous analysis of many parameters at one time on individual cells.

1.2 Mass Cytometry

Multiparameter analysis of single cells has traditionally been performed using fluorescence-based flow cytometry (FBFC)—a technique that has transformed understanding of cellular heterogeneity. As scientists begin to ask increasingly complex questions about relationships between large numbers of biological parameters, the limitations inherent to FBFC have become apparent. Specifically, the number of fluorophore tags that may be employed simultaneously in any given FBFC experiment is limited by spectral overlap, thereby prohibiting a highly parameterized analysis. Additionally, autofluorescence of cells can cause interference during analysis. The introduction of mass cytometry (MC) has provided a means for overcoming this limitation. In place of fluorophores, MC uses stable isotopes with high atomic mass as tags and harnesses the sensitivity, dynamic range, and single mass unit resolution of inductively coupled plasma mass spectrometry to allow for highly parameterized experiments (theoretically over 100 parameters). The mass cytometer (CyTOF) functions by introducing isotopically-labeled cells stochastically (but typically one at a time) into an inductively-coupled plasma torch. The torch, with a temperature of ~7000 K, vaporizes, atomizes, and ionizes every component of each cell. This process produces a discrete ion cloud for each cell which is passed through a quadrupole-like “mass filter”; a device that removes ions corresponding to isotopes of elements with a high biological abundance (carbon, nitrogen, oxygen, etc.). The ions of isotopes that are allowed through the mass filter are then detected and quantified by a time-of-flight (TOF) mass spectrometer optimized for high-precision high-speed analysis with single mass unit resolution. Unlike a traditional ICP-MS, a quadrupole mass analyzer was not used in the mass cytometer because these mass analyzers possess a long settling time (on the order of $10^2$ seconds) which makes them incompatible with simultaneous measurement of a large number of discrete isotopes contained within a transient cell event. The inclusion of a TOF detector circumvented this issue due to the high speed analysis of this technology.

MC technology has been successfully used to monitor differential responses across the human hematopoietic continuum based on 34 unique parameters and recently a study employing 42 simultaneous parameters has been described. The typical workflow for a MC experiment is
presented in Figure 1-1.

**Figure 1-1**: Mass cytometry workflow. Cells (obtained from culture, model organisms, or clinical sources) are stained with reagents bearing heavy, bioorthogonal isotopes. Following staining, the sample is injected onto the mass cytometer where a nebulization system stochastically sprays droplets of water containing ~one cell each. Droplets enter the inductively coupled plasma (ICP) torch where cells are vaporized, atomized, and ionized—a discrete cloud of ions is produced for each cell which then pass through a mass filter stage (akin to a quadrupole, not shown) which removes light elements with high biological abundance. The heavy isotopes—each corresponding to a parameter of interest, enter a time-of-flight (TOF) mass spectrometer where the identity and quantity of each isotope per cell is recorded. This data can then be plotted in a manner similar to traditional FBFC data (i.e. in two-dimensional population density maps). 9

Although unrivaled with respect to parameterization, MC does not possess the sensitivity nor rapid throughput of FBFC. While instruments employing fluorescence detection systems can analyze ~25000 cells/second with a lower limit of detection of ~500 fluorescent reagents/cell, MC is limited to analysis of ~2000 cells/second and requires at least ~1500 MC-compatible reagents/cell. 9, 13-14 Thus, if an experiment requires high-throughput analysis, or high sensitivity, FBFC is often a superior choice to MC; however, if deep phenotypic characterization is desired MC is without equal.

To fully realize the parameterization of MC requires many reagents bearing unique heavy isotopes (currently 75–209 amu) with a low biological background that report on meaningful biological parameters. To date, the most successful reagents developed have been bifunctional polymers that chelate lanthanide ions and allow for antibody conjugation (MaxPar Reagents, Figure 1-2). 15 Additionally, metal chelates that bind DNA have been exploited (Figure 1-2). 16 These transition
metal complexes are important reagents for MC technology as they are used to indicate when a single cell event has occurred on the instrument. Since individual cells stained with these intercalators possess a relatively consistent amount of either $^{191/193}$Ir or $^{103}$Rh, when a certain threshold signal for any of these mass channels has been reached, data acquisition for all other desired channels is triggered. Specifically, $^{191/193}$Ir or $^{103}$Rh signals corresponding to single cell events possess a pseudo-Gaussian shape with a full width at half maximum at 200 µs; upon detection of this signal distribution signals for other mass channels are recorded. This is akin to using light scattering as a metric for single cell event detection on a traditional FBFC instrument. Recently, the nucleoside analogue 5-iododeoxyuridine has been used in an assay for delineating cell cycle stages with the iodine atom bound to the pyrimidine ring acting as a MC-compatible reporter isotope (Figure 1-2).\textsuperscript{17} Introduction of tools beyond these three types of MC-compatible reagents would aid in unlocking additional dimensions in single cell analysis.

**Figure 1-2:** a) Generalized structure of commercially available bifunctional lanthanide-chelating polymer-antibody conjugates (MaxPar\textsuperscript{®} reagents).\textsuperscript{15} b) Structures of the heavy isotope containing nucleic acid intercalators used as standards to indicate when a cell event has occurred.\textsuperscript{16} These are used in place of light scattering for cell event detection on a traditional FBFC instrument. The structure of the unnatural nucleoside analogue 5-iododeoxyuridine (IdU) is also shown.\textsuperscript{17}
1.3 Activity-Based Probes

Although MC has already enabled important advancements in understanding complex relationships between a large number of biological parameters, these studies have been limited to measurement of relatively static epitopes, such as the presence or absence of certain proteins. Although many genome/transcriptome-based techniques for probing enzyme function exist (i.e. gene knockout mutants and small interfering RNA), these technologies are unable to report on potentially critical post-translational events/modifications that can strongly influence the localization and function of specific proteins. Thus, a great need has arisen for techniques that allow for direct interrogation of specific proteins in living systems. Activity-based protein profiling technology (ABPP), pioneered by the Cravatt group, has provided a means to realize direct interrogation of enzyme activity in a wide range of biological systems. Activity-based probes (ABPs) are typically defined as small molecules that chemically modify a biological entity using a mechanism that is dependent on a specific biochemical transformation. Usually, ABPs covalently modify the active site of a target protein (or families of related proteins), simultaneously irreversibly inhibiting enzymatic activity and installing a detectable handle (i.e. affinity tag or fluorophore). Prototypical ABPs use chemical scaffolds that contain three key features; an affinity group, a reporter group, and a mechanism-based inhibitor warhead—typically an electrophilic functionality. The affinity group and mechanism-based inhibitor warhead are often contained within the same scaffold. For example, the Withers group has reported an ABP against β-1,4-glycanase enzyme, thus demonstrating the important applications of ABPs in the discovery of new members of enzyme families. Additionally, ABPs may be used to discover inhibitors of enzyme activity through a competition assay strategy where an enzyme (or even an entire proteome) is pre-treated with an inhibitor candidate followed by exposure to an ABP. SDS-page analysis of the labeled protein/proteome as compared to an unlabeled control reveals proteins that cannot be labeled by the ABP in the presence of an unlabeled inhibitor; the reason for this is assumed to be competitive binding between the unlabeled inhibitor and ABP.
Figure 1-3: Structure of an activity based probe against β–retaining glycosidases. The dissacharide core acts as the affinity moiety to direct the probe to the active site of the target enzyme. Upon binding, the β–dinitrophenyl 2-fluoro-glycoside warhead reacts with a catalytic aspartic acid in the active site, forming a covalent conjugate with the enzyme. Finally, detection of the covalent conjugate is mediated by the biotin affinity tag which presumably sits outside the active site pocket.  

Expansion of the toolbox of reagents compatible with MC technology towards probes capable of reporting on active biology would enable studies into innumerable biological processes such as metabolism, cell growth and senescence, rates of protein synthesis, and nutrient uptake (among many others). ABPs compatible with FBFC have been routinely used to study many of these processes; however, these reagents often suffer from the same spectral overlap/quantitation issues if detected via fluorescence using FBFC, or poor resolution/potential toxicity if monitored using radiological methods (i.e. \(^{14}\text{C}\) or \(^{32}\text{P}\) labels). If fluorescence techniques are used for profiling enzymatic activity, it can become challenging to design an assay that permits detection of static biomarker epitopes (such as cell surface protein markers with fluorophore-labeled antibodies) in addition to fluorescence-detectable ABPs. This limits the ability to assign specific biochemistry to specific cell populations. For the same reasons as described in section 1.2, MC provides a solution to this problem due to its highly parameterizable nature; however, prior to the findings contained in this dissertation, no ABPs compatible with detection via MC were available.

In order for an ABP to be compatible with MC it is essential that the reporter group (analytical handle) contains a heavy bioorthogonal element that functions as a benign spectator until analysis via MC is performed. Importantly, the reporter group should not influence the biology being evaluated by the affinity/warhead components of the probe. A generalized blueprint for a MC-compatible ABP is shown in Figure 1-4.
An advantage inherent to ABPs compatible with MC is the potential for generating a series of analytically distinguishable probes that all share the same structure simply by changing the mass of the heavy bioorthogonal element in the mass tag group. Because of their identical structures, these “isotopologous” compounds should all behave exactly the same in a living system; parameters such as plasma clearance, biodistribution, toxicity, affinity for a protein target, etc. are all influenced significantly by structural changes, but should not be subject to the mass of a specific isotope of a heavy element. With this knowledge, it should be possible to perform serial labeling with isotopologous probes (SLIP) where isotopologues of a MC-compatible ABP are administered to a biological system (e.g. murine model) at different time points and the relative quantities of each isotope present in the system at the end of the experiment should provide meaningful information about the state of the system during a specific interval of time (Figure 1-5).

The SLIP approach should be translatable to study a wide range of biological processes; however, a mass tag that meets the criteria outlined in Figure 1-4 and contains an element that exists as many stable isotopes must first be developed.
1.4 Tellurium as a Mass Cytometry-Compatible Element

Tellurium provides favorable characteristics for the development of MC-compatible ABPs. The element forms telluroether and tellurophene functionalities which are small and lipophilic: properties which we hypothesized would minimize perturbation of attached biologically sensitive functionalities (i.e. activity-based groups), thus preserving their activity. This is in direct contrast to the lanthanide-chelating polymers used as mass tags in MaxPar reagents, because these groups are large and polar (Figure 1-2). Additionally, eight stable tellurium isotopes are available commercially, allowing for multiple MC-distinguishable probes to be generated using identical chemistry.

The first organotellurium compound was synthesized by Wöhler in 1840. Increasingly, organotellurium compounds are being investigated in living systems, although this area of research remains underdeveloped. Tellurium has no known biological role in prokaryotic or eukaryotic cells. Tellurium metabolism is poorly understood; however, it is thought to follow similar metabolic pathways as the lighter chalcogen, selenium. Microorganisms have been found that can methylate inorganic tellurium, producing volatile species. Alternatively, tellurium can be oxidized to soluble species that are more easily excreted, although experimental evidence for these processes is scarce due to the instability of the metabolites. The number of studies involving aryl, vinylic, alkynyl and alkyl telluroethers in biological systems is increasing, but the majority of this research has focused on the ability of aryl telluroethers to mimic glutathione peroxidase activity providing, in some cases, resistance to oxidative stress, and, in other cases, disregulation of redox homeostasis leading to apoptosis. Murine models treated with aryl- or alkyltelluroethers have exhibited diverse responses including suppression of thioredoxin activity in the brain (but increased in the liver) and enhanced memory respectively.

1.5 Activity-Based Probes Compatible with Mass Cytometry: Scope of Thesis

The development of reagents compatible with MC for monitoring active/dynamic biological processes will enable powerful new experiments with this instrumentation. The aim of this thesis is to design, synthesize, and validate ABPs containing a heavy isotope compatible with detection via MC. Upon identification of the ideal mass tag, a series of isotopologous probes can be
generated, enabling an unprecedented investigation into the dynamics of specific biological processes. The majority of this dissertation will focus on efforts to develop MC-compatible ABPs for studying tumour hypoxia; a phenomenon of great importance to cancer progression and prognosis.\textsuperscript{34-36} Prior to this work, no reliable method existed for monitoring the dynamics of tumour oxygenation \textit{in vivo}—a deficiency the SLIP strategy was poised to address.

Studies have been performed where two structurally different/analytically distinguishable probes against cellular hypoxia, pimonidazole and EF5, were injected in human tumour xenograft models at different time points to investigate hypoxia dynamics \textit{in vivo}. In this study, pimonidazole was injected at time = 0 followed by EF5 24 hours later.\textsuperscript{37} The authors reported observation of regions in the tumour that exhibited preferential labeling by one probe over the other using fluorescence microscopy; however, the aforementioned inequivalencies (see section 1.3) in pharmacokinetics of these two molecules make data interpretation challenging if not impossible when striving to make statements about temporal changes in microenvironment oxygenation. Indeed, any panel of probes which display different structural characteristics would be expected to exhibit different pharmacokinetic/dynamic properties, and thus have inequivalent access to different microenvironments within the tumour.

Our first goal was to identify a compact organic scaffold containing a heavy, bioorthogonal element that could be harnessed as a MC-compatible reporter group. In Chapter 2, we discuss why tellurium is an ideal element for this application and present a proof of concept probe against cellular hypoxia containing a telluroether reporter group. The robust literature on small chemical functionalities capable of selectively labelling cells under low pO\textsubscript{2} conditions indicated that a 2-nitroimidazole activity group was an excellent choice for our ABP.\textsuperscript{38-42} A particularly attractive attribute of 2-NI-based probes is that activating enzymes can turn over many copies of the activity group, resulting in greatly amplified signal. This is in contrast to many activity-based functionalities as most inhibit their target during the labelling process (i.e. fluorophosphonates for detecting serine protease activity, or the glycosidase probe in Figure 1-3).\textsuperscript{18-20} This telluroether-containing 2-NI was able to selectively enrich hypoxic cells in tellurium \textit{in vitro} and was the first example of an ABP compatible with MC; however, stability limitations under aerobic conditions and an insensitivity to intermediate oxygen tensions limited the usefulness of the molecule.
Chapter 3 describes our efforts to optimize the tellurium reporter group to increase its stability in aqueous buffer and O₂. We reasoned that by decreasing the amount of electron density on the tellurium atom within the activity-based scaffold, we could decrease the rate at which oxidative degradation occurred. A structure-stability study revealed that this hypothesis was sound, as 2-substituted tellurophene heterocycles exhibited enhanced performance and retained the low toxicity of the telluroether group.

By combining the findings in Chapters 2 and 3, we present an optimized MC-compatible probe against cellular hypoxia in Chapter 4. This molecule contained both the 2-nitroimidazole activity-based group and optimized tellurophene reporter. This second-generation probe did not exhibit degradation under biologically relevant conditions over extended periods of time, consistent with our structure-stability findings in Chapter 3. The new probe was able to selectively label hypoxic cells in vitro over a wide range of oxygen tensions, and its labelling ability was determined to be dependent on expression levels of cytochrome P450 oxidoreductase—a key enzyme in the metabolism of many nitroaryl compounds. Furthermore, this molecule demonstrated efficacy in vivo, labeling similar (but not identical) populations of cells as pimonidazole and EF5: two well validated FBFC-compatible probes sensitive to cellular hypoxia.

In Chapter 5, we demonstrate that the modular synthetic route used to access the second-generation probe was compatible with the synthesis of isotopologous variants—structurally identical versions of the probe with differing masses at the tellurium atom. A SLIP experiment using these isotopologues in human pancreatic cancer xenograft murine models highlighted regions experiencing fluctuating hypoxia via imaging mass cytometry—a technique that combines the quantitative, single mass unit resolution of MC with retention of spatial information lost during conventional flow-based analysis.

Finally, in Chapter 6, the advancements presented in this thesis are summarized and future directions that may prove productive are discussed.
Chapter 2
Identification of Hypoxic Cells \textit{In Vitro} Using a Probe Compatible with Mass Cytometry*

*The original research presented in this chapter has been published as: Edgar, L. J.; Vellanki, R. N.; Halupa, A.; Hedley, D.; Wouters, B. G.; Nitz, M. Identification of Hypoxic Cells Using an Organotellurium Tag Compatible with Mass Cytometry. \textit{Angew. Chem. Int. Ed.} \textbf{2014}, \textit{53}, 11473-11477. (Reference 89). The figures and text have reproduced with permission. All synthetic work and spectral characterization of compounds was performed by L. J. Edgar. Biological work was performed by L. J. Edgar, R. N. Vellanki, and A. Halupa. MC analysis was performed by L. J. Edgar and A. Halupa with assistance from T. Chen.

2.1 Tumour Hypoxia as a Target

Deficiency in molecular oxygen drastically alters cellular biochemistry. Metabolism, DNA repair, pH regulation processes, and angiogenesis are all altered in response to a low local \( \text{pO}_2 \), often leading to more aggressive cancer phenotypes.\textsuperscript{34-36} Indeed, the five year survival rate of patients presenting with severely hypoxic cancers is well below the average of 54\% for all cancers, with hypoxic pancreatic unchanged from 3\% over four decades of research.\textsuperscript{43} A major factor contributing to this poor prognosis is the ability of cells residing with a hypoxic microenvironment to resist conventional therapies such as ionizing radiation and chemotherapeutics.\textsuperscript{44-45} One reason for this is that the major effect of ionizing radiation is not directly damaging biomacromolecules (such as DNA) but rather generation of reactive oxygen species from \( \text{O}_2 \), such as superoxide, which then go on to elicit the cytotoxic effect.\textsuperscript{44} Chemotherapeutics are thought to be less effective against hypoxic tumour cells partially because of the quiescent state these populations adopt in response to low \( \text{O}_2 \) (Figure 2-1).\textsuperscript{46} Since many first line chemotherapeutics target active metabolic pathways (i.e. methotrexate (anti-folic acid metabolism), 5-fluorouracil (DNA replication), etc.) they are rendered ineffective once cellular metabolism has significantly decreased.\textsuperscript{46-47} Additionally, since hypoxic cell populations are often far from vasculature they are expected to have the most limited access to circulating therapeutic agents.\textsuperscript{44} For these reasons it is essential to identify hypoxic populations of cells in order to better understand
the adaptations they make to survive and, ultimately, how they can be selectively targeted and eliminated.

**Figure 2-1:** Illustration of the malignant progression cycle. Tumour cell proliferation leads to increased O$_2$ consumption. If vascularization is aberrant or angiogenesis cannot keep up with O$_2$ demand, hypoxic regions develop. Selection pressure for mutations that enable survival under low-O$_2$ conditions increase the proportion of cells that are resistant to conventional therapies. Adapted from reference 66.

Two major classes of tumour hypoxia have been identified; diffusion-limited (chronic) hypoxia and perfusion-limited (acute) hypoxia.$^{48}$ In the former case hypoxia is observed in cells at regions distant from vasculature. Since these cells are never in an environment that receives adequate O$_2$ they remain chronically hypoxic. In the perfusion-limited case, the distance of a cell from vasculature is less important—transient changes in the quantity and oxygenation status of blood passing through vasculature induces short-lived (acute) hypoxia. The biological consequences of this latter case are poorly understood compared to the diffusion-limited phenomenon.$^{35-36}$ Relationships between pO$_2$ and time have been
hypothesized to be highly variable in the perfusion-limited case, with rapid changes in pO₂ possibly contributing to a highly aggressive subpopulation of tumour cells that can effectively evade conventional chemotherapeutics.⁴⁹

### 2.1.1 The Significance of Dynamic Hypoxia

Cells exposed to repeated cycles of hypoxia and reoxygenation have been shown to have a long term survival advantage as compared with chronically hypoxic cells.⁴⁹ Cyclic hypoxia may be particularly important in its ability to activate pathways that promote metastasis, genomic instability, and resistance to treatment, but an understanding of what factors facilitate temporal changes in hypoxia at the cellular level is largely lacking.³⁶ Possible mechanisms of intratumoural oxygen dynamics include changes in interstitial fluid pressure, transient occlusion of vasculature, increased angiogenesis, and rate of cell turnover.⁴⁸ The fraction of cells in solid tumours experiencing dynamic oxygenation has been shown to be as high as 50% over 6 hours, but the specific clinical implications of the dynamic hypoxia timescale, which has been reported to occur over minutes or even days, are not understood, mainly due to a lack of tools for studying such processes.⁵⁰ Specifically, techniques that enable quantitative analysis of oxygenation dynamics in single cells are lacking.

### 2.2 Chemical Methods for Detecting Cellular Hypoxia

Several chemical functionalities have been identified as being able to selectively target cells which reside in an environment with a low pO₂. The most prevalent of these are the nitroimidazoles and nitrobenzyl compounds; however, recent advances in transition metal (and post-transition metal)-based complexes have provided additional functional handles for reporting on hypoxic cell populations.⁵¹-⁶⁰ Coupling these hypoxia-sensitive functional groups to modalities compatible with various analytical methods such as fluorescence imaging, flow cytometry, radiological techniques (PET, MRI, etc.) and mass spectrometry has allowed for detection of hypoxia from the gross anatomical scale all the way down to the single cell level both in vitro and in vivo.⁶¹-⁶⁹

The majority of chemical probes against cellular hypoxia take advantage of reductive metabolism to reduce a nitroaryl functionality to an electrophilic species that is capable of covalently labelling biomacromolecules.⁷⁰-⁷³ The efficiency of this process is heavily influenced by local pO₂, with
significant labelling often observed when O$_2$-tensions are near anoxic levels.$^{38-39}$ The one electron reduction potential ($E^1$) of the hypoxia-sensitive functionality is an important factor in labelling efficiency, although direct relationships between conjugate formation and electron affinity have not been fully characterized. Furthermore, the kinetics of conjugate formation are complex, with initial enzymatic reduction initiating a pathway leading to formation of the reactive electrophile which can either covalently label a target or be hydrolyzed.$^{71}$ Competition between productive conjugation and hydrolysis is an important process to consider when designing hypoxia probes as the structural changes that lead to increased electron affinity (and thus improved rates of reduction) could result in production of an electrophile so potent that hydrolysis dominates over productive conjugate formation. The ideal probe is can be reduced over a wide pO$_2$ range, but produces a labelling species that is sufficiently reactive towards biomolecules and is slowly hydrolyzed.

2.2.1 Nitroimidazoles as Redox-Sensitive Functionalities

Of all the functionalities used to target cellular hypoxia the nitroimidazoles are the most ubiquitous. Under aerobic conditions, nitroimidazoles have been used as radiosensitizers where they catalytically generate ROS as part of a “futile redox cycle” whereby the nitro group is enzymatically reduced by a single electron which is then transferred to molecular oxygen (Figure 2-2).$^{74}$ The superoxide ion is then further processed, ultimately damaging DNA, lipid, and protein. In high enough doses nitroimidazoles can also deplete cells of glutathione (GSH), resulting in cell death.$^{74}$ Under hypoxic conditions nitroimidazoles are less able to transfer the radical resulting from the initial one electron reduction step and instead are further reduced (3 e$^-$) to a hydroxylaminoimidazole.$^{70-71}$ This species can eject a hydroxide ion to afford an electrophilic nitrenium imidazole which can either react with water, low-molecular weight nucleophiles (e.g. GSH) or biomacromolecules (Figure 2-2).$^{71}$ The reaction of the nitrenium imidazole with biomacromolecules (usually thiols on exposed cysteine residues) is key for the ability of these heterocycles to act as probes for hypoxia.
Figure 2-2: Mechanisms of 2-nitroimidazole aerobic toxicity and anaerobic labelling. Adapted from reference 74.

The rate of enzymatic reduction of nitroimidazoles can be a predictive factor in evaluating how appropriate a specific scaffold is for probe design. Nitroaryls that are reduced inefficiently are not candidates for probe design as exacerbation of the proposed rate-limiting step in nitrenium ion formation (initial one electron reduction) would increase the amount of time required before sufficient conjugates form for detection. A standard method for measuring the rate of one electron reduction of the nitro group is an anaerobic xanthine oxidase (XO)-coupled assay where the UV-visible absorbance of the nitro group is monitored over time. Under anaerobic conditions, XO is able to use many nitroaryl compounds as surrogates for molecular oxygen as a final electron sink when catalysing the oxidation of xanthine to uric acid. A decrease in absorbance at 325 nm indicates loss of the nitro group and thus implies reduction has occurred. The rate of reduction correlates reasonably well with the one electron reduction potential of various nitroimidazoles (Figure 2-3, Table 2-1).
Figure 2-3: Relationship between one electron reduction potential and the rate of enzymatic reduction by xanthine oxidase. Outliers are presented in grey, and these compounds possess comparatively large (grey circles) or very small (ionisable compounds, grey squares) octanol-water partition coefficients. Data taken from Table 1-1. Figure adapted from reference 75.
<table>
<thead>
<tr>
<th>ID</th>
<th>Structure</th>
<th>$E^{1/2} (V)^a$</th>
<th>$R$ (nmol/min/U)$^b$</th>
<th>log$P^a$</th>
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<td>i</td>
<td>$O_2$</td>
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<td>N/A</td>
<td>N/A</td>
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<td>N/A</td>
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<tr>
<td>iii</td>
<td>$\begin{array}{c} \text{N} \ \text{H} \ \text{N} \ \text{H} \ \text{N} \ \text{O} \ \text{N} \end{array}$</td>
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<td>N/A</td>
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<tr>
<td>iv</td>
<td>$\begin{array}{c} \text{N} \ \text{H} \ \text{N} \ \text{H} \ \text{N} \ \text{Me} \ \text{O} \ \text{O} \ \text{N} \end{array}$</td>
<td>-0.243</td>
<td>$565 \pm 33$</td>
<td>0.80</td>
</tr>
<tr>
<td>v</td>
<td>$\begin{array}{c} \text{N} \ \text{H} \ \text{N} \ \text{H} \ \text{N} \ \text{Me} \ \text{CN} \ \text{Me} \end{array}$</td>
<td>-0.262</td>
<td>$680 \pm 147$</td>
<td>0.79</td>
</tr>
<tr>
<td>vi</td>
<td>$\begin{array}{c} \text{N} \ \text{H} \ \text{N} \ \text{H} \ \text{N} \ \text{Me} \ \text{MeO} \end{array}$</td>
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<td>$244 \pm 17$</td>
<td>3.2</td>
</tr>
<tr>
<td>vii</td>
<td>$\begin{array}{c} \text{N} \ \text{H} \ \text{N} \ \text{H} \ \text{N} \ \text{Me} \ \text{H}_2$ \ \text{N} \ \text{Me} \ \text{O} \end{array}$</td>
<td>-0.321</td>
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<td>0.56</td>
</tr>
<tr>
<td>viii</td>
<td>$\begin{array}{c} \text{N} \ \text{H} \ \text{N} \ \text{H} \ \text{N} \ \text{Me} \ \text{MeO} \end{array}$</td>
<td>-0.355</td>
<td>$21 \pm 4$</td>
<td>0.82</td>
</tr>
<tr>
<td>ix</td>
<td>$\begin{array}{c} \text{N} \ \text{H} \ \text{N} \ \text{H} \ \text{N} \ \text{Me} \ \text{OMe} \end{array}$</td>
<td>-0.360</td>
<td>$33 \pm 1$</td>
<td>0.45</td>
</tr>
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</table>
Table 2-1: The structures, reduction potentials, rates of enzymatic reduction by xanthine oxidase, and octanol-water partition coefficients of various nitroimidazoles. a: values taken from reference 75. b: values taken from reference 76.

It should be noted that compounds with especially large octanol/H₂O partition coefficients (logP values) are reduced more quickly than would be predicted (Figure 2-3, grey circles). It is likely that the increased hydrophobicity of these compounds leads to greater affinity for XO (and other reductases) and this results in a rate enhancement for reduction. One electron reduction potentials also correlate well with the ability of nitroimidazoles to act as radiosensitizers, with more easily reduced scaffolds providing a greater therapeutic benefit when administered with radiation therapy. This implies that even in vivo one electron reduction potentials provide valuable information regarding how readily a nitroimidazole probe candidate may be converted into its corresponding nitro-radical and subsequent nitrenium imidazole.
Although informative about the how suitable a particular nitroimidazole may be for enzymatic activation in vivo, reduction potentials and rates of enzymatic reduction do not report on two critical criteria: the ability of a nitroimidazole to enter a cell, and the efficiency with which a corresponding nitrenium imidazole forms productive (biomacromolecular) covalent conjugates once activated. The former is likely dependent on the same parameters that medicinal chemists often consider when selecting and optimizing molecules for use as drugs (Lipinski’s rules). For probes with basic groups it is imperative that the pK\textsubscript{a} of the basic site is low enough such that an appreciable percentage of molecules exist in the deprotonated state at physiological pH. Several nitroimidazole-based probes employ an endocyclic tertiary amine for this purpose (e.g. Pimonidazole, Figure 2-4) as the pKa of this motif is ~8.5 allowing ~10% of probe to exist in the neutral form at pH ~7.4.\textsuperscript{78} Probes bearing moderately basic groups actually may have an advantage with respect to cellular uptake as the protonated form of the probe can more easily associate with the predominantly negatively charged outer membrane of the cell and diffuse through the lipid bilayer once deprotonated. This process could aid in intracellular accumulation, thereby improving the quantity of labelling observed. In contrast to this potential advantage, probes that adopt a charged state can also accumulate preferentially in regions of low pH thereby biasing the apparent labelling of a region in a manner that may be less relevant to the oxygenation state of the region.\textsuperscript{79-80}

The efficiency of labelling with a nitrenium imidazole (Figure 2-2) is likely dependent on the interplay between reactivity and stability. The nitrenium imidazole must be reactive enough to rapidly form covalent conjugates at a location close to where reduction of the corresponding parent nitroimidazole occurred (to preserve spatial information), but not so reactive as to hydrolyze before meeting a reactive biomacromolecular partner. The reactivity of the nitrenium imidazole is likely strongly correlated to the reduction potential of the parent nitroimidazole, as more electron deficient compounds exhibit more positive reduction potentials (Table 2-1) and would also be expected to form nitrenium ions with enhanced electrophilicity and therefore greater reactivity towards nucleophiles. As such, the optimal nitroimidazole scaffold may not actually be the easiest to reduce. Thus, the most widely used nitroimidazole probes do not have the highest possible reduction
potential, with the ideal value being intermediate; around −0.390 V.\textsuperscript{77} It is because of this value of \(E_1\) that most nitroimidazole probes are based on a monosubstituted (usually at N1) 2-nitroimidazole (2-NI) scaffold. The 4- and 5-nitroimidazoles both exhibit substantially more negative \(E_1\) values unless heavily substituted with additional electron withdrawing groups, and di-/tri-substituted 2-NIs often possess reduction potentials that are too positive (Table 2-1).

### 2.2.2 2-Nitroimidazole-Based Hypoxia Probes

Because of their favourable redox properties, 2-NIs are the most common activity-based group (ABG) found in probes against cellular hypoxia for both \textit{in vitro} and \textit{in vivo} applications. Early work attached the 2-NI to a variety of scaffolds that could be detected \textit{via} antibody recognition, allowing for visualization with fluorescent antibody conjugates.\textsuperscript{38-42} This secondary detection method provided information about relative oxygenation states of individual cells both in culture and through immunohistochemical staining of tissue sections from disease models injected with an appropriate 2-NI-based probe.\textsuperscript{81} Molecules based on 2-NIs generally only efficiently label cells that reside in environments with less than 10 mmHg (~1.5% by volume) and exhibit drastic increases in labelling in increasingly hypoxic situations.\textsuperscript{39} Although few studies have reported precise pO\(_2\) vs. cell labelling relationships, one molecule, EF5 (Figure 2-4), was shown to label with greatly enhanced efficiency when pO\(_2\) levels were well below 1%.\textsuperscript{40} Significant, but less pronounced increases were reported for a pO\(_2\) regime between 1 and 10%. This trend appears to hold true for the majority of 2-NI-based probes.

It is important to note that the behaviours of structurally distinct probes differ substantially when used \textit{in vivo}.\textsuperscript{79-80} This is due primarily to differential pharmacokinetics of structurally inequivalent molecules. For example, pimonidazole—a 2-NI with a basic tertiary amine scaffold (Figure 2-4), exhibits uneven biodistribution due to pH-dependent accumulation of the molecule in more vascularized regions of tissue.\textsuperscript{80} In contrast, EF5—a probe with no ionizable groups at physiological pH, exhibits more homogeneous biodistribution and is therefore better able to report on deep-tissue hypoxia, although the increased logP value results in an decreased plasma clearance rate (5.1 vs. 11.5 hours for pimonidazole and EF5 respectively).\textsuperscript{82}
Figure 2-4: Structures and octanol-water partition coefficients for the commonly used hypoxia probes pimonidazole and EF5. Octanol-water partition coefficients obtained from reference 82.

Unsurprisingly, fluorescence has been one of the major techniques adopted for non-invasive detection of tumour hypoxia in vivo. Conjugation of 2-NI to fluorescein spironolactone through either an ionizable or hydrophobic linker has been shown to be useful in assessing cellular hypoxia both in cell culture and in vivo when monitoring hypoxia of murine retinae.79 The labelling efficiency of these two probes is significantly different, with the ionizable molecule apparently being preferentially taken up into cells in vitro, providing better signal to noise. The trade-off of this ionization-dependent accumulation is seemingly lower bio-distribution in vivo as reported (qualitatively) by the size and number of regions in murine retinae labelled as compared to the more lipophilic probe. This is consistent with differences in the observed properties of pimonidazole and EF5 (Figure 2-4).

Although very sensitive and relatively easy/inexpensive to perform, fluorescence techniques are limited for several reasons. The paramount limitation is mandatory installation of a photoactive group on the nitroimidazole ABG which severely limits the ability to fine-tune parameters such as probe molecular weight and logP. Additionally, some fluorophores are known to themselves be sensitive to enzyme-mediated reduction/metabolism which can further complicate analysis.83 As such, there has been much interest in creating probes that harness alternative reporter modalities for monitoring hypoxia, such as positron emission tomography (PET). Since several well-validated 2-nitroimidazole probes contain fluorine as part of their antigenic reporter group (i.e. EF5) incorporation of PET-detectable 18F was a logical extension since 18F-labeled molecules should not have pharmacological properties that differ from their 19F counterpart. Indeed, 18F-labelled EF5 and 18F-fluoromisonidazole (same structure as xii but with a fluorine in place of the OMe group) have proven to be effective in the clinic for non-invasive identification of hypoxic regions within a variety of tumour types.84 A disadvantage of these relatively hydrophobic probes is that
their prolonged clearance kinetics cause relatively low signal to noise when comparing healthy tissue to severely hypoxic regions. To combat this, other probes have been developed that possess reduced logP values to enhance plasma clearance rates, although the degree to which this impacts tissue distribution has not been well characterized. Several of these more hydrophilic 2-NIs contain a carbohydrate motif which drastically decreases their logP values and indeed promotes more rapid clearance—mainly through the bladder. Conjugation of a 2-nitroimidazole to O-6 on glucose through various linkers has been shown to be effective in enhancing uptake of 2-nitroimidazoles in glucose-addicted cancer cells; however, this approach does not appear to provide improved signal-to-noise in vivo when using glucose-2-NI constructs bearing the requisite $^{18}$F nucleus. Nuclei other than $^{18}$F that are PET-compatible (i.e. $^{68}$Ga) have also been included in a variety of probes against cellular hypoxia.

### 2.3 First Generation MC-Compatible Hypoxia Probe (Telox)

#### 2.3.1 Synthesis

Owing to its synthetic accessibility we initially chose the telluroether functionality for use as a MC-compatible reporter group, although very little toxicity data was available for this functionality. In order to generate a probe specific for cellular hypoxia we designed a molecule containing a 2-NI covalently tethered to a compact methyltelluroether.

A convergent synthetic route was chosen to access the desired molecule (Scheme 2-1). First, the mass tag component was assembled using metalloinsertion chemistry. Elemental tellurium was dissolved in a solution of methyllithium at room temperature, yielding the lithium salt of the methytelluride anion (I). This potent nucleophile was then treated with a functionalized alkylation reagent, 3-chloro-1-propanol, producing compound 2 in moderate yield. The hydroxyl-functionalized methyltelluroether was then converted into the corresponding $p$-nitrophenylcarbonate ester using $p$-nitrophenylchloroformate in pyridine. For the 2-NI component, azomycin (2-nitroimiazole) was treated with methyl bromoacetate under Finkelstein conditions affording compound 4 in modest yield. The methyl ester was then converted to $\beta$-amino amide through simple treatment with ethylenediamine in methanol at room temperature. Carbamylation of 5 with 3 afforded the methyltelluroether linked to the 2-NI (compound 6, referred to as “Telox” herein) in moderate yield.
Scheme 2-1: Synthesis and structure of the first-generation MC-compatible hypoxia probe “Telox”.

This compound was stable for months if stored in the dark as a solid and under an inert atmosphere at \(-20 \, ^\circ C\). At ambient light and oxygen levels the probe possessed a \(t_{1/2} > 48 \, h\) in solution (see section 2.3.6).

2.3.2 Telox Exhibits Low Toxicity

Consistent with the hypothesis that compact telluroethers exhibit lower toxicity than the corresponding aryltelluroethers Telox exhibited low toxicity in two complementary assays. First, the proliferative toxicity of Telox was measured in HC116 cells by confluency analysis (Figure 2-5).
Figure 2-5: Proliferative toxicity of Telox 2 in HCT116 cells. a) HCT116 cell confluence at various concentrations of Telox under normoxic atmosphere (21% O\textsubscript{2}). b) HCT116 cell confluence at various concentrations of Telox under hypoxic atmosphere (<0.02% O\textsubscript{2}).

This experiment suggested that proliferation was only mildly affected up to the maximum probe concentration evaluated (400 µM) under both normoxic (21% O\textsubscript{2}) and hypoxic (<0.02% O\textsubscript{2}) conditions. The second assay evaluated the metabolic toxicity of Telox using the reduction of the compound WST-1 as a metric for metabolic toxicity induced by the presence of Telox. In Jurkat cells, this experiment indicated a metabolic IC\textsubscript{50} of 200 ± 20 µM (Figure 2-6).

Figure 2-6: Metabolic toxicity of Telox. Jurkat cells were exposed to an appropriate concentration of Telox for 24 hours. Decreased normalized absorbance at 450 nm indicates Telox-promoted cellular toxicity since less WST-1 is reduced by cellular metabolism.

Taken together, these findings indicated that the toxicity of Telox, regardless of atmospheric O\textsubscript{2}-content, would likely not be a limiting factor for hypoxia-dependent labeling experiments at concentrations at or below 100 µM.
2.3.3 Telox is Efficiently Reduced by Xanthine Oxidase \textit{In Vitro}

As a surrogate for a POR enzyme, the sensitivity of Telox to enzyme-mediated reduction was evaluated using bovine xanthine oxidase (XO, Figure 2-7).\textsuperscript{75} Following the loss of the 2-NI absorption at 325 nm during XO-mediated oxidation of xanthine to uric acid demonstrated that under normoxia, no reduction of Telox was observed (data not shown); however, under hypoxic conditions a clear loss of the 2-NI occurred (Figure 2-7). Interestingly, the rate of reduction of Telox was higher than that for pimonidazole (Figure 2-4)—this is likely a consequence of Telox being able to bind to the XO active site with higher affinity than pimonidazole since the reduction potential of the 2-NI group should not be significantly perturbed by the either the β–amide or β–hydroxyl groups (we speculate that the reduction potential for both pimonidazole and Telox should be approximately $-0.38 \pm 0.01$, see entries 10 and 11 in Table 2-1). This experiment suggested that Telox was compatible with enzyme-mediated reduction and therefore should function as a hypoxia probe in cell-based assays.

\textbf{Figure 2-7}: Telox is enzymatically reduced by bovine xanthine oxidase. Top: XO-catalyzed reduction of a generalized 2-NI using xanthine as a source of electrons and FAD as a cofactor. Bottom: Change in UV absorption over time at 325 nm for the anaerobic enzymatic reduction of the 2-NI component of pimonidazole (black circles) or Telox (grey circles). [2-NI] = 100 µM, [xanthine] 500 µM, 0.2 units XO.
2.3.4 Cellular Tellurium-Accumulation is Oxygen-Dependent

Having confirmed that Telox could be enzymatically reduced \textit{in vitro}, we next evaluated the ability of the probe to label HCT116 cells under normoxic and hypoxic conditions (Figure 2-8). Under an atmosphere that contained $<0.02\%$ O$_2$, significant accumulation of tellurium was detected by ICP-MS analysis of whole cell pellets when cells were incubated for 3 hours in media containing Telox (100 $\mu$M). A substantially lower tellurium concentration was detected under all other concentrations of O$_2$, indicating that the labeling ability of Telox was indeed O$_2$-dependent. This level of O$_2$-sensitivity is similar to that of EF5, which exhibits dramatic increases in labeling only at O$_2$-tensions below 0.1%, although statistically significant labeling with EF5 has been reported at O$_2$-tensions higher than 1%.\textsuperscript{39,82} These findings indicated that Telox should be useful as a MC-compatible surrogate for widely-used probes against cellular hypoxia, such as pimonidazole and EF5.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2-8.png}
\caption{Tellurium content of whole HCT116 cell pellets as a function of O$_2$-exposure as measured by traditional ICP-MS. [Telox] = 100 $\mu$M, incubation time = 3 hours. Note that since nat\textsuperscript{Telox} (i.e. Telox with natural isotopic abundance tellurium) was used, the signal reported in this figure represents only 7.07\% total tellurium (natural abundance of $^{125}$Te).}
\end{figure}
2.3.5 Telox Can Identify Individual Hypoxic Cells in a Mixture

Our next task was to investigate whether or not Telox was capable of identifying hypoxia cells in a mixture using MC. Since tumour oxygenation is known to be heterogeneous in vivo, we reasoned that in order for Telox to be useful it would have to be capable of selectively discriminating hypoxic cells from within a heterogeneous mixture. As a simple model of this situation, HCT116 cells were incubated under normoxic (21% O₂) or hypoxic (<0.02% O₂) conditions in the presence of Telox as detailed in section 2.3.4. Following exposure to Telox, the cells were washed and fixed. To correlate the cellular tellurium content with oxygen exposure, we used metal-containing nucleic acid intercalators as internal standards for O₂-exposure. Cells incubated under normoxic conditions were treated with a rhodium-containing nucleic acid intercalator, whereas cells incubated under anoxic conditions were treated with an iridium-containing nucleic acid intercalator (Figure 2-9a). Both samples were washed separately, combined, and injected onto a second-generation mass cytometer (CyTOF) for MC analysis (Figure 2-9a). Generation of a density plot of event length versus ¹³⁰Te signal clearly indicated the presence of two distinct populations of cells (Figure 2-9b). Gating each population (high ¹³⁰Te (median ~120 counts) or low ¹³⁰Te (median ~30 counts)) and outputting ¹⁹³Ir versus ¹⁰³Rh density plots from these gates indicated that cells which contained a high relative amount of ¹³⁰Te possessed a very high ¹⁹³Ir content and a low ¹⁰³Rh content (Figure 2-9c). Conversely, cells that produced a lower ¹³⁰Te signal contained far less ¹⁹³Ir and a comparatively large amount of ¹⁰³Rh (Figure 2-9d). These results were consistent with expectations, as MC analysis was able to deconvolute the two cell populations to a high degree of resolution using tellurium content as a metric for the O₂-concentration to which cells were exposed. The non-zero ¹⁹³Ir signal observed in cells incubated under normoxic conditions was likely was likely due to leaching of less tightly bound Ir intercalator between cell populations after mixing. This is supported by the observation that cells exposed only to the Rh intercalator and not mixed with Ir-stained cells exhibit a near-zero ¹⁹³Ir signal (Figure 2-10).

Finally, in an attempt to further confirm the mode of action, we evaluated the ability of Telox to compete for the same bioreductive pathway as pimonidazole. Incubation of HCT116 cells in media that contained both probes at equal concentrations (100 µM) reduced the tellurium labeling by 1.7-fold when compared to the signal in the absence of pimonidazole as indicated by MC analysis (Figure 2-9e). This result suggested that these probes compete for at least some of the same
Figure 2-9: Telox is able to identify hypoxic cells in a mixture. a) Schematic representation of Telox cell labeling for analysis by MC on a second-generation CyTOF instrument. b) Density map of signal event length versus $^{130}$Te signal (arbitrary units). c) Density plot output from the top-right gate in (b) of the $^{193}$Ir signal versus the $^{103}$Rh signal. More than 93% of the detected events fall in the square gate. d) Density plot output from the bottom-left gate in (b) of the $^{193}$Ir signal versus the $^{103}$Rh signal. More than 89% of the detected events fall in the square gate. e) Population histograms of cell $^{130}$Te content. Oxygen concentrations are listed as numerical percentages, P = pimonidazole (100 µM). Blue and red histograms are pimonidazole-negative controls. Orange and green histograms are the pimonidazole-positive competition experiments. Note: warmer colours in density lots indicate higher cell population density. Note that since nat-Telox (i.e. Telox with natural isotopic-abundance tellurium) was used, the signal reported in this figure represents only ~34% total tellurium (natural abundance of $^{130}$Te).
Figure 2-10: $^{193}$Ir and $^{103}$Rh content of cells incubated with 100 µM Telox under normoxic (21 % O$_2$) atmosphere. a) Density plot of signal event length vs. $^{130}$Te signal (arbitrary units) for cells stained with the Ir-containing nucleic acid intercalator (Figure 2-9a). b) Density plot output from the gate in a) of $^{193}$Ir signal (arbitrary units) vs. $^{103}$Rh signal (arbitrary units). More than 99 % of cell events fall in the square gate. c) Density plot of signal event length vs. $^{130}$Te signal (arbitrary units) for cells stained with the Rh-containing nucleic acid intercalator (Figure 2-9a). d) Density plot output from the gate in c) of $^{193}$Ir signal (arbitrary units) vs. $^{103}$Rh signal (arbitrary units). More than 99 % of cell events fall in the square gate.

2.3.6 Telox Degrades Under Aerobic/Aqueous Conditions

Although useful for identifying hypoxic cells, the long-term stability of Telox was an important attribute to understand when moving forward with this molecule. Of particular interest was its stability under biologically relevant conditions—humid atmosphere and oxygen. In order to investigate this, we recorded NMR spectra of solutions of Telox that had been stored under either anaerobic or aerobic conditions. The partial $^1$H NMR spectrum of Telox in DMSO-d$_6$ at time = 0 is presented in Figure 2-11a. The resonance for the methyl group bound directly to the tellurium atom is visible as a singlet with satellites ($^{125}$Te-$^1$H coupling) at ~1.82 ppm. The partial $^1$H NMR spectrum for a sample incubated under anaerobic conditions for 48 hours is presented in Figure 2-
This spectrum appears to be essentially identical to the control (time = 0, Figure 2-11a) spectrum suggesting that under these conditions, no significant degradation of Telox occurred. In contrast to this, the $^1$H NMR spectrum of a separate sample incubated under normoxic conditions (i.e. open to atmosphere with 21% O$_2$ (moisture not excluded), Figure 2-11c) for 24 hours displayed peaks not present in the control spectrum (Figure 2-11a). The major new peaks possessed chemical shifts consistent with literature values for protons located $\beta$ to an oxidized tellurium atom—specifically an alkyltelluroxide or alkyltellurone. Thus, we hypothesized that these new resonances corresponded to the methyl group in Telox directly bound to a now-oxidized tellurium atom, although assignment of these peaks to a particular oxidized product is difficult (Figure 2-11d). Besides the telluroxide and tellurone, the hydrate of the telluroxide is also possible (Figure 2-11d). Additionally, the intensity of the residual DMSO-d$_5$ peak relative to the parent compound further suggested that significant loss of the parent compound occurred under these conditions, possibly due to precipitation or aggregation processes (compare the resonances at ~1.9 and 2.5 ppm (Telox and DMSO-d$_5$ respectively) in panels a-c in Figure 2-11).
Figure 2-11: Telox degrades under aerobic conditions as measured by $^1$H NMR. a) $^1$H NMR spectrum of Telox recorded at $t = 0$. The peak at ~1.82 ppm corresponds to the $-\text{TeCH}_3$ resonance. b) $^1$H NMR spectrum of Telox recorded at $t = 48$ hours. This sample was flushed with N$_2(g)$ prior to incubation. c) $^1$H NMR spectrum of Telox recorded at $t = 48$ hours. This sample was left open to ambient atmosphere (21% O$_2$, moisture not excluded). The peaks at ~2.12 and ~2.18 ppm likely correspond to the $-\text{Te(O)CH}_3$ (and/or $-\text{Te(OH)CH}_3$), and/or $-\text{Te(O)CH}_3$ resonances. d) Hypothesized oxidative degradation pathway for Telox (only the telluroether portion of the molecule is shown for clarity). Notes: all samples were stored at room temperature in ambient light. [Telox] = 40 mM in DMSO-d$_6$. 
2.3.7 Summary

The findings presented in this chapter suggested that the first-generation hypoxia probe, Telox, was capable of discriminating hypoxia cells from within a mixture and was compatible with analysis of samples by MC. The synthetic scheme developed provided a method for accessing adequate quantities of Telox for cell-based assays, and was practical since only two of the six transformations required flash-column chromatography for purification. Toxicity assays indicated that Telox exhibited low toxicity in human colorectal and pancreatic cancer cells, suggesting that the methyl telluroether functionality was bio-compatible at the concentrations/time scales explored in this study. The mechanism of action of this probe was found to be consistent with the well-validated hypoxia probe pimonidazole, as Telox was efficiently reduced by the enzyme xanthine oxidase and was competitive with pimonidazole when HCT116 cells were incubated with both probes.

Although potentially useful, Telox exhibited some significant limitations. First, the probe was sensitive only to the most extreme case of hypoxia (<0.02% O₂) as measured by traditional ICP-MS. Although greatly enhanced labeling is often observed with hypoxia probes constructed around a 2-nitroimidazole under near-anoxic conditions, Telox did not appear to label on a gradient of O₂ at all. This is in contrast to probes such as pimonidazole and EF5, which exhibit some labelling activity at higher O₂-tensions.39 Additionally, the long-term stability of Telox was a concern as significant oxidation of the compound was observed after exposure to normal atmosphere (21% O₂) for 48 hours as measured by 1H NMR. Sensitivity to oxidation is undesirable as some oxidized organotellurides have been shown to possess enzyme-like activities; specifically, some aryl telluroxides mimic the activity of glutathione peroxidase.30-31 Since our goal was to develop a biologically inert mass tag for incorporation into activity-based scaffolds, we reasoned that additional work was needed to identify new organotellurium functionalities that retained the low toxicity of methyl telluroethers, but possessed enhanced stability.
2.4 Chapter 2 Experimental

Section 2.3.1:

3-(methyltellanyl)propan-1-ol (2)

Elemental tellurium pellets (2.0 g, 15.67 mmol, −5 – + 50 mesh size) were pulverized into a fine powder using a mortar and pestle and immediately suspended in dry THF (40 mL) in a 250 mL oven-dried round bottom flask equipped with a magnetic stir bar. The flask was flushed with nitrogen and MeLi (9.8 mL of a 1.6 M solution in diethyl ether, 16 mmol) was added dropwise over 1 minute at room temperature with stirring. This clear yellow solution was allowed to stir for 10 minutes at room temperature. If the solution appeared deep red / brown a few extra drops of MeLi were added to revert the solution back to a clear homogeneous yellow. The solution was then frozen in liquid nitrogen and 3-chloropropan-1-ol (1.3 mL, 16 mmol) was added all at once. The solution was stirred vigorously and allowed to warm to room temperature over 2 hours at which point saturated NH₄Cl(aq) (100 mL) was added. The product was then extracted from the aqueous solution with diethyl ether (2 x 100 mL portions, dried over anhydrous MgSO₄), and dried under vacuum to afford 2 (2.28 g, 72 %) as a viscous orange oil. WARNING: This chemistry produces foul-smelling compounds. Take appropriate precautions. ¹H NMR (500 MHz, CDCl₃): δ 3.67 (t, 2H, J = 6.2 Hz, HO-CH₂-CH₂−), 2.66 (t, 2H, J = 7.4 Hz, H₃C-Te-CH₂-CH₂−), 1.97 (app. p, 2H, J = 7.4 Hz, −CH₂-CH₂-CH₂−), 1.88 (s with Te satellites, 3H, ²Jₜₜₜₜ = 10 Hz, H₃C-Te−); ¹³C NMR (125 MHz, CDCl₃): δ 63.88, 34.21, -1.27 (¹J₁₂ = 75 Hz, H₃C-Te-CH₂−), -22.22 (¹J₁₂ = 80 Hz, H₃C-Te−); ¹²₅Te NMR (126 MHz, CDCl₃): δ 106.37. HRMS m/z calcd. for C₄H₁₁O¹³⁰Te (MH⁺) 204.9872, found 204.9877.

3-(methyltellanyl)propyl (4-nitrophenyl) carbonate (3)

An oven-dried 250 mL round bottom flask was charged with 2 (2.59 g, 12.8 mmol), pyridine (2.18 mL, 27.1 mmol) and a magnetic stir bar. Dry THF (30 mL) was added and the flask was flushed with nitrogen. A solution of p-nitrophenyl chloroformate in THF (10 mL of 1.45 M, 13.5 mmol) was then added dropwise over 2 minutes via syringe with stirring. The mixture was allowed to stir a room temperature for 2 hours at which point the solvent was removed via rotary evaporation. The crude product was purified via flash chromatography (silica gel stationary phase, 10–30 %
EtOAc / pentanes mobile phase) to afford 3 (3.5 g, 75 %) as a viscous dark orange oil. \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 8.27 (d, 2H, \(J = 9.2\) Hz, Ar), 7.74 (d, 2H, \(J = 9.2\) Hz, Ar), 4.36 (t, 2H, \(J = 6.4\) Hz, \(-\text{CH}_2-\text{CH}_2-\text{OCO}^-\)), 2.71 (t, 2H, \(J = 7.6\) Hz, H\(_3\)C-Te-CH\(_2\)-CH\(_2\)-), 2.20 (app. p, 2H, \(J = 6.4\) Hz, \(-\text{CH}_2-\text{CH}_2-\text{CH}_2-\)), 1.94 (s with Te satellites, 3H, \(2^j\)\(^{\text{H}}\)-\(125\)Te = 10 Hz, H\(_3\)C-Te-); \(^13\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 155.47, 152.38, 145.32, 125.26, 121.75, 70.36, 30.56, -2.45 (\(1^j\)\(^{\text{C}}\)-\(125\)Te = 78 Hz, H\(_3\)C-Te-CH\(_2\)-), -21.95 (\(1^j\)\(^{\text{C}}\)-\(125\)Te = 80 Hz, H\(_3\)C-Te-); \(^{125}\)Te NMR (189 MHz, CDCl\(_3\)): \(\delta\) 119.46. HRMS \(m/z\) calcd. for C\(_{11}\)H\(_{17}\)N\(_2\)O\(_5\)\(^{130}\)Te (M\(^{\text{NH}_4}\)\(^+\)) 387.0200, found 387.0197.

**N-(2-aminoethyl)-2-(2-nitro-1H-imidazol-1-yl)acetamide (5)**

An oven-dried 50 mL round bottom flask was charged with a solution of methyl 2-(2-nitro-1H-imidazol-1-yl)acetate\(^91\) (500 mg, 2.7 mmol) in methanol (4.72 mL) and a magnetic stir bar. Ethylenediamine (0.722 mL, 10.8 mmol) was added dropwise to this solution over 1 minute and the mixture was allowed to stir at room temperature for 18 hours. Solvent was then removed via rotary evaporation and the resultant solid was dried under vacuum for 2 days to afford 5 (575 mg, ~ quantitative) as an amorphous pale yellow solid. \(^1\)H NMR (500 MHz, MeOD): \(\delta\) 7.45 (d, 1H, \(J = 1.2\) Hz, Ar), 7.17 (d, 1H, \(J = 1.2\) Hz, Ar), 5.17 (s, 2H, Ar-CH\(_2\)-CO\(^-\)), 3.31 (t, 2H, \(J = 6.2\) Hz, \(-\text{CH}_2-\text{CH}_2-\text{NHCO}^-\) + residual MeOD overlap), 2.75 (t, 2H, \(J = 6.2\) Hz, H\(_2\)N-CH\(_2\)-CH\(_2\)-); \(^13\)C NMR (125 MHz, MeOD): \(\delta\) 167.00, 128.00, 126.97, 51.48, 41.74, 40.47. HRMS \(m/z\) calcd. for C\(_7\)H\(_{12}\)N\(_5\)O\(_3\) (MH\(^+\)) 214.0940, found 214.0937.

**3-(methyltellanyl)propyl (2-(2-(2-nitro-1H-imidazole-1-yl)acetamido)ethyl) carbamate (6, Telox)**

An oven-dried 50 mL round bottom flask was charged with 5 (100 mg, 0.47 mmol), pyridine (0.113 mL, 1.41 mmol), and a magnetic star bar. The flask was flushed with nitrogen and dry methanol (4.0 mL) was added. A solution of 3 in methanol (1.0 mL of 470 mM, 0.47 mmol) was then added dropwise via syringe over 1 minute and the mixture was allowed to stir at room temperature for 2 hours. Solvent was then removed via rotary evaporation and the crude product was purified via flash chromatography (silica gel stationary phase, 2–6 % methanol / chloroform mobile phase) to afford 6 (Telox) (145 mg, 70 %) as a yellow-orange solid. \(^1\)H NMR (600 MHz, MeOD/CD\(_3\)CN (2:1 v/v)): \(\delta\) 7.39 (d, 1H, \(J = 1.2\) Hz, Ar), 7.17 (d, 1H, \(J = 1.2\) Hz, Ar), 5.1 (s, 2H, Ar-CH\(_3\)-CONH\(^-\)), 4.06 (t, 2H, \(J = 6.2\) Hz, \(-\text{CH}_2-\text{CH}_2-\text{O}-(\text{CONH})^-\)), 3.31 (t, 2H, \(J = 6.3\) Hz, \(-\text{CH}_2-\)
\[ \text{CH}_2\text{-NH(CO)}^- + \text{residual MeOD overlap} \], 3.20 (t, 2H, \( J = 6.3 \text{ Hz} \), -(CO)NH-\text{CH}_2\text{-CH}_2^-), 2.65 (t, 2H, \( J = 7.4 \text{ Hz} \), -Te-\text{CH}_2\text{-CH}_2^-), 2.02 (app. p, 2H, \( J = 7.0 \text{ Hz} \), -CH\text{-CH}_2\text{-CH}_2^-), 1.89 (s with Te satellites, 3H, \( J^1_{\text{H}^3\text{-Te}} = 10 \text{ Hz} \), \text{H}_3\text{C}-\text{Te}^-); ^{13}\text{C} \text{ NMR (150 MHz, MeOD)}: \delta 192.83, 166.90, 127.92, 126.97, 65.63, 51.43, 39.74, 39.28, 31.02, -2.92, -25.07; ^{125}\text{Te} \text{ NMR (126 MHz, MeOD)}: \delta 117.77. \text{HRMS m/z calcd. for C}_{12}\text{H}_{20}\text{N}_5\text{O}_5^{130}\text{Te (MH}^+\text{)} 444.0527, \text{found 444.0524.}

**Section 2.3.2:**

**Cell culture and maintenance**

HCT116 cells (colorectal carcinoma cell line (CCL-247™)) was obtained from American Type Culture Collection and cultured / maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum.

**Confluency (proliferative toxicity) assay**

HCT116 cells (25,000) were seeded in a 24 well plate and incubated overnight to allow the cells to adhere. The medium was removed and fresh medium with 50–400 µM Telox was added and incubated for 1 h. The cells were then transported to a IncuCyte™ kinetic imaging system that was maintained either at 21 % or 0.2 % O\text{2} at 37 °C. Growth profiles were monitored by 10 X objective every 4 h by IncuCyte™ ZOOM control software, using integrated confluence algorithm, until the control-untreated cells reached stationary phase. Sixteen high definition-quality images per well were collected in phase-contrast mode and averaged to provide a representative statistical measure of the well confluence.

**Metabolic toxicity assay**

A 96 well clear fluorometer plate was loaded with 200 µL of Jurkat cells (per well) at a culture density of 1 x 10\text{6} cells per mL. To each well was added an appropriate amount of a stock solution of Telox in sterile DMSO to reach a desired concentration. The concentration of DMSO was 1 % in all wells. Cells were allowed to incubate for 24 h at 37 °C under normal atmosphere, after which 20 µL of a commercially available solution of WST-1 in PBS (Roche Diagnostics, product # 05015944001) was added to each well (gentle pipetting evenly distributed the reagent throughout the well). Cells were allowed to incubate for a further 0.5 h at 37 °C under normal atmosphere, followed by subsequent measurement of the absorbance of each well at 450 nm using a TECAN
Safire 2 plate reader. Data was background corrected vs. wells that contained cell growth media (without cells), an appropriate concentration of Telox, a final concentration of DMSO = 1 %, and WST-1. Background correction wells were incubated in the same manner as described for cell-positive wells.

Section 2.3.3:

Reduction of Telox by xanthine oxidase

A septa-sealable quartz cuvette was charged with K$_2$PO$_4$ buffer (800 µL, 100 mM, pH 7.4), xanthine in K$_2$PO$_4$ buffer (100 µL, ~ 5.0 mM xanthine (saturated solution), 100 mM buffer, pH 7.4), and either pimonidazole or Telox in K$_2$PO$_4$ buffer (100 µL, 1.0 mM of the 2-NI, 100 mM buffer, pH 7.4). The cuvette was then sealed with a rubber septum and the entire solution was degassed with high purity helium gas. Xanthine oxidase (0.2 units of grade III enzyme in a (NH$_4$)$_2$SO$_4$ suspension, Sigma-Aldrich, lot # SLBB1572V) was then added via Hamilton syringe and the change in absorbance over time at 325 nm was recorded using an Agilent UV-visible photospectrometer (model # 8453).

Section 2.3.4:

Oxygen-dependent labelling

HCT 116 cells (see cell culture and maintenance in section 2.2.2) were incubated in media containing Telox (100 µM, added as a neat solution in sterile DMSO; final [DMSO] = 0.1%) for 3 hours under atmosphere containing an appropriate concentration of O$_2$ (see hypoxia exposure conditions below). Following incubation, the media was removed, cells were washed with sterile PBS, and then separated from the incubation plate via trypsinization (37 °C, 10 min) and gentle scraping. The cell suspension was pelleted and resuspended in PBS containing β-ME (1.0 mL, 100 mM). Cells were pelleted, resuspended in PBS (900 µL, no β-ME), and fixed with formaldehyde (100 µL, 37% solution) for 25 minutes. Following fixation, cells were pelleted, resuspended in PBS containing β-ME (1.0 mL, 100 mM), and pelleted once again. The resultant pellet was then dissolved in ultra pure HNO$_3$ (1.0 mL, ~ 35% solution) and half of the sample was submitted for analysis via ICP-MS. Signal for $^{130}$Te was then normalized to signal for $^{115}$In at a known concentration (5 ppb, measured from ICP-MS set-up solution, PerkinElmer) in order to account
for detector sensitivity drift. The resultant signal was further normalized to give the sample with the maximum tellurium signal a value of 1.0. All experiments were performed in duplicate.

**Hypoxia exposure**

HCT116 (500,000) cells were seeded in 60 mm plastic petri dishes (Corning Inc., NY) and incubated for 24 h at 37 °C, 21% O₂ / 5% CO₂. The cells were transferred to hypoxia chambers (H35/ H85 hypoxia workstation, Don Whitley Scientific) maintained at 1 % / 0.2 % or < 0.02 % O₂ for 3 h. Hypoxia experiments (< 0.02 % O₂) were performed by seeding the cells in 60 mm glass plates (Corning Inc., NY).

**Section 2.3.5:**

**Mass cytometry experiments**

HCT 116 cells (see sections 2.3.2 and 2.3.4) were incubated in media containing Telox (100 µM, added as a neat solution in sterile DMSO; final [DMSO] = 0.1 %) for 3 hours under atmosphere containing an appropriate concentration of O₂. Following incubation, the media was removed, cells were washed with sterile PBS, and then separated from the incubation plate via trypsinization (37 °C, 10 min) and gentle scraping. The cell suspension was pelleted and resuspended in PBS containing β-ME (1.0 mL, 100 mM). Cells were pelleted, resuspended in PBS (900 µL, no β-ME), and fixed with formaldehyde (100 µL, 37 % solution) for 25 minutes. Following fixation, cells were pelleted, resuspended in PBS containing β-ME (1.0 mL, 100 mM), and pelleted once again. Cells were then resuspended in PBS (990 µM, no β-ME) and incubated with either the Ir-containing nucleic acid intercalator (hypoxic cells) or the Rh-containing nucleic acid intercalator (normoxic cells) (see Figure 2-9a) (10 µL, 100 µM solution in sterile PBS) for 20 minutes. Cells were then pelleted and resuspended in PBS (1.0 mL, no β-ME) twice. Cell pellets were then resuspended in PBS containing ^151/153^Eu beads (1/10 dilution of CyTOF® calibration beads, DVS Sciences, 1.0–2.5 mL depending on pellet size). The two cells samples were then combined (250 µL of each) and 250 µL of the resultant sample was injected onto a CyTOF 2® instrument for MC analysis. For the data presented in Figure 2-10, cell samples were not mixed together, but rather, run separately on the CyTOF 2®. For the Pimonidazole competition experiment (see Figure 2-9e), cells were simultaneously incubated with Pimonidazole and Telox (100 µM of each), with all other
steps executed in an identical manner to the experiment described above (Note: only the Ir-
intercalator was used for this experiment since all samples were run separately).
Chapter 3
Organotellurium Scaffolds Optimized for Activity-Based Probe Design*

*The original research presented in this chapter has been published as: Park, H.; Edgar, L. J.; Lumba, M. A.; Willis, L. M.; Nitz, M. Organotellurium Scaffolds for Mass Cytometry Reagent Development. Org. Biomol. Chem. 2015, 13, 7027-7033. (Reference 113) The figures and text have reproduced with permission. Synthetic work was performed by H. Park, L. J. Edgar., and M. A. Lumba. Biological work was performed by L. Willis. Degradation experiments were designed by H. Park, L. J. Edgar, and M. Nitz, and performed by H. Park.

3.1 Optimized Organotellurium Functionalities: Introduction

Although Telox demonstrated an important concept, the telluroether functionality was unoptimized, having moderate stability under biological conditions. This issue motivated us to more thoroughly investigate organotellurium chemical space with the goal of identifying a more appropriate mass-tag scaffold for incorporation into new ABPs compatible with MC. This chapter describes our efforts to synthesize a small library of differently-functionalized organotellurides and evaluate their stability under aerobic/aqueous conditions. The toxicities of compounds found to be stable under conditions that mimic cell-based assays are also investigated. We discuss three major classes of tellurium-containing functional groups: methyl telluroethers, trifluoromethyl telluroethers, and tellurophene heterocycles. Aryl telluroethers were not investigated because of their aforementioned glutathione peroxidase-like activity.\textsuperscript{30-31} Aryl telluroethers have also been shown to be unstable in ambient light.\textsuperscript{94}

3.2 Generation of a Library of Organotellurium Compounds

3.2.1 Synthesis of Methyltelluroethers

To ensure that the sensitivity towards oxidation exhibited by Telox was not a special case, our first goal was to construct a small library of methyl telluroethers with various attached functional groups. If oxidation promoted by aerobic atmosphere is a general phenomenon exhibited by methyl telluroethers then this class of organotelluride would be inappropriate for ABP design.
Two synthetic routes were pursued for accessing methyl telluroethers; nucleophilic substitution (Scheme 3-1, top), and Michael-type addition chemistry to an $\alpha,\beta$-unsaturated ester (Scheme 3-1, bottom).

In the first case, nucleophilic lithium methyl tellurolate was generated using a modified procedure first established by N. Khun et. al. Addition of the desired electrophile afforded compounds 2, 7, and 8 with yields ranging from moderate to excellent. In the second case, methyltellurol was generated by quenching lithium methyl tellurolate with H$_2$O followed by addition of the Michael-acceptor methyl acrylate, providing compound 9 in good yield. Because a common method for attaching reporter groups to activity-based scaffolds is through an amide linkage, we saponified compound 8 and subsequently coupled the resultant carboxylate to benzyl amine using standard conditions (Scheme 3-1). Finally, we synthesized a carbamate-containing methyl telluroether (17) to better represent the structure of the first-generation hypoxia probe without including any potential confounding effects from inclusion of a 2-NI functionality. We reasoned that if the carbamate linkage was contributing to instability (or even toxicity), the simple benzylcarbamate 17 should highlight this. We accessed 17 through simple carbamylation of benzylamine with 3 (Scheme 3-2, see section 2.3.1 for the synthesis of 3). The compounds described in this section are summarized in Table 3-1.

Scheme 3-1: Synthesis of methyl telluroethers. Yields: 2 (74%), 7 (66%), 8 (91%), 9 (85%), 10 (81%).
3.2.2 Synthesis of Trifluoromethyltelluroethers

Tellurium atoms in the −2 oxidation state are expected to be soft and electron-rich. Thus, it is not unexpected that oxidation of the element is a relatively favourable process. In order to combat this, we reasoned that a functionality with less electron-density formally localized on the tellurium nucleus could potentially be less susceptible to oxidation by molecular oxygen. Trifluoromethyl groups are potent withdrawers of electron density from atoms they are bound to, and we reasoned that by replacing the −CH₃ group in methyl telluroethers with −CF₃, sufficient electron density could be removed from the tellurium atom to decrease the rate at which it oxidizes. To realize this, we adopted a synthetic procedure developed by Tyrra et al. (Scheme 3-3).⁹⁶ Trifluoromethyltrimethylsilane (TMS−CF₃) was treated with tetramethylammonium fluoride in the presence of elemental tellurium, generating the tetramethylammonium salt of trifluoromethyltelluride in situ. Addition of methyl 4-bromobutyrate afforded compound 11 in low yield. Inefficient generation of trifluoromethyltelluride from TMS−CF₃ is the likely source of this low yield since this reaction requires strictly anhydrous conditions to proceed efficiently and tetramethylammonium fluoride is often contaminated with trace amounts of H₂O. In order to demonstrate chemical compatibility with common transformations used to attach reporter groups to activity-based scaffolds, we once again opted to saponify the methyl ester and subsequently couple the resultant carboxylate to benzylamine. This provided access to compound 12.

Scheme 3-2: Synthesis of compound 17.

Scheme 3-3: Synthesis of trifluoromethylethers 11 and 12.
3.2.3 Synthesis of Tellurophenes

Tellurophenes have not been evaluated in biological systems, and only recently has the first water soluble tellurophene been reported.\textsuperscript{97} Tellurophenes possess interesting photophysical properties and have been investigated as light harvesting agents for solar cell applications and in materials chemistry.\textsuperscript{98-100} The lighter chalcogen analogue, selenophenes, have been investigated in biological systems with promise as antioxidant molecules. Computational analysis has revealed that the ground state aromaticity of tellurophenes should enhance their stability more than comparable selenophenes.\textsuperscript{101} We hypothesized that the aromatic nature of the tellurophene would provide greater chemical stability over the telluroether derivatives under the desired biological conditions, primarily because the electron density on the tellurium atom should be delocalized around the ring, and thus, make oxidation of tellurium a higher energy process.

Our strategy for accessing functionalized tellurophenes was addition of $\text{Te}^{2-}$ to a monofunctionalized diacetylene in a protocol modified from the initial report by Barton and Roth.\textsuperscript{102} In synthesizing these tellurophenes, the generation of $\text{Te}^{2-}$ was a key step. Commonly, an aqueous suspension of $\text{Te}^0$ is treated with $\text{NaBH}_4$; however, we found the use of a basic Rongalite ($\text{HOCH}_2\text{SO}_2\text{Na}$) solution reproducibly generated $\text{Te}^{2-}$ and gave higher yields of the desired tellurophene.\textsuperscript{103-104} Trialkylsilyl-protected diacetylenes are the source of tellurophene ring carbon in this reaction and can be generated in excellent yield using the Cadiot-Chodkiewicz cross-coupling reaction (Scheme 3-4).\textsuperscript{105} Bromination of triisopropylsilyl acetylene using $N$-bromosuccinimide and silver nitrate afforded the (bromoethynyl)triisopropylsilane building block 13 required for Cadiot-Chodkiewicz chemistry.\textsuperscript{106} This compound was then coupled to an appropriate terminal alkyne partner using catalytic $\text{CuCl}$ in 30% $\text{BuNH}_2(\text{aq})$. The triisopropylsilyl-protected diacetylene compounds were then deprotected using tetrabutylammonium fluoride and subsequently cyclized, using the previously discussed conditions, yielding tellurophenes 14 and 15 in good yield (Scheme 3-4). Once again, to demonstrate that tellurophenes were compatible with standard amide-bond forming chemistry, we coupled compound 15 to benzylamine, producing compound 16 in good yield.
Scheme 3-4: Synthesis of monofunctionalized tellurophenes 14-16. Yields: 13 (98%), 14 (66%), 15 (70%), 16 (81%).
3.3 Aerobic Stability of Functionalized Organotellurium Compounds

Having synthesized the desired organotellurides (Table 3-1), the next task was to evaluate their stabilities under aerobic conditions. NMR was the analytical method of choice for monitoring stability since the technique is quantitative (with inclusion of an appropriate internal standard) and provides detailed structural information. Additionally, the non-destructive nature of NMR would allow for monitoring a single sample at multiple time points.

![Structures and compound IDs for the organotellurides synthesized in section 3.2.](image)

**Table 3-1:** Structures and compound IDs for the organotellurides synthesized in section 3.2.

### 3.3.1 Aerobic Stability of Methyltelluroethers

The relative chemical stabilities of the methyl telluroethers (compounds 2, 7-10, and 17) were quantified using $^1$H NMR by integration of the –TeCH$_3$ signals with respect to a residual DMSO-d$_5$ internal standard. Samples were prepared as solutions in DMSO-d$_6$ and placed under a slow, continuous stream of dry ambient atmosphere in a clear glass desiccator (Figure 3-1). This setup allowed the stability of the compounds to be monitored without interference from atmospheric water. Aliquots of each sample were taken periodically over a 24 h period for analysis via $^1$H NMR.
All methyl telluroethers exhibited aerobic instability over the course of the 24 h incubation. This is consistent with our observations for Telox (6). The amide- and carbamate-containing compounds (10 and 17 respectively) appeared to be the most stable, with <20 % degradation over a 24 h period (Figure 3-2, grey triangles and grey squares). Compounds 2 and 9 degraded more rapidly than all other methyl telluroethers, with ~75% and ~85% of each compound lost respectively over 24 h (Figure 3-2, black circles and grey circles).

During incubation the initially yellow solutions became colourless with the formation of a white precipitate at varying rates. This phenomenon has been previously observed and is thought to be the telluroxide species forming polymeric structures or the formation of TeO$_2$. In addition, a new downfield $^1$H NMR resonance (~2.5 ppm, singlet) was observed for compound 10 after prolonged exposure (≥8 hours) to aerobic atmosphere, consistent with our observations for the aerobic degradation of Telox where new singlet peaks appeared downfield to the original –TeCH$_3$ resonance between ~2.1 and ~2.2 ppm (see section 2.3.6). Alkyl tellurides are also known to be susceptible to homolytic bond cleavage and it is possible that this process could produce dimethyl ditelluride and dimethyl telluride, although these compounds are volatile and would be difficult to detect under these conditions. Of particular interest was the production of methyl acrylate during the degradation of compound 9. This is presumably the product of a $\beta$–elimination reaction, where methyltelluride is ejected from the acrylate scaffold—the reverse of the synthetic pathway used to access 9. This result suggests that $\beta$–carbonyl telluroethers are particularly unstable under
these conditions and are poor choices for our mass tag application. The organic components resulting from degradation of the other compounds could not be identified and may be lost due to their potentially low molecular weight and thus, probable volatility.

These results clearly indicated that while select higher molecular weight methyl telluroethers exhibited reduced susceptibility to O$_2$-promoted degradation, all of the methyl telluroethers investigated did not possess adequate aerobic stability for use at extended time points in MC-compatible ABP assays.

![Graph showing aerobic stability of compounds](image)

**Figure 3-2**: Aerobic stability of compounds 2, 7-10, and 17.

### 3.3.2 Aerobic Stability of Trifluoromethyltelluroethers

We next moved to evaluate the aerobic stability of the trifluoromethyl telluroethers (11-12) using the same conditions as described in section 3.3.1 (Figure 3-1). Over the course of the experiment, no significant degradation was observed for these compounds (Figure 3-3). This result supported our hypothesis that reducing electron density at the tellurium centre made these compounds less susceptible to oxidative degradation.
Figure 3-3: Aerobic stabilities of compounds 11 and 12. Data for the structurally-related methyl telluroether-containing compounds 8 and 10 is included for comparison.

These results suggested that trifluoromethyl telluroethers may be candidates for use as chemically/biologically-benign mass tags in MC-compatible ABPs; however, the aqueous stability of these scaffolds still required investigation (see section 3.3.4).

3.3.3 Aerobic Stability of Tellurophenes

The stabilities of the tellurophenes were studied using the protocol described in section 3.3.1 (Figure 3-1). All three tellurophene-containing compounds (14-16) exhibited excellent stability under these conditions, with no significant degradation observed over the course of the experiment (Figure 3-4). This was in contrast to the structurally related methyl alkyl telluroethers 2, 8, and 10, all of which displayed instability under these conditions as previously described (section 3.3.1). These results supported our hypothesis that delocalization of electron density off of the tellurium nucleus would help reduce its susceptibility towards oxidative degradation. This strategy for enhancement of stability is complementary to the purely inductive withdrawal of electron density mediated by the trifluoromethyl group in compounds 11 and 12.
Figure 3-4: Aerobic stabilities of compounds 14-16. Data for the structurally-related methyl telluroether-containing compounds 2, 8, and 10 are included for comparison.

3.3.4 Aerobic/Aqueous Stability of Lead Scaffolds

Since both the trifluoromethyl telluroethers and tellurophenes exhibited excellent aerobic stability, we next moved to study the stability of these compounds under buffered aqueous conditions. We chose to focus on the amide-linked compounds 12 and 16 since these were structurally similar to scaffolds that would be implemented in activity-based probe design (i.e. linkage of the mass tag through an amide to an activity-based (reactive) group). We prepared solutions of 12 and 16 in a 1:1 (v/v) solution of DMSO-d₆/PBS-d buffer. The compounds were kept in an environment exposed to air and ambient light at room temperature. $^{19}$F NMR was used to monitor compound 12 using a trifluoroacetate acid internal standard while $^1$H NMR was used to follow the stability of compound 16 using the DMSO-d₅ internal standard as described previously. Interestingly, the trifluoromethyl telluroether (12) exhibited 60% degradation after 24 hours; however, under the same conditions the tellurophene 16 was completely stable (Figure 3-5).
Figure 3-5: Stabilities of lead scaffolds under aerobic buffered-aqueous conditions.

We speculated that while the trifluoromethyl functionality does indeed reduce the reactivity of the tellurium nucleus towards oxidation, the tradeoff of this is enhanced susceptibility of the carbon bearing the –TeCF₃ group towards nucleophilic attack. The reasons for this are likely twofold. First, the electron-withdrawing nature of the –TeCF₃ functionality would be expected to enhance the electrophilicity of the carbon in question. Second, the suppressed pKa of the resultant trifluoromethyl telluride anion (as compared to a methyl telluride anion) leaving group would be expected to enhance reactivity towards Sₘ₂-type substitution processes (Scheme 3-5).

Scheme 3-5: Possible pathway leading to degradation of trifluoromethyl telluride under buffered-aqueous conditions. Nucleophiles present in aqueous buffer such as H₂O, Cl⁻, etc. may displace the trifluoromethyl telluride group in an Sₘ₂-type process. The methyl telluroethers are likely less susceptible to this process because more electron density is located on the carbon directly-bonded to the tellurium atom and the pKa of the resultant methyl telluride leaving group would be expected to be higher than for trifluoromethyl telluride. Proposed dipole directions and intensities are displayed by arrows beginning with crosses (large arrow = more intense dipole).
3.4 Toxicity of Functionalized Organotellurium Compounds

The lead tellurophene scaffold (16) exhibited excellent stability under aerobic buffered-aqueous conditions; however, the toxicity of this functionality was not known. To the best of our knowledge, tellurophenes had never been studied in the context of biology. Organotellurium compounds are often described as toxic, with aryl telluroethers often showing cellular toxicity below 100 µM across a range of cell lines under different assay conditions. Here, we investigated the toxicity of compounds 10, 12, 16, and 17 in Jurkat cells after a 24 h incubation period—once again using the metabolic probe WST-1 as a reporter for metabolic toxicity (Figure 3-6, see section 2.3.2). Compounds 10, 12 and 17 were expected to degrade over the course of the toxicity assay as indicated by our NMR stability studies (sections 3.3.1 and 3.3.4), and as such, these experiments reflect not only the relative toxicities of these compounds but also their degradation products.

Compound 17 had an apparent IC$_{50}$ value of 610 µM, but with a large experimental error due to the low solubility of this compound at higher concentrations. Compounds 10 and 12 were more toxic, displaying IC$_{50}$ values <200 µM; however, the tellurophene (16) was significantly less toxic with an IC$_{50}$ of ~280 µM. These data suggest that, in general, the alkyl telluroethers and the tellurophenes are less toxic than the previously investigated aryl telluroethers. Since we expect MC-compatible probes to be used at or below a concentration of ~100 µM, the tellurophene scaffold was a promising choice for activity-based probe design.
Figure 3-6: Toxicities of select organotellurium compounds as reported by a WST-1 assay.

3.5 Summary

In this chapter we have demonstrated that a variety of functionalized organotellurium compounds can be accessed using a combination of established and modified synthetic protocols. The methyl telluroethers can be obtained through either nucleophilic substitution or Michael-type addition chemistries. Trifluoromethyl tellurides were accessed through in situ generation of nucleophilic trifluoromethyl telluride using the trifluoromethyl anion source trifluoromethyltrimethylsilane and tetramethylammonium fluoride followed by nucleophilic substitution chemistry. Although low-yielding compared to the synthesis of methyl telluroethers, this method provided sufficient quantities of the desired trifluoromethyl telluride for characterization of stability and toxicity. Finally, we were able to synthesize several differently functionalized tellurophenes by modifying established protocols. This modular synthetic route allowed for rapid access to the requisite diacetylene compounds and produced useful quantities of various tellurophenes in high yields. A particularly attractive attribute of this protocol was its potential compatibility with the synthesis of isotopically enriched variants of these tellurophenes. Since commercially available isotopically enriched tellurium is provided as the metalloid in its zero-oxidation state (i.e. elemental tellurium), only synthetic protocols that use tellurium in this form will be useful for the SLIP experiments described in section 1.3.
The aerobic stabilities of the three classes of organotelluriums followed the expected trend. The methyl telluroether group, with a large amount of electron density localized on the tellurium atom, was shown to be consistently susceptible to oxidation by atmospheric O₂ regardless of the other functional groups present on the molecule—although differently functionalized methyl telluroethers did exhibit different rates of oxidation. In contrast, the trifluoromethyl telluroethers exhibited greatly enhanced stability to O₂, with no significant degradation observed over the course of the experiment. This is consistent with our hypothesis that by decreasing the amount of electron density on the tellurium atom, the rate of oxidation can be drastically decreased. The tellurophenes also displayed markedly enhanced stability under aerobic conditions as compared to the methyl telluroethers. Just like the trifluoromethyl telluroethers, no significant degradation was observed over the course of our experiments. This is likely due to delocalization of tellurium atom electron density over the five-atom aromatic system, thereby making oxidation at the nucleus energetically less accessible.

In contrast to their observed aerobic stability, the trifluoromethyl telluroethers were found to be highly unstable in buffered aqueous solution. We hypothesized that this was due to nucleophilic substitution chemistry, where the trifluoromethyl telluride group was displaced by nucleophiles present in the buffered solution (Scheme 3-5). Pleasingly, the tellurophenes exhibited robust stability even under these buffered-aqueous conditions, and also possessed low metabolic toxicity as reported by a WST-1 assay. The synthetic accessibility of the tellurophene functional group coupled with its potential compatibility with the generation of isotopologues, robust aerobic/buffered-aqueous stability, and low cellular toxicity provided strong motivation to pursue tellurophenes as MC-compatible, biologically benign reporter groups for ABP development.
3.6 Chapter 3 Experimental

Section 3.2.1:

3-methyltellanyl-1-ethanol (7)

Tellurium metal (granular, -5 - +50 mesh, 500 mg, 3.9 mmol) was ground to a fine powder using a mortar and pestle and suspended in THF (50 mL). Methyl lithium (2.5 mL, 4.0 mmol) was added drop-wise to the suspension until the solution became a homogenous yellow solution at room temperature. The resulting mixture was cooled to −196 °C in a liquid nitrogen bath. Upon freezing, 2-chloro-ethanol (0.26 mL, 3.9 mmol) was added in one portion and the reaction was warmed to room temperature. The reaction mixture was stirred at room temperature for 2.5 hours. Once the reaction was complete by TLC, sat. NH₄Cl (100 mL) was added to the mixture. The solution was extracted into diethyl ether (2 x 100 mL). The combined organic layer was washed with brine (1 x 100 mL), dried over MgSO₄, filtered and concentrated. The crude compound was purified by column chromatography on silica gel (10% EtOAc in Pentane) and dried under vacuum to give a viscous yellow oil. Yield: 488 mg (66%). WARNING: This chemistry produces foul-smelling compounds. Take appropriate precautions. ¹H NMR (500 MHz, CDCl₃): δ 3.78 (s, 2H, -CH₂OH), 2.80 (t, 2H, J = 6.8 Hz, -CH₂CH₂OH), 1.88 (s, 3H, -TeCH₃); ¹³C NMR (125 MHz, CDCl₃): δ 62.59 (-CH₂OH), 8.40 (-CH₂-Te), −22.41 (-Te-CH₃). HRMS m/z calcd. for C₃H₁₂N⁴O¹⁶²¹⁰Te [M+NH₄⁺] 207.99826, found 207.99829.

3-(methyltellanyl)propan-1-ol (2)

See section 2.3.1.

Methyl 4-methyltellanyl-butanoate (8)

Tellurium metal (granular, -5 - +50 mesh, 500 mg, 3.9 mmol) was ground to a fine powder using a mortar and pestle and suspended in THF (50 mL). Methyl lithium (2.5 mL, 4.0 mmol) was added drop-wise to the suspension until the solution turned yellow at room temperature. The resulting mixture was cooled to −196 °C in a liquid nitrogen bath. Upon freezing, methyl-4-chlorobutyrate (0.478 mL, 3.9 mmol) was added in one portion and the reaction was warmed to room temperature. The reaction mixture was stirred at room temperature for 2.5 hours. Once the reaction was
complete by TLC, sat. NH₄Cl (100 mL) was added to the mixture. The solution was extracted into diethyl ether (2 x 100 mL). The combined organic layer was washed with brine (1 x 100 mL), dried over MgSO₄, filtered, concentrated and dried under vacuum to give a viscous dark yellow oil. Yield: 877 mg (91%). **WARNING:** This chemistry produces foul-smelling compounds. Take appropriate precautions. ¹H NMR (500 MHz, CDCl₃): δ 3.64 (s, 3H, -COOCH₃), 2.60 (t, 2H, J = 7.6 Hz, -TeCH₂-), 2.39 (t, 2H, J = 7.4 Hz, -CH₂COOCH₃), 2.01 (m, 2H, -CH₂CH₂CH₂-), 1.86 (s, 3H, -TeCH₃); ¹³C NMR (125 MHz, CDCl₃): δ 173.09 (C=O), 51.36 (-COOCH₃), 35.71 (-CH₂C(O)-), 26.76 (-CH₂CH₂C(O)-), 1.92 (-TeCH₂CH₂-), −22.52 (-TeCH₃). HRMS m/z calcd. for [M+H⁺] 246.99723, found 246.99690.

**Methyl 3-methyltellanyl-propionate (9)**

Tellurium metal (granular, -5 - +50 mesh, 500 mg, 3.9 mmol) was ground to a fine powder using a mortar and pestle and suspended in THF (50 mL). Methyl lithium (2.5 mL, 4.0 mmol) was added drop-wise to the suspension until the solution turned yellow at room temperature. Water (0.18 mL) was added to the solution, inducing a color change to a dark brown mixture. The resulting mixture was cooled to −196 °C in a liquid nitrogen bath. Upon freezing, methyl acrylate (0.355 mL, 3.9 mmol) was added in one portion and the reaction was warmed to room temperature. The reaction mixture was stirred at room temperature for 0.5 hours. Once the reaction was complete by TLC, sat. NH₄Cl (100 mL) was added to the mixture. The solution was extracted into diethyl ether (2 x 100 mL). The combined organic layer was washed with brine (1 x 100 mL), dried over MgSO₄, filtered, concentrated and dried under vacuum to give a viscous dark yellow oil. Yield: 775 mg (85%). **WARNING:** This chemistry produces foul-smelling compounds. Take appropriate precautions. ¹H NMR (500 MHz, CDCl₃): δ 3.68 (s, 3H, -COOCH₃), 2.86 (m, 2H, -CH₂COOCH₃), 2.76 (m, 2H, -CH₂CH₂Te-), 1.92 (s, 3H, -Te-CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 173.87 (C(O)), 52.11 (-C(O)CH₃), 37.17 (-CH₂C(O)OCH₃), −4.72 (-TeCH₂CH₂-), −21.35 (-TeCH₃). HRMS m/z calcd. for [M+H⁺] 232.98158, found 232.98149.

**N-Benzyl-4-(methyltellanyl)butanamide (10)**

Compound 4 (400 mg, 1.64 mmol) was dissolved in THF (25 mL) and stirred vigorously. To the mixture, 1 M NaOH (25 mL) was added and a biphasic mixture was generated. This solution was stirred for 1 hour. The reaction was then diluted with H₂O (50 mL) and 1 M citric acid was added.
until the reaction mixture was acidic by litmus paper. The resulting mixture was extracted into diethyl ether (3 x 100 mL) and washed with brine (2 x 100 mL). The solvent was removed from the combined organic layers by rotary evaporation. This compound was re-dissolved in DCM (5 mL) and added to a new round bottom flask where DCC (355 mg, 1.72 mmol) was added and stirred for 5 minutes. Once the mixture became a milky solution, NHS (198.2 mg, 1.72 mmol) was added to the mixture and stirred for an additional 5 minutes. To this resulting solution, a mixture of benzylamine (215 µL, 1.97 mmol) and TEA (275 µL, 1.97 mmol) in DCM (5 mL) were added at room temperature. The reaction was stirred overnight. Once the reaction was complete by TLC, the stir bar was removed and the solvent was removed by rotary evaporation. The resulting product was re-dissolved in cold EtOAc (100 mL) where white precipitate formed in the solution; this precipitate was removed by filtration. This process was repeated 3 times to remove the precipitate. The filtrate was washed with 0.5 M citric acid (2 x 100 mL), NaHCO₃ (2 x 100 mL) and brine (1 x 100 mL). The organic layers were combined, dried with MgSO₄, filtered, concentrated and dried under vacuum to give a light yellow solid product. Yield: 440 mg (81%). ¹H NMR (500 MHz, CDCl₃): δ 7.33 (m, 5H, aryl), 5.92 (s, 1H, -NH-), 4.40 (d, 2H, J = 5.8 Hz, aryl-CH₂NH-), 2.62 (t, 2H, J = 7.4 Hz, -TeCH₂-), 2.29 (t, 2H, J = 7.3 Hz, -COCH₂CH₂CH₂Te-), 2.06 (m, 2H, -COCH₂CH₂CH₂Te-), 1.86 (s, 3H, -TeCH₃). ¹³C NMR (125 MHz, CDCl₃): δ 172.25 (C(O)), 138.63 (aryl), 129.08 (aryl), 128.17 (aryl), 127.89 (aryl), 43.99 (aryl-CH₂-NH-), 38.66 (-CH₂CH₂CH₂Te-), 27.78 (-CH₂CH₂CH₂Te-), 2.90 (-CH₂TeCH₃), -21.95 (-TeCH₃). HRMS m/z calcld. for C₁₂H₁₈N₁⁶O₁³⁰Te [M+H⁺] 322.04451, found 322.04378.

3-(Methyltellanyl)propyl benzylecarbamate (17)

The p-NP-carbamate intermediate was synthesized from 2 using the procedure described in section 2.3.1 (Angew. Chem. Int 2014, 53, 11473-11477., reference 89). In an oven dried 25 mL round bottom flask, benzylamine (42 µL, 0.38 mmol) and pyridine (92 µL, 114 mmol) were added to methanol (5 mL). Upon mixing, a dilute solution of the p-NP-carbamate intermediate (139 mg, 0.38 mmol in 1 mL methanol) was added dropwise via syringe over 1 minute. The reaction mixture was stirred for 3 hours at room temperature. The solvent (methanol) was removed by rotary evaporation and the crude compound was purified by flash chromatography (5-20% EtOAc/Pentanes on silica gel stationary phase) to afford light yellow crystals of 8. Yield: 98 mg (77%). ¹H NMR (500 MHz, CDCl₃): δ 7.34 (m, 5H, aryl), 4.97 (s, 1H, -NH-), 4.37 (d, 2H, J = 5.7
Hz, aryl-CH₂NH), 4.14 (t, 2H, J = 6.2 Hz, -OCH₂CH₂CH₂Te-), 2.64 (t, 2H, J = 7.4 Hz, -OCH₂CH₂CH₂Te-), 2.06 (m, 2H, -OCH₂CH₂CH₂Te-), 1.90 (s, 3H, -TeCH₃). ¹³C NMR (125 MHz, CDCl₃): δ 156.77 (C(O)), 138.35 (aryl), 130.80 (aryl), 130.57 (aryl), 128.59 (aryl), 127.42 (aryl), 68.86 (-OCH₂CH₂CH₂Te-), 30.76 (-CH₂CH₂CH₂Te-), 2.64 (t, 2H, J = 7.4 Hz, -OCH₂CH₂CH₂Te-), 2.06 (m, 2H, -OCH₂CH₂CH₂Te-), 1.90 (s, 3H, -TeCH₃).

HRMS m/z calcd. for $^{12}$C$_6$H$_{13}$F$_3$¹⁴N$^{16}$O$^{130}$Te [M+NH$_4^+$] 355.06598, found 355.03791.

Section 3.2.2:

**Methyl 4-((trifluoromethyl)tellanyl)butanoate (11)**

Tellurium metal (granular, -5-+50 mesh, 500 mg, 3.9 mmol) was ground to a fine powder using a mortar and pestle and suspended in 7 mL of DME. The solution was cooled to −60 °C using a 40% ethylene glycol / 60% ethanol dry ice cooling bath. Upon cooling, trimethyl(trifluoromethyl)silane (0.356 mL, 2.61 mmol) and tetramethylammonium fluoride (243 mg, 2.61 mmol) were added to the reaction mixture. The reaction was stirred vigorously for 1 hour at −60 °C and for 3 hours at room temperature. Once the reaction was complete, the yellow supernatant was decanted off and the solid residues remaining were washed with DME. The supernatant and the washes were combined and concentrated. To the concentrated crude mixture, 3 mL of DME and methyl 4-bromobutyrate (0.230 mL, 1.82 mmol) were added. The reaction mixture was stirred over-night at room temperature. Once the reaction was complete, the DME was removed by rotary evaporation and the remaining crude mixture was taken up in EtOAc. This organic layer was washed with water (3x), brine (1x), dried over MgSO₄, filtered, and concentrated. The crude mixture was purified by column chromatography (Toluene on a silica gel stationary phase). Yield: 270 mg (50%). ¹H NMR (500 MHz, CDCl₃): δ 3.68 (s, 3H, -C(O)OC₃H₃), 3.13 (t, 2H, J = 7.7 Hz, -TeCH₂-), 2.47 (t, 2H, J = 7.7 Hz, -CH₂C(O)OCH₃), 2.26 (p, 2H, J = 7.1 Hz, -CH₂CH₂CH₂-); ¹³C NMR (125 MHz, CDCl₃): δ 172.82 (C(O)), 103.79-95.40 (q, J = 351.5 Hz, -Te-CF₃), 51.763 (-C(O)OCH₃), 35.41 (-CH₂CH₂CH₂Te-), 27.08 (-CH₂CH₂CH₂Te-), 8.20 (-TeCH₂-). HRMS m/z calcd. for $^{12}$C$_6$¹H$_{13}$¹⁹F$_3$¹⁴N$^{16}$O$^{130}$Te [M+NH$_4^+$] 317.99551, found 317.99529.

**N-Benzyl-4-((trifluoromethyl)tellanyl)butamide (12)**

Compound 11 (400 mg, 1.4 mmol) was dissolved in 25 mL of THF and stirred vigorously. To the mixture, 25 mL of 1 M NaOH was added and a biphasic mixture was generated. This solution was
stirred for 1 hour. Upon completion, the reaction was diluted and 1 M citric acid was added until the reaction mixture was acidic. The resulting mixture was extracted into ethyl acetate (3 x 100 mL) and washed with brine (2 x 100 mL). The combined organic layers were combined and the solvent was removed by rotary evaporation. This compound was re-dissolved in DCM (5 mL) and added to a new round bottom flask where DCC (303 mg, 1.47 mmol) was added and stirred for 5 minutes. Once the mixture became a milky solution, NHS (169 mg, 1.47 mmol) was added and the mixture was stirred for an additional 5 minutes. To this solution, a mixture of benzylamine (180 µL, 1.68 mmol) and TEA (235 µL, 1.68 mmol) in 5 mL of DCM was added at room temperature. The reaction was stirred overnight. The solvent was then removed by rotary evaporation. The crude product was re-dissolved in cold EtOAc (100 mL) where a white precipitate formed which was removed via filtration. The filtrate was washed with 0.5 M citric acid (2 x 100 mL), NaHCO₃ (2 x 100 mL) and brine (1 x 100 mL). The organic layers were combined, dried with MgSO₄, filtered, concentrated, and dried under vacuum to give a yellow solid product. Yield: 157 mg (30%). ¹H NMR (500 MHz, CDCl₃): δ 7.33 (m, 5H, aryl-), 5.79 (s, 1H, -NH-), 4.41 (d, 2H, J = 5.7 Hz, aryl- CH₂NH-), 3.14 (t, 2H, J = 6.9 Hz, -CH₂Te-), 2.34 (m, 2H, -CH₂CH₂CH₂Te-), 2.28 (p, 2H, J = 6.8, -CH₂CH₂CH₂Te-). ¹³C NMR (125 MHz, CDCl₃): δ 171.87 (C(O)), 138.40 (aryl), 129.19 (aryl), 128.26 (aryl), 128.07 (aryl), 104.84-96.44 (-CF₃), 44.15 (aryl-CH₂-NH-), 37.94 (- CH₂CH₂CH₂Te-), 27.75 (-CH₂CH₂CH₂Te-), 9.05 (-CH₂CH₂CH₂Te-). HRMS m/z calcd. for ¹²C₁₂¹H₁₅¹⁹F₃¹⁴N₁⁶¹⁰O¹³⁰Te [M+H⁺] 376.01625, found 376.0175.

Section 3.2.3:

**(Bromoethynyl)triisopropylsilane (13)**

N-bromosuccinimide (5.15g, 29 mmol), silver nitrate (4.28g, 25.2 mmol) and TIPS-acetylene (5.6 mL, 25.2 mmol) were added to 200 mL of acetone. The solution mixture was stirred vigorously for 3 hours at room temperature. Once the reaction was complete, 150 mL of water was added to the mixture. The solution was extracted into hexane (3 x 125 mL). The combined organic layer was washed with brine (2x), dried over MgSO₄, filtered, concentrated and dried under vacuum to give a clear oil product. Yield: 6.51 g, 98%. *Org. Lett.* 2011, 13, 537- 539.

**Hepta-4,6-diynoic acid intermediate**
Cadiot-Chodkiewicz coupling was completed according to literature. (J. P. Marino, H. N. Nguyen, *J. Org. Chem.*, 2002, 67, 6841-6844.) CuCl (15 mg, 0.15 mmol) was added to an aqueous solution of 30% BuNH₂ (25 mL) at room temperature which generated a transparent blue solution. A few hydroxylamine hydrochloride crystals were added to this solution mixture to discharge the color. 4-pentyloic acid (901 mg, 9.2 mmol) was added to the mixture at once, resulting in a yellow suspension. This solution was cooled using an ice-water bath. Upon cooling, 2-bromo-1-triisopropylsilyl acetylene (2 g, 7.6 mmol) was added drop-wise. Additional crystals of hydroxylamine hydrochloride were added to maintain the yellow solution when blue-green color changes occurred. The reaction was stirred vigorously for 0.5 hours. Once the reaction was complete by TLC, the solution was extracted with EtOAc (2 x 100 mL). The combined organic layer was washed with 1M HCl (1 x 100 mL), brine (1 x 100 mL), dried with MgSO₄, filtered, concentrated and dried under vacuum to give a dark brown crude crystalline product. This product is directly deprotected. The product 6-(triisopropylsilyl)hepta-4,6-diynoic acid (388 mg, 1.39 mmol) was dissolved in THF (15 mL). This solution mixture was cooled using an ice-water bath. While cooling, tetrabutylammonium fluoride (1.39 mL, 1M in THF) was added dropwise until the solution reached room temperature. The reaction was stirred vigorously for 3 hours. Once the reaction was complete, the solution was extracted with EtOAc (3 x 100 mL). The combined organic layer was washed with 1M citric acid (3 x 100 mL), brine (3 x 100 mL), dried with MgSO₄, filtered, concentrated and dried under vacuum to give a brown crude oil product. The compound was taken directly to the next step of 2-(tellurophene-2-yl)propanoic acid synthesis.

**2-(Tellurophen-2-yl)propanoic acid (15)**

Tellurium metal (granular, -5 - +50 mesh, 3.0 g, 23.5 mmol) was ground to a fine powder using a mortar and pestle. The tellurium powder was added to an aqueous solution of 1 M NaOH (30 mL). To the reaction mixture, sodium hydroxymethylation sulfinate (6.0 g, 38.9 mmol) was added and stirred vigorously. The reaction solution was heated using an oil bath, to 95 °C for 0.5 hours and the solution turned a deep purple color. The reaction solution was cooled to 60 °C and stirred for an additional 5 mins. In 5 mL of ethanol, hepta-4,6-diynoic acid (388 mg, 3.17 mmol) was added to the reaction mixture. This solution mixture was stirred for 1.5 hours at 60 °C. The reaction was then exposed to oxygen by removing the septum and allowing in atmosphere. The reaction was allowed to cool to room temperature and stirred for 15 mins. Upon cooling, the reaction was diluted
with a sat. NH₄Cl solution (100 mL). The solution was extracted with EtOAc (2 x 100 mL). The combined organic layer was washed with 1 M HCl (2 x 100 mL), brine (2 x 100 mL), dried over MgSO₄, filtered, concentrated, and dried under vacuum to give a dark yellow solid crude product. The crude product was purified by flash chromatography (5%-50% EtOAc/Hexanes on silica gel) to give a light yellow solid (563 mg, 70%). ¹H NMR (500 MHz, CDCl₃): δ 8.71 (dd, 1H, J = 6.9, 1.2 Hz, -HCTe-), 7.59 (m, 1H, -HCHCTe-), 7.38 (m, 1H, -TeCCH-), 3.22 (t, 2H, J = 7.3, Tephene-CH₂-), 2.73 (t, 2H, J = 7.3, -CH₂CH₂COOH); ¹³C NMR (125 MHz, CDCl₃): δ 178.94 (C=O), 148.69 (-TeCCH-), 137.42 (-HCHCTe-), 136.15 (-TeCCH-), 125.29 (-HCTe-), 37.95 (Tephene-CH₂-), 32.01 (-CH₂CH₂COOH). HRMS m/z calcd. for C₇H₉O₁₃Te [M+H⁺] 254.96593, found 254.96665.

**Hexa-3,5-diyne-1-ol intermediate**

Cadiot-Chodkiewics coupling. CuCl (7.5 mg, 0.08 mmol) was added to an aqueous solution of 30% BuNH₂ (25 mL) at room temperature that generated a transparent blue solution. A few hydroxylamine hydrochloride crystals were added to this solution mixture to discharge the color. 3-Butyn-1-ol (1 g, 3.83 mmol) was added to the mixture resulting in a yellow suspension. This solution was cooled using an ice-water bath. Upon cooling, 2-bromo-1-triisopropylsilyl acetylene (832 mg, 3.19 mmol) was added drop-wise. Additional crystals of hydroxylamine hydrochloride were added to prevent the solution from turning a blue-green color. The reaction was stirred vigorously for 0.5 hours. Once the reaction was complete by TLC, the solution was extracted with EtOAc (2 x 100 mL). The combined organic layer was washed with 1 M HCl (1 x 100 mL), brine (1 x 100 mL), dried with MgSO₄, filtered, concentrated and dried under vacuum to give a neat dark brown oil. This product is directly deprotected. The product, 6-(triisopropylsilyl)hexa-3,5-diyne-1-ol (873 mg, 1.44 mmol) was dissolved in THF (15 mL). This solution mixture was cooled using an ice-water (1:1) bath. Upon cooling, tetrabutylammonium fluoride (1.44 mL, 1 M in THF) was added dropwise and the solution was allowed to warm to room temperature. The reaction was stirred vigorously for 3 hours. Once the reaction was complete by TLC, the solution was extracted with EtOAc (3 x 100 mL). The combined organic layer was washed with 1 M citric acid (3 x 100 mL), brine (3 x 100 mL), dried with MgSO₄, filtered, concentrated and dried under vacuum to give a brown oil. This crude product was immediately taken to the next step for the synthesis of 2-(tellurophen-2-yl)ethanol since the compound possess limited stability as a free diacetylene.
2-(Tellurophen-2-yl)ethan-ol (14)

Tellurium metal (granular, -5 - +50 mesh, 3.0 g, 23.5 mmol) was ground to a fine powder using a mortar and pestle. The tellurium powder was added to an aqueous solution of 1 M NaOH (30 mL). To the reaction mixture, sodium hydroxymethylsulfinate (6.0 g, 38.9 mmol) was added and stirred vigorously. The reaction solution was heated using an oil bath, to 95 °C for 0.5 hours and the solution turned a deep purple color. The reaction solution was cooled to 60 °C and stirred for an additional 5 mins. In ethanol (5 mL), hexa-3,5-diyn-1-ol (600 mg, 6.37 mmol) was added to the reaction mixture. This solution mixture was stirred for 1.5 hours at 60 °C. At this point, the reaction was exposed to oxygen by removing the septa and exposing the reaction to atmosphere. The reaction was cooled to room temperature and allowed to stir for 15 mins. Upon cooling, the reaction was diluted with a sat. NH₄Cl solution (100 mL). The solution was extracted with EtOAc (2 x 100 mL). The combined organic layer was washed with 1 M HCl (2 x 100 mL), brine (2 x 100 mL), dried with MgSO₄, filtered concentrated, and dried under vacuum to give a dark yellow oil crude product. The crude product was purified by flash chromatography (10%-30% EtOAc/Hexanes on silica gel stationary phase) to give a light yellow oil (949 mg, 66%).

1H NMR (400 MHz, CDCl₃): δ 8.71 (dd, 1H, J = 6.9, 1.2 Hz, -HCTe-), 7.65 (m, 1H, -HCHTe-), 7.44 (m, 1H, -TeCCH-), 3.82 (t, 2H, J = 6.0 Hz, Tephene-CH₂-), 3.13 (t, 2H, J = 6.4 Hz, -CH₂CH₂OH), 2.68 (s, 1H, -OH). 13C NMR (100 MHz, CDCl₃): δ 145.37 (-TeCCH-), 136.34 (-HCHTe-), 135.48 (-TeCCH-), 124.89 (-HCTe-), 63.64 (-CH₂CH₂OH), 38.85 (-Tephene-CH₂-). HRMS m/z calcd. for C₆H₉O₁₆Te[M+H⁺] 226.97101, found 226.97161.

N-Benzyl-3-(tellurophen-2-yl)propanamide (16)

Compound 6 (100mg, 0.4 mmol) was dissolved in dissolved in DCM (5 mL) and DCC (86 mg, 0.42 mmol) was added and stirred for 5 minutes. Once the mixture became a milky solution, NHS (48 mg, 0.42 mmol) was added and stirred for an addition 5 minutes. To this resulting solution, a mixture of benzylamine (52 µL, 0.48 mmol) and TEA (67 µL, 0.48 mmol) in 5 mL of DCM was added at room temperature. The reaction was stirred overnight. Upon completion, the stir bar was removed and the solvent was removed by rotary evaporation. The resulting product was redissolved in cold EtOAc (150 mL) where white precipitate crashed out of solution. The precipitate, presumed to be DCU, was removed by filtration and the filtrate was washed with 0.5
M citric acid (2 x 150 mL), NaHCO₃ (2 x 150 mL) and brine (1 x 150 mL). The organic layers were combined, dried with MgSO₄, filtered, concentrated and dried under vacuum to give a yellow solid. The product was purified by flash chromatography (5%-25% EtOAc/Hexanes) to give a yellow solid (440 mg, 81%). ¹H NMR (500 MHz, CDCl₃): δ 8.71 (dd, 1H, J = 6.9, 1.3 Hz, -HCTe-), 7.57 (m, 1H, -HCHCTe-), 7.31 (m, 7H, -TeCCH & aryl), 5.76 (s, 1H, -NH-), 4.41 (d, 2H, J = 5.7 Hz, aryl-CH₂NH-), 3.25 (t, 2H, J = 7.6 Hz, Tephene-CH₂CH₂-), 2.53 (t, 2H, J = 7.6 Hz, Tephene-CH₂CH₂-). ¹³C NMR (125 MHz, CDCl₃): δ 171.31 (C=O), 148.96 (-TeCCH-), 137.85 (-HCHCTe-), 136.73 (-TeCCH-), 135.54 (aryl), 128.84 (aryl), 128.56 (aryl), 127.7 (aryl), 127.39 (aryl), 124.87 (aryl), 43.58 (aryl-CH₂NH-), 39.88 (Tephene-CH₂CH₂-), 32.33 (Tephene-CH₂CH₂-). HRMS m/z calcd. for [¹²C₁₄¹⁴H₁₆¹⁴N₁⁶O₁₃⁰Te] [M+H⁺] 344.02886, found 344.02941.

**Sections 3.3.1–3.3.4:**

**Monitoring organotelluride stability via nuclear magnetic resonance:**

The organotellurium compound of study was dissolved in DMSO-d₆ in a relative 3:1 concentration ratio with 1,3,5–trioxane, the secondary internal standard of study. The compounds were kept in 20 mL vials sealed with parafilm. The film layer was punctured 4 times to create 4 small holes for oxygen to enter. The vials were kept in a moisture free environment for 24 hours. House air was filtered using sulfuric acid, potassium hydroxide and calcium sulfate to remove moisture. Each sample was contained inside a clear glass desiccator. The desiccator was maintained moisture free by spreading a layer of calcium sulfate on the bottom. No efforts were made to exclude ambient light. Aliquots were taken from each sample and ¹H NMR was taken at 0, 4, 8, 12 and 24 Hours. An organotellurium proton signal that could be integrated with confidence and the DMSO absorbance at 2.5 ppm were integrated. The ratio between the DMSO and the organotellurium protons were normalized to generate degradation plots (i/istd).

Experimental error was calculated taking into consideration the confidence of integration. Each NMR peak was integrated to its minimum value (Xppm-Yppm) centered on the peak of interest. The confidence of integration was taken by taking a second integration of (X+0.01ppm–Yppm) and a third integration of (Xppm-Yppm-0.01ppm). Taking the error of deviation of these three values takes into consideration potential integration bias, integration changes that may arise from instrumental drift, and peak broadening due to the observed precipitates.
Section 3.4:

WST – 1 Assay Protocol:

Jurkat cells were maintained in RPMI media supplemented with 10% (FBS) at 37 °C with 5% CO₂. Each compound was dissolved in DMSO at 100 mM to generate a stock solution, which was used immediately. Compounds were diluted in fresh media to 2-8 mM, depending on the solubility of the compound, and then two-fold serial dilutions were prepared in media. In 12-well plates, 250 µL of diluted compound was mixed 250 µL Jurkat cells at 10⁶ cells/mL and incubated at 37 °C for 24 h. Cell viability was measured using the WST-1 reagent (Roche Diagnostics, Laval, Quebec) as per manufacturer's instructions.
Chapter 4
A Tellurophene-Based Cellular Hypoxia Probe Exhibits Globally-Enhanced Performance*

*The original research in this chapter is in preparation for publication as: Edgar, L. J.; Vellanki, R. N.; McKee, T.; Hedley, D.; Wouters, B. G.; Nitz, M. Isotopologous Organotellurium Probes Reveal Dynamic Hypoxia \textit{in vivo} with Cellular Resolution. (Submitted). All synthetic work and spectral characterization of compounds was performed by L. J. Edgar. Biological work (both \textit{in vitro} and \textit{in vivo}) was performed by R. N. Vellanki and L. J. Edgar. MC analysis was performed by L. J. Edgar with technical assistance from O. Loboda and J. Watson.

4.1 Harnessing the Optimized Organotellurium Scaffold for Design of a Second-Generation Hypoxia Probe

Our discovery that tellurophenes substituted at the 2-position exhibited robust stability and low toxicity under biologically relevant conditions encouraged us to design a second-generation MC-compatible ABP against cellular hypoxia. We reasoned that by attaching a tellurophene to the 2-NI group, a stable, cell-permeable probe could be generated. Additionally, after optimizing the synthesis of this molecule, isotopologous variants should be accessible thus enabling our ultimate goal of studying changes in tumour oxygenation using the SLIP strategy (see section 1.3). This chapter describes our efforts to synthesize such a probe and characterize its activity both \textit{in vitro} and \textit{in vivo}.

We began by designing a simple, low molecular-weight tellurophene-2-NI conjugate (Telox 2) by applying some of the chemical transformations described in Chapter 3. After generating adequate quantities of this molecule, we verified that it was stable under biologically-relevant conditions. Following this, Telox 2 was characterized \textit{in vitro} using human pancreatic cancer cells. We discovered that the compound exhibited low toxicity \textit{in vitro} using two complementary assays, and that it was capable of identifying hypoxic cells in a mixture with a signal-to-noise ratio $\sim$10-fold greater than our first generation probe (Telox). Furthermore, we discovered that Telox 2 was capable of labeling cells on a gradient of $pO_2$—a property our previous generation molecule lacked. Labeling was found to be dependent on the activity of a specific oxidoreductase enzyme suggesting that its mechanism of action was similar to other molecules in this class. Following this
validation in vitro, we next moved to evaluate the ability of Telox 2 to label hypoxic cells in vivo. Here, we demonstrated that Telox 2 labelled hypoxic tumour cells in a manner similar to pimonidazole in vivo using human pancreatic cancer xenograft-bearing mouse models, and that at the doses used, no significant toxicity was observed.

### 4.2 Synthesis of a Tellurophene-Bearing Hypoxia Probe

Our first task was to design a low-molecular weight probe that replaced the methyl telluroether found in Telox with a tellurophene. We reasoned that the simple tellurophenes synthesized previously (see section 3.2.3) represented a tractable starting point for elaboration with the 2-NI functionality. Since the pKa of the 1H proton on 2-NI is significantly suppressed due to the electron-withdrawing character of the 2-NO₂ group, 2-NI is compatible with Mitsunobu chemistry (Mitsunobu-compatible nucleophiles must have a pKa lower than ~15, preferably below 11).¹¹⁴ With this knowledge, we decided to take the primary alcohol-bearing tellurophene 14 and treat it with azomycin (2-NI) in the presence of PPh₃ and diisopropyl azodicarboxylate (DIAD) (Scheme 4-1). This pathway cleanly afforded the tellurophene-2-NI conjugate 18 (referred to herein as Telox 2) and also demonstrated for the first time that tellurophenes are compatible with the Mitsunobu reaction.

![Scheme 4-1](image)

**Scheme 4-1:** Synthesis of the second-generation MC-compatible hypoxia probe Telox 2. Yields: 13 (98%), 14 (66%), 18 (95%).
4.3 Telox 2 Exhibits Long-Term Stability Under Aerobic/Aqueous Conditions

With a promising compound in hand, we next moved to evaluate the stability of Telox 2 under conditions that emulated a cell-based assay. Two methods were used to monitor stability over time: NMR and UV-visible spectroscopy. Our initial experiments involved taking $^1$H NMR spectra of a solution of Telox 2 in 0.01% DMSO-d$_6$/D$_2$O at various time points and looking for changes in the intensities/positions of resonances relative to trace DMSO-d$_5$ (Figure 4-1). No special precautions, apart from keeping the NMR tube (3 mm) capped, were taken to exclude light or atmosphere. Under these conditions, no changes were observed in the spectrum of Telox 2 from $-2$ to 14 ppm over one week on the benchtop. This result suggested that Telox 2 was stable after long-term incubation in aqueous solution, consistent with our findings on tellurophene stability in Chapter 3 (see section 3.3.3).

**Figure 4-1:** $^1$H NMR spectra of a solution of Telox 2 recorded over a week indicated that the molecule was stable under aqueous conditions. Resonance at $\sim$2.5 ppm is trace DMSO-d$_5$. [Telox 2] = 200 µM. All spectra recorded on a 500 MHz spectrometer.
To complement these findings, we prepared a solution of Telox 2 in 0.01% DMSO/PBS and incubated it in a loosely capped vial (to slow the rate of solvent evaporation) at room temperature in ambient light over 3 days. UV-vis spectra of this solution were recorded every 24 hours and the absolute absorption of both the tellurophene and 2-NI components of Telox 2 were determined. The tellurophene and 2-NI absorptions were assigned by recording UV-visible spectra of 14 (i.e. tellurophene without the 2-NI group) and azomycin (i.e. 2-NI without the tellurophene) respectively. We determined that $\lambda_{\text{max}}$ was ~282 and ~325 nm for the tellurophene and 2-NI respectively. This experiment indicated that essentially no loss of either chromophore occurred over the 3 day incubation suggesting that Telox was stable under aerobic conditions in buffered-aqueous solution, again, consistent with our findings in Chapter 3 (Figure 4-2, see section 3.3.4).

![Graph showing UV-visible spectra of Telox 2](image)

**Figure 4-2:** The UV-visible spectrum of Telox 2 did not change significantly after incubation of the molecule in buffered-aqueous solution without rigorous exclusion of aerobic atmosphere and light. [Telox 2] = 200 µM.

### 4.4 Telox 2 Exhibits Low Toxicity

With the knowledge that Telox 2 was stable under biologically relevant conditions, we proceeded to evaluate the toxicity of the probe using two complementary assays. In the first assay, Jurkat
cells were incubated with an appropriate concentration of Telox 2 for 24 hours and then exposed to the metabolic toxicity reporter WST-1 for 30 minutes. As described previously, WST-1 reports on reductive metabolism of cells—the reduced WST-1 metabolite exhibits strong absorbance between 420 and 480 nm, and the intensity of absorbance in this region can be used as a metric for the number of metabolically active cells present in a sample. As expected based on toxicity data for compound 16 (see section 3.4), Telox 2 exhibited low metabolic toxicity under these conditions (Figure 4-3), with an LD₅₀ of 264 µM—substantially higher than the concentration of probe expected to be used for future experiments. Interestingly, this result suggested that the tellurophene functionality may have an intrinsic toxicity that is independent of other groups, as the measured LD50 for Telox 2 was very close to that for other tellurophenes irrespective of other functionalization.

Figure 4-3: Telox 2 exhibits low metabolic toxicity in Jurkat cells as reported by WST-1. Incubation time = 24 hours for all concentrations of Telox 2. All samples were incubated in normoxic (21% O₂) atmosphere.

Parallel to our metabolic toxicity assay, we investigated the effect of Telox 2 on cellular proliferation in an experiment similar to that described in section 2.3.2. Panc1 cells were exposed to an appropriate concentration of Telox 2 and the rate of cellular proliferation over time was monitored using an IncuCyte kinetic imaging system. This experiment indicated that proliferation was only affected at high concentrations of the probe under both normoxic (21% O₂) and hypoxic (1% O₂) conditions. Interestingly, Telox 2 appeared to have an enhanced negative effect on cellular proliferation under normoxic conditions, where a 100 µM dose of probe significantly slowed cell division, whereas a 200 µM dose was required under hypoxic conditions. This may be partially
due to the ability of the 2-NI group to catalyze the generation of reactive oxygen species in the presence of O₂, potentially inducing DNA damage in rapidly dividing cells (cells are expected to divide more rapidly under normoxic as compared to hypoxic conditions).\textsuperscript{74} Taken together, these results indicated that Telox 2 exhibited both low metabolic and proliferative toxicities. Additionally, these experiments suggested that Telox 2 should be used at a concentration well below 100 μM—the lowest dose at which a toxic effect was observed (Figure 4-4A).

![Figure 4-4: Proliferative toxicity of Telox 2 under (A) normoxic (21% O₂) and (B) hypoxic (1% O₂) conditions.](image)

### 4.5 Differently Oxygenated Cells Can be Identified in a Mixture Using Telox 2

The favourable stability and toxicity profiles of Telox 2 encouraged us to move forward with the molecule as a candidate second-generation hypoxia probe. In order to compare the hypoxic cell labelling performance of Telox 2 with Telox, we performed an experiment similar to that presented in section 2.3.5, where cells incubated under atmospheres of differing O₂-content were mixed together and an attempt was made to identify the hypoxic and normoxic cell populations as a function of cellular tellurium content. We chose to continue using the human pancreatic cancer cell line Panc1 to investigate the performance of Telox 2 \textit{in vitro} since it is known to form solid tumours with a high hypoxic fraction.\textsuperscript{115} In culture, cells were incubated with Telox 2 under either near-anoxic (<0.02% O₂), hypoxic (1% O₂), or normoxic (21% O₂) conditions (Figure 4-5A). We included the intermediate (1%) pO₂ condition to determine if Telox 2 suffered from the same issue as Telox, where only binary detection of O₂-exposure was possible.\textsuperscript{89} In the event that Telox 2 was capable of labeling moderately hypoxic cells we expected to observe more tellurium in this
population than in cells exposed to normoxia, but substantially less than those exposed to near-anoxic conditions, as this profile would be consistent with the behaviours of other 2-NI-based probes. Following incubation, the cells were washed, fixed, and stained with one of three combinations of heavy isotope-containing nucleic acid intercalators: $^{103}$Rh only (21% $\text{O}_2$), $^{193}$Ir and $^{103}$Rh (1% $\text{O}_2$), or $^{193}$Ir only (<0.02% $\text{O}_2$) (Figure 4-5A). The purpose of staining with intercalators was twofold: first, to provide the requisite transition metal signal for cell event detection, and second, to act as internal standards such that cellular oxygenation could be inferred from the relative intensities of $^{103}$Rh and $^{193}$Ir signals. The three samples were then combined and injected as a mixture onto a second-generation mass cytometer (CyTOF 2, Fluidigm®). A density-map of $^{130}$Te (the most abundant isotope of tellurium) vs. event length revealed three populations of cells (Figure 4-5B). $^{103}$Rh vs. $^{193}$Ir output from each of these three populations retrieved the original nucleic acid staining combination expected for each population demonstrating that Telox 2 was capable of labeling cells in an oxygen concentration-dependent manner (Figure 4-5C-E) and was compatible with delineation of cell populations from within a mixture. Importantly, these results demonstrated that Telox 2 was capable of labelling cells exposed to an intermediate amount of $\text{O}_2$ (1%)—a physiologically-important oxygenation regime.
Figure 4-5: Identification of hypoxic cells in a mixture using Telox 2 and mass cytometry. Panc1 cells were exposed to atmosphere containing an appropriate oxygen-content (reported as % of total atmosphere by volume) for 3 h followed by trypsinization, washing, and fixation. Individual cell cultures were then incubated with one or two transition metal-containing nucleic acid intercalators (either $^{103}\text{Rh}$ and/or $^{191,193}\text{Ir}$) followed by additional washing steps. All 3 samples were then mixed together and analyzed via MC. Cartoons of expected ICP-MS readouts for a cell from each culture are presented on the right. (B) Density map of Event Length vs. $^{130}\text{Te}$ signal (arbitrary units). (C) Output of $^{193}\text{Ir}$ vs. $^{103}\text{Rh}$ (arbitrary units) from the low $^{130}\text{Te}$ gate. More than 82% of total cell events fall within the gated population. (D) Output of $^{193}\text{Ir}$ vs. $^{103}\text{Rh}$ (arbitrary units) from the intermediate $^{130}\text{Te}$ gate. More than 85% of total cell events fall within the gated population. (E) Output of $^{193}\text{Ir}$ vs. $^{103}\text{Rh}$ (arbitrary units) from the high $^{130}\text{Te}$ gate. Approximately 93% of total cell events fall within the gated population.
4.6 Labeling with Telox 2 is Linear with Both Concentration and Time

The labelling profiles of Telox 2 with respect to concentration and time are important to understand before attempting to use this compound in vivo. An understanding of labelling with respect to concentration is important because it allows for selection of a working concentration that provides both optimal signal-to-noise (i.e. tellurium signal at <0.02 vs. 21% O₂) and, when considered in concert with toxicity data, a value well below the LD₅₀. Understanding labelling with respect to time is necessary to allow for selection of incubation times that provide desirable signal-to-noise for a selected concentration of probe. We continued to use mass cytometry for these experiments to ensure that results were reflective of single cell tellurium content.

Telox 2 exhibited robust, linear labeling with respect to concentration (Figure 4-6). Although the lowest concentration (1 µM) exhibited the greatest signal-to-noise (~32-fold), no significant ¹³⁰Te signal was observed at 1% pO₂, indicating that a higher dose was needed to apply the probe to labelling cells on a gradient of hypoxia. We chose 10 µM as the working concentration for all future experiments since this concentration exhibited the second-best signal-to-noise (~28-fold based on median values) and was well below any concentration that exhibited toxicity (see section 4.4).

Figure 4-6: ¹³⁰Te signal vs. concentration for Telox 2. Panc1 cells were exposed to an appropriate concentration of Telox 2 under an appropriate atmosphere (21, 1, or < 0.02% O₂; light grey, dark grey, and black circles respectively) for 3 hours followed by trypsinization, washing, fixation, and staining with the ¹⁹¹/¹⁹³Ir-containing nucleic acid intercalator. Cells were then analyzed via mass
cytometry. Values are reported as median $^{130}$Te signals (arbitrary units). $n = 3$.

The labelling profile with respect to time was linear over the course of 24 hours (Figure 4-7). At 10 µM Telox 2, the incubation time that resulted in the greatest signal-to-noise (based on median values) was 3 hours. Taken together, these results indicated that for *in vitro* experiments in Panc1 cells, Telox 2 should be used at a dose of 10 µM and exposure time should be 3 hours to maximize signal-to-noise.

**Figure 4-7:** $^{130}$Te signal vs. exposure time for Telox 2. Panc1 cells were exposed to 10 µM Telox 2 for an appropriate amount of time under atmosphere containing either 21 or <0.02% O$_2$ (light grey and black circles respectively) followed by trypsinization, washing, fixation, and staining with the $^{191/193}$Ir-containing nucleic acid intercalator. Cells were then analyzed via mass cytometry. Values are reported as median $^{130}$Te signals (arbitrary units). $n = 3$.

4.7 Telox 2 Labels Cells on a Gradient of Oxygen

Unlike Telox, our observation that Telox 2 was capable of identifying cells exposed to intermediate hypoxia encouraged us to rigorously characterize the ability of the probe to label cells on a gradient of exposure to O$_2$. To achieve this, we incubated Panc1 cells under a range of atmospheres of varying pO$_2$ in the presence of the optimized dose of Telox 2 (10 µM) for the optimized incubation time (3 hours). To ensure that incubation media was properly equilibrated with the desired atmosphere, cells were grown in flasks equipped with a stirring mechanism that agitated media, thereby forcing equilibration of dissolved gasses. Cell cultures were grown in these flasks using standard conditions (see section 4.12) under normoxic atmosphere. Telox 2-containing media was pre-equilibrated with atmosphere (with a specific pO$_2$) and once cell cultures were placed in their
appropriate air-tight incubator, growth media was replaced with pre-equilibrated Telox 2-containing media. These measures were essential to ensure that cells were exposed to a constant amount of O₂, irrespective of media-atmosphere equilibration times and local O₂-consumption. After 3 hours, cells were washed, fixed, and analyzed via MC—again, to ensure that data was reflective of single cell tellurium content. This experiment clearly demonstrated that Telox 2 was able to label cells on a continuum of exposure to O₂ and that, moving forward, experiments where detection of intermediate hypoxia is desired should be possible (Figure 4-8).

**Figure 4-8:** $^{130}$Te signal vs. atmosphere O₂ content (as a % of total atmosphere by volume) for Telox 2. Panc1 cells were exposed to 10 µM Telox 2 for 3 hours under atmosphere containing an appropriate O₂ content followed by trypsinization, washing, fixation, and staining with the $^{191/193}$Ir-containing nucleic acid intercalator. Cells were then analyzed via mass cytometry. Values are reported as median $^{130}$Te signals (arbitrary units).

### 4.8 Cellular Tellurium-Accumulation is Dependent on Cytochrome P450 Oxidoreductase Expression

In order to help verify the mechanism of action of Telox 2 we obtained HCT116 cells engineered to have either enhanced or reduced expression of a key enzyme known to be involved in 2-NI reduction under low-pO₂ conditions; cytochrome p450 oxidoreductase (POR). These mutant cell lines, generously donated by Professor William R. Wilson, were grown using standard conditions (see section 4.12) and exposed to the optimized dose of Telox 2 for 3 hours.¹¹⁶ We expected to observe greatly enhanced accumulation of tellurium in cells that overexpressed POR since more
enzyme would be available to catalyze the reduction required to activate the 2-NI as a labeling agent (see section 2.2.1). In contrast to this, decreased labeling was expected for cells with a compromised ability to express POR. As expected, greatly enhanced labeling was observed in POR-overexpressing cells; however, cells unable to produce wild-type quantities of POR did not exhibit the drastically decreased labeling we expected as compared to the control (Figure 4-9). We propose that this is a consequence of a compensatory mechanism, where expression of alternative reductase enzymes may be induced to compensate for the lack of POR, although further study would be required to verify this. Since 2-NIs are known to be reduced by a wide-variety of different reductase enzymes, this regime would still allow for activation to the 2-nitreneium ion and subsequent labeling. The observed reductase enzyme-dependence of labeling supported our hypothesis that Telox 2 harnesses similar metabolic pathways for activation as other well-validated 2-NI-containing molecules.116

![Figure 4-9](image)

**Figure 4-9:** HCT116 cells with either wild type (WT), knocked-out (KO), or overexpressed (OE) cytochrome P450 oxidoreductase were exposed to 10 µM Telox 2 for 3 hours under either normoxic (21% O₂, black bars) or anoxic (<0.02% O₂, grey bars) atmosphere. n = 3.

4.9 Cellular Conjugates Formed by Telox 2 are Stable for Days After Reoxygenation

In order to successfully execute the SLIP experiment described in section 1.3, an understanding of how long Telox 2-macromolecule conjugates persist inside cells after forming under hypoxic conditions must be understood. The reason for this is that if the rate of conjugate degradation is
rapid, then detection of the isotope of tellurium corresponding to the initial dose of probe (i.e. isotopologue #1) could be challenging at time points after administration of the second dose (i.e. isotopologue #2). Ideally, conjugates formed by Telox 2 would persist in cells for long periods of time after forming so that the dynamics of hypoxia could be measured over longer periods of time, since changes in cellular oxygenation over a period of days may be clinically-relevant. To evaluate this, we incubated Panc1 cells with Telox 2 using the optimized conditions (see section 4.6) under near-anoxic atmosphere to promote maximum labelling. After incubation, we replaced Telox 2-containing media with fresh media and incubated cells at normoxia for various amounts of time. We reasoned that this protocol would mimic the in vivo scenario where cells are hypoxic at time point 1, and then reoxygenate at time point 2. Our goal was to determine the half-life of Telox 2 conjugates under these conditions in order to provide an estimate of compatible temporal-spacing between isotopologue dosing. Pleasingly, we discovered that conjugates persisted over long periods of time, with a half-life of ~22 h (Figure 4-10). Even after 2 days, sufficient tellurium was present in reoxygenated cells for detection via MC. This result suggested that conjugates formed by Telox 2 would be detectable in an extended SLIP experiment, since robust detection was possible after a long period of time.

**Figure 4-10:** Rate of Telox 2 conjugate degradation in vitro. Panc1 cells were exposed to Telox 2 (10 µM) for 3 hours under anoxic (< 0.02% O₂ by volume) atmosphere. Telox 2-containing media was then removed from each culture replaced with fresh media that did not contain Telox 2. Cells were allowed to incubate in the fresh media under normal O₂-content atmosphere (21% O₂) for an appropriate amount of time followed by trypsinization, washing, and fixation. Samples were then incubated with the $^{191/195}$Ir nucleic acid intercalator for 10 min. at room temperature followed by additional washing steps. Cells were then analyzed via mass cytometry. Values are reported as median $^{130}$Te signals (arbitrary units).
4.10 Characterization of Telox 2 In Vivo

Having thoroughly characterized the performance of Telox 2 in vitro, we were poised to answer the important question of whether or not the probe could detect hypoxic cells in vivo. To study this, we adopted a strategy where Telox 2 was co-administered with either pimonidazole or EF5 to human pancreatic tumour xenograft-bearing mice (Figure 4-11). Our hypothesis was that if Telox 2 functioned as a hypoxia-specific probe in vivo, it should label similar populations of cells as pimonidazole and EF5—both well-validated hypoxia probes. Due to differences in pharmacokinetics, and possibly activation by specific reductase enzymes with differing activities, we did not expect perfect co-labelling of Telox 2 with these probes. Indeed, previous work has demonstrated that pimonidazole and EF5 label different populations of cells in vivo even though both molecules are designed to specifically target hypoxic cells.\(^{37}\)

The first experiment involved injecting a Panc1 tumour xenograft-bearing mouse with both pimonidazole and Telox 2. The dose of 60 mg/kg of each probe was chosen as this is a common value for dosing of several hypoxia probes in the literature.\(^{80}\) After 3 hours, the mouse was sacrificed and the tumour was excised/digested into single cells. In order to evaluate cellular pimonidazole and Telox 2 content, we were required to employ two different analytical technologies for analysis of single cells. Since pimonidazole is commonly detected using fluorescence-based flow cytometry, we first stained cells with a commercial FITC-tagged antibody against pimonidazole conjugates and sorted these cells via fluorescence-activated cell sorting (FACS) into four separate fractions (Figure 4-11A). Since FACS is a non-destructive technique, samples of varying enrichment in pimonidazole conjugates could be separately analyzed via MC in an assay that would report on cellular tellurium content. Here, our hypothesis was that cells exhibiting higher FITC fluorescence (i.e. higher pimonidazole-conjugate content) should also exhibit enrichment in tellurium. Indeed, analysis of each fraction obtained through FACS on a CyTOF 2\(^{2}\) revealed pimonidazole-dependent enrichment of\(^{130}\)Te, suggesting that the two probes labelled similar populations of cells in vivo (Figure 4-11A).

Having demonstrated that Telox 2 labelled similar populations of cells as pimonidazole, we next moved to determine if this observation was consistent in a co-injection experiment involving both Telox 2 and EF5. To evaluate this, a Panc1 tumour xenograft-bearing mouse was injected with both EF5 and Telox 2. After 15.5 hours, the mouse was injected with the MC-detectable cell
proliferation probe 5-iododeoxyuridine (IdU, see section 1.2), followed by sacrifice 30 minutes later. We expected this experiment to answer three key questions: first, does Telox 2 label similar populations of cells as EF5; second, are Telox 2 conjugates detectable after 16 hours \textit{in vivo}; and finally, does hypoxic cell labelling occur mainly in populations of non-proliferating cells as previously described? Since an MC-compatible antibody against EF5 conjugates had been previously reported, we were able to analyze all parameters simultaneously using only MC. The excised tumour was digested into single cells and stained with a $^{159}$Tb-labelled metal chelating polymer-antibody conjugate against EF5 for detection via MC. If Telox 2 labelled similar populations of cells as EF5, then cells exhibiting a high $^{159}$Tb signal should also exhibit enrichment in $^{130}$Te. Additionally, the cells with the highest $^{159}$Tb and $^{130}$Te content should possess comparatively less $^{127}$I since severely hypoxic cells are expected to divide more slowly than cells in an aerobic environment. A density map of cell proliferation (as a function of IdU incorporation, reported by $^{127}$I) vs. EF5 labeling revealed an apparent continuum of hypoxia within the tumour (Figure 4-11B). The most hypoxic cells ($^{159}$Tb signal > 200 counts (cts)) possessed a low IdU signal ($^{127}$I signal < 100 cts), consistent with the expectation that severely hypoxic cells proliferate slowly. Conversely, highly proliferative cells possessed a comparatively low signal for EF5 ($^{159}$Tb signal < 100 cts). Gating on the most and least hypoxic populations as reported by $^{159}$Tb (Figure 4-11B; red and blue gates respectively) and retrieving their respective $^{130}$Te content indicated that Telox 2 enrichment was observed in cells that were strongly positive for EF5, but was less pronounced for non-hypoxic/proliferating cells. Again, the imperfect correlation between the two hypoxia probes underscored the difficulties that may be encountered when employing structurally, and therefore pharmacologically, distinct probes even when they are designed to label the same targets. The strong $^{130}$Te signal observed in the hypoxic gate suggested that even after 16 hours, conjugates formed by Telox 2 \textit{in vivo} were easily detectable \textit{via} MC. Taken together, this initial \textit{in vivo} characterization provided strong evidence that Telox 2 appeared to label hypoxic populations of cells within a human tumour model in a manner similar to well-validated probes. Additionally, conjugates formed by Telox 2 \textit{in vivo} appeared to persist for long periods of time, indicating that detection of the first isotope of tellurium in a SLIP experiment should be possible even at extended time points.
Figure 4-11: Telox 2 labels similar populations of cells as pimonidazole and EF5 in vivo (A) Panc1 xenograft-bearing SCID mice were injected (via tail vein) with a cocktail of Telox 2 and Pimo (60 mg/kg of each) and were sacrificed after 3 h. Tumours were excised, digested into a single cell suspension, washed, fixed, and stained with the $^{191/193}$Ir-containing nucleic acid intercalator and a FITC-tagged anti-Pimo antibody. Cells were then injected onto a fluorescence activated cell sorter and populations of increasing Pimo content (FITC fluorescence intensity) were isolated. Samples obtained via FACS were then individually injected onto a mass cytometer for analysis of $^{130}$Te content (arbitrary units). (B) Panc1 xenograft-bearing SCID mice were injected (via tail vein) with a cocktail of Telox 2 and EF5 (60 mg/kg of each probe). After 15.5 h the mice were injected with 5-iododeoxyuridine (10 mg/kg, intraperitoneal) and sacrificed 30 min. later. Tumours were excised, digested into a single cell suspension, washed, fixed, and stained with the $^{193/191}$Ir-containing nucleic acid intercalator and a $^{159}$Tb-tagged anti-EF5 antibody. Samples were then injected onto a mass cytometer for analysis. $^{159}$Tb, $^{127}$I, and $^{130}$Te signals are all reported as intensities with arbitrary units.
4.10.1 Murine Plasma Clearance Profile of Telox 2

In order to obtain meaningful data from a SLIP experiment, it is necessary to understand how much time must pass before a second dose (i.e. second isotopologue) of Telox 2 can be administered without interference from the first dose (i.e. first isotopologue). In a situation where the second dose is administered before the first dose has cleared the system, co-labelling of cell populations would be expected and this would greatly complicate interpretation of data. Thus, we measured the circulating half-life ($t_{1/2}$) of Telox 2 in murine plasma—we assume this to be a reasonable metric for systemic clearance of the molecule. This experiment used liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC/MS/MS) to measure the quantity Telox 2 present in murine plasma at various time points as compared to an internal standard. The reason this analytical technique was chosen over simple measurement of murine plasma tellurium content (i.e. using ICP-MS) was that unlike ICP-MS, LC/MS/MS reports on molecular structure and therefore can indicate how much active probe is present in circulation at a given time. ICP-MS would only report on the quantity of tellurium present regardless of its structural context. Thus, tellurium-containing metabolites of Telox 2 would be detected even if they did not possess the ability to label hypoxic cells. Our LC/MS/MS measurements indicated that Telox 2 possessed a circulating half-life of ~3 hours (Figure 4-12). This data suggested that administration of a second isotopologue of Telox 2 could be performed 12-16 hours (~4 x $t_{1/2}$) after the initial pulse, as the majority of the first tellurium isotope would have cleared circulation at that time.
Figure 4-12: Murine plasma clearance rate of Telox 2. Telox 2 was injected (via tail vein) into healthy white female mice (~20 g). Blood samples were obtained at various time points and centrifuged to separate plasma. Plasma samples were analyzed via quantitative LC/MS/MS using an appropriate tellurium-containing internal standard (compound 16). Error bars represent standard deviation from triplicate biological replicates.

4.10.2 Telox 2 Exhibits Low Toxicity in Mice

Although we measured low metabolic and proliferative toxicity for Telox 2 in vitro, it was important to understand if the probe exhibited toxic effects in vivo. To achieve this, we measured the mass of healthy and Panc1 xenograft-bearing mice over a 7 day period after injection with 60 mg/kg Telox 2. No statistical differences were observed in the masses of injected mice as compared to control mice (i.e. received only vehicle) in either healthy or tumour model groups. This data indicated that Telox 2 exhibited low toxicity in vivo.

Figure 4-13: In vivo toxicity of Telox 2. Female NOD/SCID mice (6-7 weeks old) with or without PANC-1 tumour xenografts were weighed and then injected with either vehicle (see section 5-VI, 100 µL injection volume) or vehicle + Telox 2 (60 mg/kg) (see section 5-VI, 100 µL injection volume). After seven days mice were weighed again. Error bars represent the standard deviation in mouse weight (n = 4).
4.11 Summary

In this chapter, we built upon the findings discussed in Chapters 2 and 3 to design, synthesize, and validate a second-generation tellurium-containing probe against cellular hypoxia both in vitro and in vivo. The modular synthetic route described in section 4.2, which built off the findings presented in section 3.2.3, provided an efficient, high-yielding route to a tellurophene-2-NI construct we branded “Telox 2”. This synthesis harnessed inexpensive, commercially available reagents to provide gram-quantities of the next-generation probe using Cadiot-Chodkiewicz coupling chemistry followed by tellurium installation, and finally a Mitsunobu reaction to install the 2-NI group (Scheme 4-1). A significant advantage of this synthetic pathway was that flash column chromatography purification was only required after the final step. The stability of Telox 2 was found to be excellent, as no detectable degradation was observed after incubation of the probe under biologically relevant conditions for up to a week. The exceptional stability was verified using two independent assays (1H NMR and UV-vis spectroscopy) and was consistent with our findings in sections 3.3.3 and 3.3.4.

Having identified a stable probe candidate, we demonstrated that Telox 2 exhibited low toxicity in two separate human cell lines using two separate assays; metabolic toxicity as reported by WST-1, and proliferative toxicity as indicated by the rate of cell division in the presence of the probe. Both assays confirmed that concentrations where toxicity was observed (LD50 ~ 264 µM) were well above the concentration that would be used in future assays. We next demonstrated, using the human pancreatic cancer cell line Panc1, that Telox 2 labelled cells in an O2-dependent manner and that labelling was linear with the concentration of probe and with time. From these experiments, we identified an optimal working concentration and incubation time which we applied to study the ability of Telox 2 to discriminate hypoxic populations of cells within a mixture. Similar to Telox, Telox 2 was able to easily delineate populations of cells based on O2-exposure, as demonstrated by an assay employing nucleic acid internal standards as described previously (see section 2.3.5). Pleasingly, the new probe was able to identify cellular exposure to O2 on a gradient rather than only in the binary manner exhibited by Telox. Labelling with Telox 2 was also shown to be dependent on the expression of cytochrome p450 oxidoreductase—an enzyme known to be partially involved in reduction of the 2-NI functionality. We also discovered that conjugates
formed by Telox 2 in cells were stable over long periods of time, therefore allowing for analysis of hypoxia at extended time points.

Having thoroughly characterized Telox 2 in vitro, we proceeded to investigate the performance of the probe in vivo. First, we investigated the ability of the probe to label hypoxic cancer cells in human pancreatic tumour xenograft-bearing mice in a manner consistent with existing well-validated probes against cellular hypoxia—specifically pimonidazole and EF5. By combining fluorescence-activated-cell-sorting and mass cytometry we discovered that Telox 2 did indeed label similar populations of cells as pimonidazole. Additionally, cells labelled with EF5 also exhibited enrichment in tellurium, suggesting that Telox 2 could be used as a MC-compatible surrogate for both of these probes. Telox 2 was also found to exhibit low in vivo toxicity as monitored by mouse body weight over one week.

To realize our ultimate goal of employing a SLIP strategy using isotopologues of Telox 2 to monitor the dynamics of tumour microenvironment oxygenation, we measured the plasma half-life of Telox 2 to ensure we understood when the second isotopologue could be administered without interference from the first. All of our findings up to this point suggested that Telox 2 would be compatible with monitoring dynamic hypoxia provided we could access isotopically enriched variants.
4.12 Chapter 4 Experimental

General:

Cell Culture and Maintenance

The human cell lines, HCT 116 (ATCC® CCL-247™) colon and PANC-1 (ATCC® CRL1469™) pancreatic adenocarcinoma cell lines were obtained from American Type Culture Collection (Manassas, VA). HCT 116 cells were maintained in RPMI (Sigma) supplemented with 10% fetal bovine serum (FBS) (Corning). HCT116 cells (either overexpressing or not expressing cytochrome P450 oxidoreductase (EC 1.6.2.4, Gene ID 5447)) were donated by Professor William R. Wilson’s laboratory. PANC-1 cells were maintained in DMEM medium (Sigma) supplemented with 10% FBS and 5% sodium pyruvate. All cells were maintained at 37 °C with 21% O₂, 5% CO₂ (v/v). Cells were routinely tested and found to be negative for mycoplasma. All cell suspensions from in vitro samples were centrifuged at 450 rcf.

Single cell mass cytometry experiments

After incubation under appropriate conditions the cell suspension was pelleted and resuspended in cold PBS (1.0 mL). Cells were pelleted, resuspended in PBS (900 µL), and fixed with formaldehyde (100 µL, 3.7 % solution) for 25 minutes. Following fixation, cells were pelleted, resuspended in PBS (1.0 mL), and pelleted once again. Cells were then resuspended in PBS (990 µL) and incubated with the ¹⁹¹/¹⁹³Ir-containing nucleic acid intercalator (10 µL, 100 µM solution in PBS) for 10 minutes at room temperature. For the experiment presented in Figure 4-5A, cells were stained with either the ¹⁹¹/¹⁹³Ir-containing nucleic acid intercalator (anoxic cells) (1 mL, 1 µM solution in PBS), the ¹⁰³Rh-containing nucleic acid intercalator (normoxic cells) (1 mL, 1 µM solution in PBS), or both intercalators (hypoxic cells) (1 mL, 1 µM solution in PBS of each) for 10 minutes at room temperature. Cells were then pelleted and resuspended in PBS (1.0 mL) twice and then pelleted and resuspended once in distilled H₂O (from a Milli-Q® purification system). Cell pellets were then resuspended in distilled H₂O containing a mass cytometry bead standard (1/10 dilution of CyTOF® calibration beads, Fluidigm, 0.5-2.5 mL depending on pellet size) and then analyzed using a second-generation mass cytometer (CyTOF 2®). For the experiment presented in Figure 4-5A the three samples were combined (equal volumes of each) and the
resultant mixture was analyzed using a second-generation mass cytometer (CyTOF 2®). Single cells were generally identified as events with a $^{193}$Ir signal of $\sim 10^3$ counts and an event length of $\sim 20-50$.

**Section 4.2:**

**2-Nitro-1-(2-(tellurophen-2-yl)ethyl)-1H-imidazole (18, Telox 2)**

An oven-dried 50 mL round bottom flask was charged with $^{nat}$Te-2-(tellurophen-2-yl)ethan-1-ol (natural abundance tellurium) (500 mg, 2.23 mmol), dry tetrahydrofuran (15 mL), triphenylphosphine (1.17 g, 4.47 mmol), azomycin (505 mg, 4.47 mmol), and a dry magnetic stir bar. The reaction vessel was flushed with nitrogen and cooled on an ice bath, Diisopropyl azodicarboxylate (0.822 mL, 4.47 mmol) was then added dropwise over 5 minutes and the reaction was allowed to stir, warming gradually to room temperature, for 18 hours. The reaction was then concentrated via rotary evaporation and the product directly purified via flash chromatography (silica gel stationary phase, 10-50 % EtOAc/Pentanes, product Rf $\sim 0.5$ on silica-coated thin layer chromatography plate with 1:1 Pentanes/EtOAc mobile phase, staining with KMnO$_4$) to afford the title compound (674 mg, 95 %) as a yellow solid. $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 8.79 (dd, 1H, $J = 6.6, 1.2$ Hz), 7.55 (dd, 1H, $J = 7.2, 4.2$ Hz), 7.24 (m, 1H), 7.07 (d, 1H, $J = 1.0$ Hz), 6.93 (d, 1H, $J = 1.0$ Hz), 4.65 (t, 2H, $J = 7.0$ Hz), 3.41 (dt, 2H, $J = 7.0, 1.1$ Hz) ppm. $^{13}$C NMR (125.8 MHz, CDCl$_3$): $\delta$ 142.9, 137.5, 137.3, 128.2, 126.4, 126.3, 52.8, 37.3 ppm.* HRMS m/z calcd. For $^{12}$C$_9$H$_{10}^{14}$N$_3^{16}$O$_2^{130}$Te (M•H$^+$) 321.98350, found 321.98405.

*Note:* the carbon attached to three quadrupolar $^{14}$N nuclei in the 2-nitroimidazole is not observable via $^{13}$C NMR.

**Section 4.4:**

**Metabolic toxicity assay**

A 96 well clear fluorometer plate was loaded with 200 $\mu$L of Jurkat cells (per well) at a culture density of 1 x $10^6$ cells per mL. To each well was added an appropriate amount of a stock solution of Telox 2 in sterile DMSO to reach a desired concentration. The concentration of DMSO was 1 % in all wells. Cells were allowed to incubate for 24 h at 37 °C under normal atmosphere, after
which 20 µL of a commercially available solution of WST-1 in PBS (Roche Diagnostics, product #05015944001) was added to each well (gentle pipetting evenly distributed the reagent throughout the well). Cells were allowed to incubate for a further 0.5 h at 37 °C under normal atmosphere, followed by subsequent measurement of the absorbance of each well at 450 nm using a TECAN Safire 2 plate reader. Data was background corrected vs. wells that contained cell growth media (without cells), an appropriate concentration of Telox 2, a final concentration of DMSO = 1 %, and WST-1. Background correction wells were incubated in the same manner as described for cell-positive wells.

**Confluency (proliferative toxicity) assay**

Panc-1 cells (25,000) were seeded in a 24 well plate and incubated overnight to allow the cells to adhere. Media was then removed and fresh media with 0-400 µM Telox-2 was added. Cells were incubated for 1 h and then transported to an IncuCyte™ kinetic imaging system that was maintained either at 21% or 1% O₂ at 37 °C. Growth profile was monitored by 10 X objective every 4 h using IncuCyte™ ZOOM control software with an integrated confluence algorithm until the control (untreated) cells reached stationary phase. Sixteen images per well were collected in phase-contrast mode and averaged to provide a representative statistical measure of the well confluence. Phase-contrast images were used to qualitatively monitor associated morphological changes in control and Telox-2 treated cells over the same timecourse.

**Section 4.4:**

**Generation of Murine Xenograft Models:**

University Health Network institutional guidelines and Animal Research Committee-approved protocols were followed for mouse studies. For these experiments, 4-6 week old female NOD SCID mice were obtained from Charles River (Wilmington, MA,) and housed in clean specific pathogen free (SPF) rooms in groups of 5 in cages. Panc-1 cells were harvested, washed in ice-cold serum free RPMI or DMEM media, counted and re-suspended in the same medium at a concentration of 2.0×10⁶ cells/mL. An equal volume of matrigel was mixed with the above cell suspension to afford a final volume of 0.15 mL. This sample was then injected subcutaneously into the right flank of each mouse. Tumor growth was monitored over time and volumes were
calculated as length × (width)$^2$/2. Tumors arose ~25 days after cell implantation. Final tumour volumes were 832±180 mm$^3$.

**Experiments using murine xenograft models**

Mice bearing subcutaneous tumour xenografts were injected with a 60 mg/kg body weight dose (per probe) of Telox 2 (vehicle: 20% DMSO, 20% Kolliphor® EL, 60% saline (v/v/v), 100 µL injection volume) and/or EF5 (6% EtOH in a ~275 mM aqueous solution of glucose, 100 µL injection volume) or Telox 2 and Pimo (20% DMSO, 20% Kolliphor® EL, 60% saline (v/v/v), 100 µL injection volume) via the tail vein. For mice co-injected with Telox 2 + EF5 the vehicle was 14% DMSO, 14% Kolliphor® EL, 4.3% EtOH and 67.7% ~275 mM aqueous glucose solution (v/v/v) (injection volume = 140 µL). After an appropriate incubation period mice were sacrificed and the tumours digested into single cell suspensions (see below).

**Enzymatic dissociation of xenograft tissue**

Tissue disassociation protocols were adapted from established procedures. Approximately 500 mg of tumor tissue was first washed with serum free RPMI/DMEM media and minced with a surgical blade in 1 mL of tissue disaggregation buffer (TDB) containing the enzymes collagenase IV, DNAse, and protease. The tissue suspension was then transferred to a 50 mL disassociation flask containing 11 mL of TDB and stirred (200 rpm) at 37 °C for 45 min. The cell suspension was then filtered through a 70 mm cell strainer, mixed with growth medium (DMEM) containing 5% FBS and centrifuged at 350 g for 5 min. The cell pellet was then resuspended and processed for CyTOF analysis using a protocol identical to that described above (only the $^{191/193}$Ir nucleic acid intercalator was used).
Chapter 5
Isotopologous Hypoxia Probes Report on Dynamic Hypoxia in vivo with Spatiotemporal Resolution*

*The original research in this chapter is in preparation for publication as: Edgar, L. J.; Vellanki, R. N.; McKee, T.; Hedley, D.; Wouters, B. G.; Nitz, M. Isotopologous Organotellurium Probes Reveal Dynamic Hypoxia in vivo with Cellular Resolution. (Submitted). All synthetic work and spectral characterization of compounds was performed by L. J. Edgar. Biological work (both in vitro and in vivo) was performed by R. N. Vellanki and L. J. Edgar. MC analysis was performed by L. J. Edgar with assistance from O. Loboda and J. Watson. Software for processing images was developed by T. McKee.

5.1 Introduction: Imaging Hypoxia Using Tellurium as a Reporter

Although fluorescence- and mass-based cytometry methods can both report important information about populations of cells, a key piece of data is lost during flow analysis—spatial information. The physical distances between cells in different populations (and even within the same population) can provide valuable information regarding the pathology of a tissue. Specifically, parameters such as the distance of a cell from vasculature and proximity to necrosis both have important implications on the oxygenation status of cells, but this information is lost during the tumour digestion step required before flow-based techniques can be used.\textsuperscript{10,121-125} To combat this limitation, recent work has focused on adapting MC for compatibility with an imaging platform. An imaging mass cytometry (IMC) system would allow for retention of important spatial information, while retaining the highly-parameterizable attributes of MC, giving it significant advantages over fluorescence-imaging techniques. In the initial report of this technology, Giesen et. al. reported simultaneous imaging of 32 parameters in human breast cancer tissue sections with subcellular resolution.\textsuperscript{126} This study highlighted the cellular heterogeneity inherent to tumour tissues and, after software-mediated single cell segmentation, was able to delineate cell subpopulations and cell-cell interactions.

The IMC system combines a pulsed-UV-laser-mediated tissue ablation system front-end with a CyTOF 2\textsuperscript{®} detector back-end (Figure 5-1). Small portions of a thin (5 µm thick) tissue section, stained with MC-compatible reagents, are first aerosolized by the laser and the resultant aerosol is directed into the CyTOF system via a constant laminar flow of noble gas (i.e. He).\textsuperscript{127} Careful
optimization of the laser pulse-frequency and rate of delivery of matter to the CyTOF resulted in a system capable of reporting on the isotopic content of a single “pixel” (0.5-1 µm in diameter) of tissue. Plotting the intensity of a specific mass channel in a two-dimensional matrix can then produce an “isotopic image” of the tissue section.

**Figure 5-1:** The imaging mass cytometer functions by first ablating “pixels” of a thin (5 µm thick) tissue section using a pulsed-UV-laser. The ablated matter is then directed into a CyTOF 2® where it is atomized and ionized by an ICP-torch. The TOF mass spectrometer then records the isotopic composition of the matter from each pixel which can later be plotted in a matrix to produce an image.

In this chapter we explore the compatibility of Telox 2 with IMC and apply our findings to the study of hypoxia dynamics in human tumour tissue using isotopically-enriched variants of the probe (isotopologues). We reasoned that given the cost associated with generating isotopologues of Telox 2 it would be advantageous to employ IMC as opposed to MC for analysis of tumour tissue given the advantages inherent to imaging vs. single cell data.

### 5.2 Telox 2 is Compatible with Imaging Mass Cytometry

To understand if sufficient tellurium accumulation occurred *in vivo* for visualization *via* IMC, we performed a simple experiment where a Panc1 tumour xenograft-bearing mouse was injected with both Telox 2 and EF5 (Figure 5-2) at the same dose used in previous experiments (60 mg/kg of each probe, see section 4.10, Figure 4-11B).
Figure 5-2: Workflow for initial imaging mass cytometry experiment. Both Telox 2 and EF5 were administered at a dose of 60 mg/kg. $^{159}$Ln(III) = $^{159}$Tb for anti-EF5 and $^{168}$Er for anti-Ki-67.

After 16 hours (~4 circulating half-lives of the probe, see section 4.10.1, Figure 4-12) the animal was sacrificed and the tumour excised, embedded in OCT medium, frozen in liquid N$_2$, and cryosectioned into 5 µm thick slices. We stained one of these tissue sections with antibodies against both the nuclear cell proliferation marker Ki-67 (labelled with $^{168}$Er) and the MC-compatible anti-EF5 antibody (labelled with $^{159}$Tb) described previously (see section 4.10). We then stained an adjacent tissue section with Hoechst dye (nuclear stain) and a Cy5.5-labelled antibody against EF5. Our goal was to use fluorescence microscopy to guide our IMC experiments since this technology is currently much lower-throughput than fluorescence techniques making ablation of an entire tumour section impractical. Regions of severe hypoxia were clearly visible in the fluorescence microscopy image (Figure 5-3A and B, pink). We chose a region that contained both EF5-positive and negative areas by fluorescence and submitted it for IMC analysis. The image we obtained from IMC analysis agreed with the fluorescence data, with the $^{159}$Tb signal correlating reasonably well with the Cy5.5 fluorescence signal (compare the pink channel in Figure 5-3B with the green and red channels in Figure 5-3C). We also observed good correlation between $^{125}$Te and $^{159}$Tb signals, suggesting that Telox 2 and EF5 labelled similar regions of cells—consistent with our single cell data obtained via MC (see section 4.10, Figure 4-11). We chose to monitor the $^{125}$Te isotope because no other stable isotope shares the 125 mass channel, and thus, we anticipated there would be minimal background. The low signal intensity for this channel is reflective of the low natural abundance of the $^{125}$Te isotope (7.07%). We observed that the majority of the Ki-67-positive cells were located outside of the hypoxic region (i.e. $^{159}$Tb / $^{125}$Te-positive, Figure 5-3C, blue channel), consistent with previous work suggesting that cellular proliferation and hypoxia are often mutually exclusive (see EF5 vs. IdU plot in Figure 4-11). Taken together, these results
indicated that Telox 2 was compatible with imaging of tissue sections *via* IMC and that labelling with Telox 2 correlated reasonably well with EF5 in two-dimensional space. We expected that experiments using isotopically-enriched variants of Telox 2 would exhibit drastically enhanced signal intensities since we obtained data using tellurium at natural abundance, with only 7.07% of total tellurium registering on the 125 mass channel.
Figure 5-3: Imaging mass cytometry analysis of a tissue section exhibiting regions both positive and negative for EF5 via fluorescence microscopy. A) Fluorescence microscopy image of the whole tumour section. Blue = Hoechst dye (nuclear stain), pink = Cy5.5 (anti-EF5 antibody). B) Magnified view of region B in panel A. C) Image produced via imaging mass cytometry. Red = $^{159}$Tb (anti-EF5-antibody), green = $^{125}$Te (7.07% of total Telox 2), blue = $^{168}$Er (anti-Ki-67 antibody).
5.3 Synthesis of Isotopologues of Telox 2

In order to differentiate between conjugates formed by Telox 2 at different time points, variants of Telox 2 each containing a different isotope of tellurium are required. Since the chemical transformations and target structures are identical, exactly the same synthetic pathway can be applied to the synthesis of these “isotopologues”—the only difference being the isotopic content of elemental tellurium used during the tellurophene ring cyclization step (see synthesis of 14 in Scheme 4-1).

The precise mass of the isotope of tellurium to be used is important to consider. Because the sampling time is longer in IMC, the amount of xenon that enters the CyTOF back-end is increased as compared to traditional single cell MC analysis. Because 130/128 Xe isotopes are common contaminants in argon used to fuel the ICP torch, a high background signal is always present on the 130 and 128 mass channels. The consequence of this is incompatibility of the 130/128 Te isotopes with the IMC instrument. This is unfortunate since these are the most abundant naturally occurring isotopes of tellurium. Nevertheless, all other isotopes of tellurium should be readily compatible with IMC. Because of these reasons, we selected the 122 Te and 125 Te isotopes for experiments involving isotopologues of Telox 2. Since 125 Te does not share a mass with any other stable isotope of any other element on the periodic table and 122 Te only shares its mass with a low-abundance (∼4.6%) isotope of tin, we reasoned that there should be essentially no background signal in tissue when performing IMC experiments.

Samples of 122 Te0 and 125 Te0 were purchased from a commercial source. Upon receiving these samples we observed a black coating on the material. This indicated that oxidation on the surface of the elemental tellurium power had occurred. Our preliminary synthetic work suggested that this oxidized form of tellurium was incompatible with tellurophene cyclization chemistry. We therefore opted to reduce this black (likely mixed oxides of tellurium) coating using hydrazine monohydrate based on some literature precedence (Scheme 5-1).

Scheme 5-1: Reduction of oxidized ²⁹⁸Te(O)ₙ using excess hydrazine monohydrate.
This procedure efficiently produced the reduced form (zero oxidation state) of the element, allowing for the procedure developed in section 4.2 to be applied (Schemes 4-1 and 5-2). We were able to access sufficient quantities of $^{122}\text{Te}$lox 2 and $^{125}\text{Te}$lox 2 (i.e. isotopologues with $>91\%$ enrichment of $^{122}\text{Te}$ and $^{125}\text{Te}$ respectively) to proceed with \textit{in vivo} experiments, although the yield was reduced as compared to Telox 2 synthesized using a sample of the element at natural abundance (see section 4.2). We hypothesize that the lower yield was due to the calculation of the number of moles of elemental tellurium being used was based on metal as provided, prior to reduction, which likely contained substantial amounts of oxide.

**Scheme 5-2:** Synthesis of isotopologues of Telox 2. The two-step yield from isotopically-enriched elemental tellurium ($^{A}\text{Te}^0$) was $\sim 65\%$ for both the $^{122}\text{Te}$ and $^{125}\text{Te}$ isotopes. $A = 122$ or 125.

Confirmation of isotopic enrichment in the final isotopologue products was provided by both DART mass spectrometry and $^1\text{H}$ NMR spectroscopy. Since $^{125}\text{Te}$ is NMR active ($I = \frac{1}{2}$), coupling ($\sim 100$ Hz) was observed in the $^1\text{H}$ NMR spectrum of $^{125}\text{Te}$lox 2 between the 5-position tellurophene proton and the $^{125}\text{Te}$ nucleus (Figure 5-4, bottom spectrum). In contrast, the NMR-inactive $^{122}\text{Te}$ nucleus in $^{122}\text{Te}$lox 2 did not split the resonance at $\sim 8.9$ ppm, as expected (Figure 5-4, top spectrum).
Figure 5-4: Truncated $^1$H NMR spectra of $^{122}$Telox 2 (top) and $^{125}$Telox 2 (bottom). Both spectra were taken in CDCl$_3$ and recorded on 500 and 600 MHz spectrometers respectively. The small peak at ~8.8 ppm in the spectrum of $^{125}$Telox 2 corresponds to the 5-position proton in molecules containing $^{124}$Te and $^{126}$Te (these isotopes are NMR-inactive and comprise ~7% combined impurity in the sample of isotopically-enriched $^{125}$Te as reported by the commercial supplier).

5.4 Tellurium is Observed at Sites Distant from Vascularization

Armed with isotopologues of Telox 2, we next moved to investigate the performance of a single isotopologue ($^{125}$Telox 2) \textit{in vivo}. We had two major goals here: the first was to measure the signal intensity we could expect from a sample where the majority of the tellurium signal was concentrated on a single mass channel (125); the second was to determine if we could correlate labelling with Telox 2 to morphological features in tissue—specifically, how labelling was affected by the presence or absence of vasculature. To achieve this, a Panc1 tumour xenograft-bearing mouse was injected with $^{125}$Telox 2 at the same dose used in previous experiments (see sections 4.10 and 5.2) and then placed in a chamber containing atmosphere with 7\% O$_2$ (v/v) (Figure 5-5). It has been shown previously that by manipulating the O$_2$-content of atmosphere, hypoxia can be either exacerbated or rescued—indeed this has been used clinically to reduce the hypoxic fraction of cells within a tumour prior to radiation therapy by administering 95\% O$_2$ (v/v) to patients.\textsuperscript{129} In this experiment we expected that by forcing the xenograft model to breathe low-O$_2$-content atmosphere, intratumoural hypoxia may be exacerbated thus leading to increased labelling with $^{125}$Telox 2. After 3 hours in the low-O$_2$ chamber the animal was sacrificed, the

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tumour excised, embedded in OCT medium, frozen in liquid N\textsubscript{2}, and sectioned into 5 µm thick slices (Figure 5-5). We stained one of these tissue sections with the antibody against Ki-67 (\textsuperscript{168}Er) as before, in addition to the \textsuperscript{191/193}Ir nucleic acid intercalator (an IMC-compatible surrogate for Hoechst dye, Figure 1-2).

**Figure 5-5:** Exacerbation of hypoxia in a Panc1 tumour xenograft-bearing mouse model. \textsuperscript{125}Telox was administered at a dose of 60 mg/kg.

An adjacent tissue section was stained with hematoxylin and eosin (H&E) to better visualize tissue morphology (Figure 5-6A). We reasoned that if we could identify a vascularized region using the H&E stained section, IMC analysis of this region could reveal interesting distributions of Telox 2 with respect to the vascularized zones. Indeed, ablation of what appeared to be a chordal blood vessel (Figure 5-6A (dashed black oval), running perpendicular to the plane of the tissue section) revealed a strong correlation between cellular \textsuperscript{125}Te content and distance of a cell from the blood vessel (Figure 5-6B). Cells located further away from the blood chord generally possessed more \textsuperscript{125}Te—this was consistent with expectations since O\textsubscript{2} can only diffuse a finite distance within tissue before it is consumed. Hence, cells farthest away from the O\textsubscript{2}-source (blood) should have the least access to O\textsubscript{2}, and thus be labelled strongly by \textsuperscript{125}Telox 2. Using a single-cell segmentation software protocol, the average tellurium content of individual cells in this image could be calculated and plotted as a function of distance from vasculature (Figure 5-7A and B). This plot clearly indicated that maximum labelling with \textsuperscript{125}Telox 2 occurred at a distance of ~250-300 µm from vasculature (Figure 5-7B). This is beyond the hypothetical diffusion distance limit of O\textsubscript{2} in tissues and as such, these cells would be expected to be severely hypoxic. Upon transitioning from the viable cell population to necrosis, a decrease in the average \textsuperscript{125}Te signal was observed, consistent with expectations that reduced enzymatic activity in necrotic zones should cause fewer Telox 2 conjugates to form (Figure 5-7B). Once again, cells positive for Ki-67 were anti-localized
with Telox 2-positive cells and these proliferating cells were positioned closer to the blood vessel (Figure 5-6B)—consistent with the expectation that cell populations closer to a nutrient source would have greater proliferative potential than those residing in a nutrient-deprived hypoxic niche.
Figure 5-7: (A) Software-processed image of data presented in B. Segmented cells are shaded on a thermal scale that represents the intensity of the $^{125}$Te mass channel (thermal legend on the right). The blood chord is outlined with a magenta oval. Scale bar = 100 µm (B) $^{125}$Te intensity as a function of distance from the blood chord. The vertical axis represents the average $^{125}$Te signal for each segmented cell within 20 µm-thick circular slices of increasing distance from the blood chord. Error bars represent the standard deviation of $^{125}$Te for all cells within the corresponding 20 µm-thick circular slice.
5.5 Temporally-Spaced Dosing of Isotopologues of Telox 2 Reports on Induced Hypoxia

With the knowledge that Telox 2 was able to label hypoxic microenvironments \textit{in vivo} as reported by cellular proliferation and tissue morphology (Figures 5-6 and 5-7), and was compatible with detection \textit{via} IMC, our next task was to determine if temporal changes in tumour hypoxia could be detected using isotopologues of the probe under controlled conditions. To achieve this, we designed an experiment that involved temporally-spaced dosing of Telox 2 isotopologues in a Panc1 tumour xenograft-bearing mouse coupled with a reduction in O$_2$-exposure immediately following dosing of the second isotopologue (Figure 5-8). Our goal was to demonstrate that the difference in the quantities of tellurium isotopes would mirror the controlled change in O$_2$-exposure, with more cells exhibiting a higher signal for the second isotope as hypoxia should be exacerbated when the animal model breathes low-O$_2$ atmosphere (7\% (v/v)). To realize this, the animal model was initially injected with both $^{125}$Telox 2 (60 mg/kg) and EF5 (60 mg/kg) and left to breathe normal atmosphere (21\% O$_2$ (v/v)) for 24 hours (Figure 5-8). We chose to include EF5 for the same reasons described in section 5.2. Following this, the animal was injected with $^{122}$Telox (60 mg/kg) and immediately transferred to a chamber containing atmosphere with 7\% O$_2$ (v/v). We chose to wait 24 hours because our LC/MS/MS data (see section 4.10.1) suggested that nearly all of the first isotopologue would have cleared circulation by that time point (~8 circulating half-lives, Figure 4-12). Nearly-complete clearance of the first isotopologue before administration of the second is essential to avoid cross-labelling. After 24 hours in the low-O$_2$ chamber, the animal was sacrificed, the tumour excised, embedded in OCT medium, frozen in liquid N$_2$, and sectioned into 5 \( \mu \)m thick slices. We stained one of these tissue sections with the following MC-compatible antibodies: anti-cross vimentin ($^{156}$Gd), Ki-67 ($^{168}$Er), anti-human pan-keratin ($^{162}$Dy), and anti-mouse CD31 ($^{165}$Ho) in addition to the $^{191}/^{193}$Ir nucleic acid intercalator. Additionally, two more (adjacent) tissue sections were prepared; one was stained with Hoechst dye and a Cy5.5-labelled antibody against EF5, the other was stained with hematoxylin and eosin.
**Figure 5-8:** SLIP approach to monitoring temporally-controlled induced hypoxia in a Panc1 tumour xenograft-bearing mouse model. The tissue section submitted for IMC analysis was stained with a cocktail of MCAPCs (anti-cross vimentin (\(^{156}\)Gd), Ki-67 (\(^{168}\)Er), anti-human pan-keratin (\(^{162}\)Dy), and anti-mouse CD31 (\(^{165}\)Ho) in addition to the \(^{191/193}\)Ir nucleic acid intercalator).

Once again, we used fluorescence microscopy to guide our IMC experiment, choosing a region to image that contained significant hypoxia (as reported by the Cy5.5 anti-EF5 antibody) and a large population of viable cells (as reported by H&E staining). In keeping with previous data (see section 5.2), labelling with Telox 2 correlated reasonably well with EF5, and the data clearly showed that more regions were positive for the second isotopologue of Telox 2 (122 mass channel) than the first (125 mass channel) (Figure 5-9). This finding was consistent with expectations since induction of hypoxia *via* reduced O\(_2\)-breathing during exposure to the second isotopologue should result in enhanced labelling with $^{122}$Telox 2 as compared to $^{125}$Telox 2. Once again, proliferating cells, as reported by Ki-67 expression, generally anti-correlated with hypoxic zones. It was possible to assign $^{122}$Te-$^{125}$Te (i.e. the difference in the intensities of these isotopes) values for cells that exhibited significant labelling with either $^{125}$Telox 2 or $^{122}$Telox 2 (>50 counts of either isotope) *via* software-mediated single cell segmentation. In this way, microenvironments within the tumour that experienced the most significant change in oxygenation were easily visualized with single cell resolution (Figure 5-10). The majority of cells analyzed in this manner exhibited a value for $^{122}$Te-$^{125}$Te > 0, consistent with enhanced labelling with $^{122}$Telox 2 as induced by low O\(_2\)-breathing. Regions of necrosis were easily excluded by the algorithm during data processing.
Figure 5-9: Analysis of tumour tissue from a model that had been subjected to reduced O$_2$-breathing (7% O$_2$ (v/v)) after administration of the second isotopologue. (A) Optical imaging of tissue morphology/necrosis (H&E). (B) IMC image of the same region as in A in an adjacent tissue section stained with MC-compatible reagents. Red = $^{122}$Te, Green = $^{125}$Te, Blue = $^{193}$Ir. Scale bars = 100 µm. Individual channels, images for parameters not shown here, and fluorescence microscopy images are presented in the appendix.
Figure 5-10: The single cell segmentation algorithm allows for visualization of changes in cellular oxygenation as reported by the value of $^{122}\text{Te} - ^{125}\text{Te}$ for the same sample as in Figure 5-9B. The thermal scale bar represents the value of $^{122}\text{Te} - ^{125}\text{Te}$. More positive values suggest a more severe transition of a cell to a hypoxic state after administration of the second isotopologue. Negative values suggest reoxygenation. Grey masks represent necrotic zones.

We next sought to confirm these findings using an orthogonal method for inducing tumour hypoxia. Rather than manipulating the O$_2$-content of atmosphere, we chose to employ the vasodilator hydralazine. This drug has been shown to be effective at reducing tumour oxygenation, presumably by lowering the rate at which blood flows through tumour vasculature and potentially causing collapse of capillaries within regions of high interstitial pressure.$^{131}$ Previous work has demonstrated that administration of EF5 followed by injection with hydralazine 10 minutes later
reproducibly resulted in an increase in the number EF5-positive tumour regions *in vivo* as measured by fluorescence microscopy.\textsuperscript{132} We hypothesized that a similar result would be obtained if Telox 2 was used in place of EF5. This experiment was performed using the same protocol as described previously (Figure 5-8); however, rather than placing the animal model in a chamber of altered atmospheric composition, hydralazine was injected 30 minutes after administration of the second isotopologue (Figure 5-11). Dosing with hydralazine *after* administration of the second isotopologue was important because if injections were performed in the reverse order it is likely that a reduced quantity of \textsuperscript{122}Telox 2 would be delivered to the tumour for the same reasons that influence delivery of O\textsubscript{2} (i.e. reduced blood flow through the tumour). After 24 hours, the tumour was harvested and processed using the same protocol as described above.

Figure 5-11: SLIP approach to monitoring temporally-controlled pharmacologically-induced hypoxia in a Panc1 tumour xenograft-bearing mouse model. The tissue section submitted for IMC analysis was stained with a cocktail of MCAPCs (anti-cross vimentin (\textsuperscript{156}Gd), Ki-67 (\textsuperscript{168}Er), anti-human pan-keratin (\textsuperscript{162}Dy), and anti-mouse CD31 (\textsuperscript{165}Ho) in addition to the \textsuperscript{191/193}Ir nucleic acid intercalator).

Once again, fluorescence microscopy was used to guide analysis by IMC. The image produced by IMC displayed enhanced labelling with \textsuperscript{122}Telox 2 as compared to \textsuperscript{125}Telox 2, consistent with expectations based on the isotopologue dosing regime (Figures 5-12 and 5-13).
Figure 5-12: Analysis of tumour tissue from a model that had been injected with the vasodilator hydralazine 30 min. after administration of the second isotopologue. (A) Optical imaging of tissue morphology/necrosis (H&E). (B) IMC image of the same region as in A in an adjacent tissue section stained with MC-compatible reagents. Red = $^{122}$Te, Green = $^{125}$Te, Blue = $^{193}$Ir. Scale bars = 100 µm. Individual channels, images for parameters not shown here, and fluorescence microscopy images are presented in the appendix.
Figure 5-13: The single cell segmentation algorithm allows for visualization of changes in cellular oxygenation as reported by the value of $^{122}\text{Te} - ^{125}\text{Te}$ for the same sample as in Figure 5-12B. The thermal scale bar represents the value of $^{122}\text{Te} - ^{125}\text{Te}$. More positive values suggest a more severe transition of a cell to a hypoxic state after administration of the second isotopologue. Negative values suggest reoxygenation. Grey masks represent necrotic zones.
These results provided evidence that the SLIP approach was a viable method for monitoring controlled induction of cellular hypoxia in vivo through both manipulation of O₂-breathing and pharmacological intervention. Although promising, additional experiments were needed to determine if the strategy could also report on hypoxic rescue, and more importantly, O₂-dynamics in a model that had not been subject to artificial manipulation of hypoxia.

5.6 Temporally-Spaced Dosing of Isotopologues of Telox 2 Reports on Hypoxic Rescue

The results presented in section 5.5 demonstrated that our SLIP strategy for monitoring dynamic hypoxia within a human tumour model was viable for detection of newly hypoxic cells; however, information regarding the sensitivity of the strategy towards detection of cells that had recently reoxygenated was lacking. To determine if our methodology could report on this important process, we designed an experiment nearly identical to that described in section 5.5 (Figure 5-8) but with one key difference: instead of placing the animal model in a low-O₂ chamber (7% O₂ (v/v)) after administration of the second isotopologue, the animal was transferred to a chamber containing a high atmospheric O₂ content (95% (v/v)) (Figure 5-14). We reasoned that by doing this, some hypoxic regions within the tumour may become oxygenated since erythrocytes entering the tumour should be more heavily saturated with O₂ under this hyperbaric regime. Following exposure to both isotopologues, the animal was sacrificed, the tumour harvested, sectioned, and stained as described previously (see section 5.5). Once again, we used fluorescence microscopy to guide our selection of a region to submit for analysis by IMC.

**Figure 5-14:** SLIP approach to monitoring temporally-controlled hypoxic rescue in a Panc1 tumour xenograft-bearing mouse model. The tissue section submitted for IMC analysis was stained with a cocktail of MCAPCs (anti-cross vimentin (¹⁵⁶Gd), Ki-67 (¹⁶⁸Er), anti-human pan-keratin (¹⁶²Dy), and anti-mouse CD31 (¹⁶⁵Ho) in addition to the ¹⁹¹/¹⁹³Ir nucleic acid intercalator).
The image produced via IMC clearly showed that the majority of viable cells positive for tellurium were enriched in the $^{125}$Te isotope (corresponding to the first isotopologue) as compared to $^{122}$Te (Figures 5-15 and 5-16). This result was consistent with a situation where microenvironments exhibiting hypoxia during circulation of the initial isotopologue (the first ~24 hours) were enriched in $^{125}$Te, but upon exposure of the model to the second isotopologue and high-O$_2$-content atmosphere, comparatively fewer regions were strongly enriched in $^{122}$Te. This result suggested that the SLIP strategy was capable of not only highlighting newly hypoxic cells (see section 5.5), but also could delineate cells that experienced recovery from hypoxia.
Figure 5-15: Analysis of tumour tissue from a model that had been subjected to increased O₂-breathing after administration of the second isotopologue. (A) Optical imaging of tissue morphology/necrosis (H&E). (B) IMC image of the same region as in A in an adjacent tissue section stained with MC-compatible reagents. Red = $^{122}_{\text{Te}}$, Green = $^{125}_{\text{Te}}$, Blue = $^{193}_{\text{Ir}}$. Scale bars = 100 µm. Individual channels, images for parameters not shown here, and fluorescence microscopy images are presented in the appendix.
Figure 5-16: The single cell segmentation algorithm allows for visualization of changes in cellular oxygenation as reported by the value of $^{122}\text{Te} - ^{125}\text{Te}$ for the same sample as in Figure 5-15B. The thermal scale bar represents the value of $^{122}\text{Te} - ^{125}\text{Te}$. More positive values suggest a more severe transition of a cell to a hypoxic state after administration of the second isotopologue. Negative values suggest reoxygenation. Grey masks represent necrotic zones.
5.7 Regions of Dynamic Hypoxia are Highlighted Without Artificial Manipulation of Oxygen-Content

Having demonstrated that the SLIP approach was able to report on controlled changes in hypoxia in vivo (see sections 5.5 and 5.6) we moved to measure hypoxia dynamics in a tumour model that was a closer analogue of a clinical situation. Since clinically/physiologically relevant hypoxia is not always induced by artificial manipulation of a patient (i.e. reduced O₂-breathing or vasodilation) it was essential to determine if our approach to monitoring dynamic hypoxia could report on native changes in oxygenation without exogenous intervention. We performed an experiment identical to those presented in sections 5.5 and 5.6, except the animal was not subjected to an atmosphere of altered O₂-content or pharmacological intervention (i.e. treatment with hydralazine) (Figure 5-17). Tumour sections were processed as previously described (see section 5.5) and the region to be imaged was chosen using fluorescence microscopy as before.

**Figure 5-17:** SLIP approach to monitoring native dynamic hypoxia in a Panc1 tumour xenograft-bearing mouse model. The tissue section submitted for IMC analysis was stained with a cocktail of MCAPCs (anti-cross vimentin (¹⁵⁶Gd), Ki-67 (¹⁶⁸Er), anti-human pan-keratin (¹⁶²Dy), and anti-mouse CD31 (¹⁶⁵Ho) in addition to the¹⁹¹/¹⁹³Ir nucleic acid intercalator).

Indeed, IMC data revealed regions that presented with either higher $^{125}$Te, $^{122}$Te, or near-equal quantities of both isotopes suggesting reoxygenation, reduced oxygenation, or no change during the chase-phase respectively (Figures 5-18 and 5-19).
**Figure 5-18:** Analysis of tumour tissue from a model that had not been subjected to artificial modulation of O$_2$. (A) Optical imaging of tissue morphology/necrosis (H&E). (B) IMC image of the same region as in A in an adjacent tissue section stained with MC-compatible reagents. Red = $^{122}$Te, Green = $^{125}$Te, Blue = $^{193}$Ir. Scale bars = 100 µm. Individual channels, images for parameters not shown here, and fluorescence microscopy images are presented in the appendix.
Figure 5-19: The single cell segmentation algorithm allows for visualization of changes in cellular oxygenation as reported by the value of $^{122}\text{Te} - ^{125}\text{Te}$ for the same sample as in Figure 5-18B. The thermal scale bar represents the value of $^{122}\text{Te} - ^{125}\text{Te}$. More positive values suggest a more severe transition of a cell to a hypoxic state after administration of the second isotopologue. Negative values suggest reoxygenation. Grey masks represent necrotic zones.
Of particular interest was our observation of a region bordering necrosis that appeared to experience rapid turnover of hypoxic cells (Figure 5-20). We speculate that proliferating cells displace cells close to the necrotic region, forcing them into an O$_2$-deficient microenvironment, thereby inducing hypoxia as reported by the $^{122}$Te ox 2 isotopologue.

**Figure 5-20:** A highly proliferative non-hypoxic niche appears to displace adjacent cells into a hypoxic microenvironment. (A) Magnified view of the region of interest from Figure 5-19B. (B) Cellular proliferation as reported by the anti-Ki-67 antibody (labelled with $^{168}$Er). The dashed-white rectangle highlights the newly hypoxic region bordering necrosis in A. The white arrow indicates the proposed direction of cellular displacement as a result of proliferation. The necrotic zone is labelled as “N”.

We also investigated native dynamic hypoxia in a pancreatic tumour model derived from a patient cell line (OCIP-51) known to be particularly hypoxic.$^{133}$ Analysis of this tumour *via* IMC revealed regions that appeared to develop severe hypoxia after the second isotopologue was administered, with particular microenvironments exhibiting very large values for $^{122}$Te–$^{125}$Te as reported by software-mediated single cell segmentation (Figures 5-21 and 5-22). Other regions within this same tumour appeared to exhibit moderate reoxygenation after administration of the second isotopologue as reported by negative $^{122}$Te–$^{125}$Te values (Figure 5-22).
Figure 5-21: Analysis of tumour tissue from a patient-derived tumour model (OCIP-51) that had not been subjected to artificial modulation of O₂. (A) Optical imaging of tissue morphology/necrosis (H&E). (B) IMC image of the same region as in A in an adjacent tissue section stained with MC-compatible reagents. Red = $^{122}$Te, Green = $^{125}$Te, Blue = $^{193}$Ir. Scale bars = 100 µm. Individual channels, images for parameters not shown here, and fluorescence microscopy images are presented in the appendix.
Figure 5-22: The single cell segmentation algorithm allows for visualization of changes in cellular oxygenation as reported by the value of $^{122}\text{Te} - ^{125}\text{Te}$ for the same sample as in Figure 5-21B. The thermal scale bar represents the value of $^{122}\text{Te} - ^{125}\text{Te}$. More positive values suggest a more severe transition of a cell to a hypoxic state after administration of the second isotopologue. Negative values suggest reoxygenation. Grey masks represent necrotic zones.

Robust visualization of changes in tumour microenvironment oxygenation states in two distinct human tumour xenograft models provided strong evidence that the SLIP approach using Telox 2 isotopologues may be a generally applicable strategy for monitoring dynamic native tumour hypoxia \textit{in vivo}. The results presented in this section are of particular importance because they were obtained without deliberate manipulation of tumour oxygenation, and thus would be expected
to mirror a more clinically relevant situation. The microenvironments experiencing the most significant changes in oxygenation over the time course of these experiments may possess particular pathological importance and further investigation in highly parameterized assays is warranted.

5.8 Summary

By modifying our synthetic protocol, we were able to use isotopically enriched samples of elemental tellurium to access variants of Telox 2 containing mostly either $^{122}\text{Te}$ or $^{125}\text{Te}$. Experiments with pancreatic tumour xenograft-bearing mouse models revealed that robust signal could be obtained from tissue exposed to isotopically enriched Telox 2 as reported by IMC. We also discovered that labelling was more intense in cells at sites distant from vasculature, likely because of the limited diffusion distance of $\text{O}_2$ in tissue.\textsuperscript{130}

In order to demonstrate that dynamic changes in tumour oxygenation could be reliably monitored using isotopologues of Telox 2, we subjected tumour-bearing mouse models to various conditions that were known to produce specific artificial changes in tumour oxygenation. The first set of mice were initially treated with $^{125}\text{Telox 2}$ and then subjected to either atmosphere of reduced $\text{O}_2$-content, or the hypoxia-inducing vasodilator hydralazine shortly after administration of $^{122}\text{Telox 2}$. IMC analysis of these tumours clearly showed that more microenvironments were positive for $^{122}\text{Te}$ as compared to $^{125}\text{Te}$, consistent with deliberate induction of hypoxia after dosing with the second isotopologue. Conversely, in a mouse model treated with high-$\text{O}_2$-content atmosphere after administration of the second isotopologue, reduced $^{122}\text{Te}$ accumulation was observed, suggesting that the SLIP strategy could report not only on induction of hypoxia, but also hypoxic rescue.

Finally, in models not treated with conditions that could artificially influence tumour oxygenation, we observed specific microenvironments that appeared to change oxygenation status over the course of a SLIP experiment. These final experiments provided strong evidence that this strategy for monitoring intratumoural hypoxia dynamics could be useful for studying clinically-relevant oxygenation regimes, as demonstrated by detection of hypoxia dynamics in the patient-derived OCIP-51 tumour model.

The trends observed in $^{122}\text{Te}^{125}\text{Te}$ values for each experiment presented in this section are summarized in Figure 5-23, where histograms reporting the number of cells (segmented by
software) possessing a specific $^{122}\text{Te}^{125}\text{Te}$ value for each Panc1 tumour are overlaid. Median $^{122}\text{Te}^{125}\text{Te}$ values for tumours forced into a hypoxic state (see section 5.5) are higher than for the tumour that was not artificially manipulated (see section 5.7) or the tumour that was reoxygenated with high O$_2$-content atmosphere (see section 5.6).

![Figure 5-23: Histograms of differential tellurium content ($^{122}\text{Te}^{125}\text{Te}$) from Panc1 tumours subjected to: hypoxia-inducing conditions (red and orange), hypoxia-relieving conditions (blue), or complete normoxia (green). Only cells that were significantly enriched (> 50 counts) in either Te isotope are reported.](image-url)
5.9 Chapter 5 Experimental

General:

Tissue Embedding and Sectioning

Immediately after excision, tumors were embedded in OCT compound (Tissue-Tek® Sakura® Finetek) and flash frozen in liquid nitrogen. Samples were then stored at −80 °C until cryosectioning was performed. Cryosectioning: 5 µm-thick tissue sections were prepared from embedded tumours using a cryomicrotome. These sections were then mounted on 1 mm-thick glass microscope slides and dried for 30 min on a slide warmer at 37°C followed by fixation/staining or storage at −80°C.

Imaging Mass Cytometry Staining Procedure for Tissue Sections

Tissue sections were fixed with 4% paraformaldehyde for 10 min at 4°C. A hydrophobic barrier was drawn around the tissue sections to keep staining buffers localized on the mounted tissue. Sections were then soaked in PBS for 10 min followed by incubation with blocking buffer (1% BSA: Non-Fat dried milk in PBS) for 30 min at 25 °C. A cocktail of the following metal-chelating polymer-antibody conjugates was prepared (2 µL of each antibody in 200 µL (total volume) PBS per tissue section): 156Gd-anti-cross Vimentin (clone RV202) (Fluidigm Cat#3156023A), 162Dy-anti-Human Pan-Keratin (clone C11) (Fluidigm Cat#3162027A), and 168Er-anti-Human Ki-67 (clone Ki-67) (Fluidigm Cat#3168001B). Sections were exposed to the MCPAC cocktail and incubated for 16 h at 4°C. Slides were then washed with PBS (3x) and exposed to the 191/193Ir nucleic acid intercalator (500 nM in PBS) for 15 min. The intercalated tissue was washed with PBS (2x), distilled water (2x) and then air-dried before IMC analysis.

Optical Imaging of Tissue Sections (Including Staining Protocol)

Tissue sections were fixed and blocked as previously described (see sections 5-VII and 5-IX for sectioning and fixation/blocking protocols respectively). A subset of sections were stained with hematoxylin and eosin (H&E). Fluorophore-conjugated antibodies (mouse anti-EF5 (clone Elk3-51)-Cy5.5 (non-commercial); used at 1:250 dilution and rabbit anti-mouse polyclonal CD31 (Sc-1506-R; used at 1:1000 dilution, Santa Cruz, Santa Cruz, California, USA)) diluted in incubation
buffer were added to tissue sections followed by incubation for ~16 h at 4 °C. Sections were then washed in PBS (3x, 15 min) and subsequently stained with DAPI (4′, 6-diamidino-2-phenylindole) at r.t. for 5 min. After washing (1x PBS) and drying, sections were mounted using an anti-fade system. Whole sections were then scanned using an Aperio Scanscope CS system.

**Imaging Mass Cytometry Data Acquisition**

IMC data was collected using a laser ablation chamber (LAC) similar to that previously described. The LAC front-end was connected to a second-generation mass cytometer (CyTOF 2) back-end for detection of heavy isotopes.

**Imaging Mass Cytometry Data Analysis and Image Visualization**

IMC data was output as a multidimensional matrix where each Cartesian coordinate (two-dimensional) contained a signal corresponding to the quantity of each heavy isotope detected in a 1 µm² ablated “pixel” of tissue. The signal for each isotope for each Cartesian coordinate was then plotted as a two-dimensional image using customized software (this produced the “raw” IMC image data). For single cell segmentation, IMC multi-channel images were converted to a series of 32 bit floating point tiff files (in units of ions / pixel) and loaded into Definiens TissueStudio software. Ir nucleic acid intercalator signal was used to perform nuclear and cellular segmentation, and mean ions / pixel / cell, and total ions / cell were quantified for all markers. A threshold was set to determine Telox positivity, and cells with intensities above this threshold (for Te) were identified. For SLIP experiments, cells above the threshold for either tellurium isotope were included for analysis, and the absolute difference (122Te − 125Te) of tellurium isotopes was calculated for each cell. Position and shape of each cell, as well as absolute Te isotope difference, was overlaid on the original image, and a histogram was calculated to compare treatment groups. Distance to nearest blood vessel was quantified for each cell and compared to mean Te uptake per cell.
Section 5.3:

Reagents and General Conditions:

Solvents were removed under vacuum at approximately 40 °C. Isotopically-enriched tellurium metal (\(^{125}\text{Te}^0 \text{ at } >92\% \text{ enrichment, and }^{122}\text{Te}^0 \text{ at } >91\% \text{ enrichment}) was purchased from Trace Sciences International and treated with hydrazine monohydrate (Sigma Aldrich\(^\circledR\), 5 mL / 50 mg \(^{125}\text{Te}^0\), 18 h, r.t.; removed via rotary evaporation followed by vacuum drying) prior to use—this was done to ensure that all tellurium metal was in the reduced (\(^0\text{Te}\)) form prior to performing tellurophene cyclization chemistry. EF5 (2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)acetamide) and the anti-EF5 Cy5.5-tagged antibody were (non-commercial) gifts from Professor Cameron J. Koch. Dry THF (Acros Organics), methanol (Acros Organics), azomycin (2-nitro-1H-imidazole, Matrix Scientific), and all other reagents (Sigma Aldrich\(^\circledR\), GFS Organic Chemicals\(^\circledR\), BDH Chemicals) were used as received.

\(^{125}\text{Te} (>92\% \text{ enriched})-2-(\text{tellurophen}-2-\text{yl})\text{ethanol-ol}

The previously described procedure (see section 4.2) was followed, with the exception of \(^{125}\text{Te}^0\) (>92\% enriched) being used instead of \(^{\text{nat}}\text{Te}^0\). The reaction was performed on a 50 mg scale of \(^{125}\text{Te}\) (pre-reduced via the protocol above). The title compound was used in the next step (synthesis of \(^{125}\text{Te} (>92\% \text{ enriched})-2\text{-nitro-1-}(2-(\text{tellurophen}-2-\text{yl})\text{ethyl})-1H\text{-imidazole}) immediately after isolation via extraction (as previously described in section 4.2).

\(^{122}\text{Te} (>92\% \text{ enriched})-2-(\text{tellurophen}-2-\text{yl})\text{ethanol-ol}

The previously described procedure (see section 4.2) was followed, with the exception of \(^{122}\text{Te}^0\) (>91\% enriched) being used instead of \(^{\text{nat}}\text{Te}^0\). The reaction was performed on a 50 mg scale of \(^{122}\text{Te}\) (pre-reduced via the protocol above). The title compound was used in the next step (synthesis of \(^{122}\text{Te} (>91\% \text{ enriched})-2\text{-nitro-1-}(2-(\text{tellurophen}-2-\text{yl})\text{ethyl})-1H\text{-imidazole}) immediately after isolation via extraction (as previously described in section 4.2).

\(^{125}\text{Te}- (>92\% \text{ enriched})-2\text{-nitro-1-}(2-(\text{tellurophen}-2-\text{yl})\text{ethyl})-1H\text{-imidazole (}^{125}\text{Telox 2)}

The same protocol as that for the synthesis of \(^{\text{nat}}\text{Te}-2\text{-nitro-1-}(2-(\text{tellurophen}-2-\text{yl})\text{ethyl})-1H\text{-imidazole (}^{\text{nat}}\text{Telox 2}) was followed but with 50 mg (1/10 the scale) of 2-(\text{tellurophen}-2-\text{yl})ethano-
1-ol with >92% $^{125}$Te enrichment (62% yield from $^{125}$Te$^0$). $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 8.79 (ddd, 0.92H*, $J = 102.2$ ($^1$H $^{125}$Te), 6.8, 1.3 Hz) and 8.79 (dd, 0.08H*, $J = 6.8$, 1.3 Hz), 7.56 (m, 1H), 7.25 (m, 1H (overlapping with trace CHCl$_3$)), 7.08 (d, 1H, $J = 1.1$ Hz), 6.94 (d, 1H, $J = 1.1$ Hz), 4.64 (t, 2H, $J = 6.9$ Hz), 3.41 (m, 2H) ppm. $^{125}$Te NMR (189.6 MHz, CDCl$_3$): $\delta$ 784.6 (s) ppm. HRMS m/z calcd. for $^{12}$C$_9$H$_{10}$N$_3$O$_2$^{125}$Te (M•H$^+$) 316.98170, found 316.98224.

*Note:* coupling of tellurophene H-5 to $^{125}$Te results in a resonance corresponding to ~92% of all molecules present. Tellurophene H-5 in molecules *without* $^{125}$Te results in a resonance corresponding to ~8% of all molecules present. These observations are consistent with the reported enrichment of the sample of $^{125}$Te$^0$ used (>92%).

$^{122}$Te-$>$92% enriched 2-nitro-1-(2-(tellurophen-2-yl)ethyl)-1H-imidazole ($^{122}$Telox 2)

The same protocol as that for the synthesis of $^{nat}$Te-2-nitro-1-(2-(tellurophen-2-yl)ethyl)-1H-imidazole ($^{nat}$Telox 2) was followed but with 50 mg (1/10 the scale) of 2-(tellurophen-2-yl)ethanol with >91% $^{122}$Te enrichment (60% yield from $^{122}$Te$^0$). $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 8.79 (dd, 1H, $J = 6.85$, 1.3 Hz), 7.56 (dd, 1H, $J = 6.8$, 3.9 Hz), 7.25 (m, 1H), 7.07 (d, 1H, $J = 1.1$ Hz), 6.93 (d, 1H, $J = 1.1$ Hz), 4.64 (t, 2H, $J = 6.9$ Hz), 3.41 (dt, 1H, $J = 6.9$, 1.1 Hz) ppm. HRMS m/z calcd. for $^{12}$C$_9$H$_{10}$N$_3$O$_2$^{122}$Te (M•H$^+$) 313.98036, found 313.98006.
Chapter 6
Summary and Future Directions

6.1 Summary

The work presented herein has described several major advancements. First, we demonstrated that by coupling a simple methyl telluroether scaffold to a 2-NI an ABP against cellular hypoxia could be generated (Telox) that was compatible with MC and capable of identifying a hypoxic cell population from within a mixture. Although an important proof-of-concept, Telox exhibited limited stability when stored in solution in the presence of O₂. To improve upon this first-generation probe we performed experiments exploring some fundamental properties of organotellurium compounds. This work advanced our understanding of the synthetic tractability, aerobic/aqueous stability, and toxicity of several classes of organotellurides. We discovered that many organotellurides exhibited low toxicity in cell culture, but only tellurophenes were resistant to degradation under biologically relevant conditions. With this knowledge, we designed and synthesized a second-generation probe that included both a tellurophene and 2-NI (Telox 2). Telox 2 exhibited drastically enhanced solution stability and sensitivity for detecting hypoxia on a gradient of O₂, rather than in the binary manner exhibited by the first-generation probe. Careful in vitro characterization of Telox 2 revealed that it was a promising candidate for compatibility with a SLIP experiment for measuring the dynamics of tumour hypoxia in vivo. Indeed, after administering temporally-spaced doses of isotopologous variants of Telox 2, we were able to detect microenvironments within human tumour tissue (derived from mouse xenograft models) that exhibited drastic changes in cellular oxygenation over the course of 24-48 hours, even in tumour models that had not had oxygenation artificially manipulated.

6.2 Future Directions

Although we achieved our goal of using isotopologues of an ABP to monitor the dynamics of a biological process, Telox 2 exhibited some limitations. The most important of these is the long circulating plasma half-life of the probe. In order to monitor dynamic hypoxia on shorter timescales, it is critical to have a probe that clears circulation rapidly so that administration of a
second isotopologue can be performed at an earlier time point. Furthermore, if more than two isotopologues are to be used, the temporal spacing between doses may need to be reduced to ensure that sufficient quantities of the conjugates formed by the isotopologues administered at earlier time points are retained for detection. It is unclear at this time if conjugates formed by Telox 2 in vivo have a long enough $t_{1/2}$ for robust detection at extended time points. For example, if an experiment involving 5 isotopologues of Telox 2 was to be performed using the same conditions as described in section 5.7, 120 hours would pass before the tumour would be analyzed (5 doses, 24 hours between each dose). It is possible that insufficient quantities of the first, second, and potentially third isotopes would be retained, and the signals for those mass channels could therefore be below the limit of detection of current instrumentation.

Both pimonidazole and EF5 exhibit markedly shorter circulating $t_{1/2}$s in rodents than Telox 2, suggesting that 2-NI-based probes do not possess intrinsically long circulating $t_{1/2}$s, and that careful modification of the structure of Telox 2 could produce a probe that clears more quickly. Generally, hydrophobic molecules clear circulation more slowly than polar/charged compounds. Pimonidazole, which contains a basic nitrogen, possesses a circulating $t_{1/2}$ of ~22 minutes in rodents; in contrast, the circulating $t_{1/2}$ for less polar EF5 is ~40 minutes. Given that Telox 2 is less polar than EF5 (as indicated by logP values), it is unsurprising that the circulating $t_{1/2}$ for Telox 2 is longer (~180-240 min.). There are at least two strategies that could potentially decrease the circulating $t_{1/2}$; identification of a more polar tellurium-containing functional group, or inclusion of a linker with polar functionalities. For example, a tellurophene bearing a trifluoromethyl group at the 2, 4, or 5 position should possess increased polarity (Figure 6-1). Inspiration for this modification can be found by examining the structure of EF5, which is polyfluorinated. Alternatively, direct fluorination of the tellurophene ring at any of the aforementioned positions may also provide the desired increase in polarity, although nucleophilic aromatic substitution chemistry is common on fluorinated aromatic rings—this may present problems in the form of hypoxia-independent accumulation of tellurium (Figure 6-1). Unfortunately, at this time, there is no literature precedence for constructing either trifluoromethylated or fluorinated tellurophenes and therefore new chemistry may need to be developed to access these modified mass tags.
The second strategy—inclusion of a polar linker—may also be a viable, and more synthetically-accessible strategy for the development of a third-generation probe. Taking inspiration from the structure of pimonidazole, it may be beneficial to introduce a piperidine-type functionality between the tellurophene and 2-NI since the endocyclic nitrogen in these groups exists mainly in the protonated state at physiological pH (Figure 6-1). Although greatly enhanced renal clearance of a cation-bearing probe would be expected, the trade-off of this (as discussed in section 2.2.1) is potential sequestering of the molecule in regions of reduced pH. This would result in labelling bias, since regions of low pH would be expected to have a higher local concentration of the molecule, and thus could present with increased tellurium accumulation in a manner not entirely dependent on the pO$_2$ of the region.

![Figure 6-1](image)

**Figure 6-1**: Proposed structures for third-generation MC-compatible ABPs sensitive towards cellular hypoxia. Reduced circulating t1/2s could be promoted by a more polar tellurophene mass tag (I), enhanced polarity by inclusion of a second nitroimidazole (II), or installation of a cationic linker between the 2-NI and tellurophene (III).

Once a probe with a shorter circulating $t_{1/2}$ has been identified and validated both *in vitro* and *in vivo*, a panel of up to 6 isotopologues could theoretically be synthesized (only the $^{130/128}$Te isotopes would be incompatible as discussed in section 5.3). Temporally-spaced administration of these isotopologues could allow for monitoring of complex hypoxia dynamics, including “cycling” of oxygenation states—a phenomenon that would require at least 3 isotopologues to detect. Additionally, experiments involving pharmacological or radiological treatment between doses of isotopologues could reveal important information regarding the effects of various therapies on the oxygenation state of specific regions within hypoxic tumours.

Ultimately, a method that allows for not only detection of hypoxia, but also rigorous absolute quantification of the amount of O$_2$ present at a particular time, would enable unprecedented interrogation of the biological consequences of hypoxia dynamics. Realization of this goal would require extraordinary effort. First, the probe used for this purpose would have to distribute through
a tumour with near-perfect uniformity to ensure that all microenvironments are exposed to the same amount of probe (hypoxia-sensitive functionalities label in a concentration-dependent manner). Second, a method for absolute quantification of conjugates formed would be needed. This could be achieved if an appropriate internal standard for tellurium is developed for the IMC instrument. Additionally, information regarding the rate at which conjugates degrade after forming in vivo would be needed to compensate for possible decreased signals for isotopologues administered at early time points. Finally, a method for correlating a specific tellurium signal to a specific concentration of O₂ would be required (i.e. a reliable standard curve).

The strategy for monitoring a dynamic biological process developed in this dissertation should not be limited to the study of cellular hypoxia. Important dynamics for a diverse array of processes could potentially be investigated using MC-compatible ABPs containing tellurium. For example, changes in the sialic acid content of cell-surface glycans could potentially be monitored using isotopologues of a tellurium-containing N-acetyl mannosamine derivative (ManTe, Figure 6-2) since unnatural derivatives of N-acetyl mannosamine have been shown to be converted into unnatural variants of sialic acid mediated by metabolic promiscuity. A SLIP experiment employing isotopologues of ManTe may allow for identification of cells that exhibit a high turnover of cell surface glycans, or even discrimination of important hyper-sialylated cancer cells within complex populations. Other applications of the SLIP approach could include monitoring rates of protein synthesis using an unnatural amino acid containing tellurium (TePhe, Figure 6-2). It is possible that under phenylalanine (Phe)-deficient conditions, cells may use TePhe in place of Phe, and the quantity of tellurium incorporated into protein may be a useful metric for quantifying rates of protein turnover.

![Figure 6-2: Extensions of isotopologous tellurium-containing ABPs for monitoring diverse dynamic biological processes.](image)
Fundamentally, most ABPs that rely on fluorophores for detection should be adaptable for detection via MC/IMC simply by replacing the fluorescent analytical handle with a tellurophene. In principle, this should allow for translation of the SLIP approach to address a wide range of time-dependent biological problems.
Appendix I: Additional Tumor Tissue Images

Figure A-1: 125 amu channel from IMC analysis of tumour tissue from the hydralazine-treated mouse model. This channel corresponds to $^{125}$Telox 2. Lighter colours represent more intense signal. Scale bar = 100 µm.
Figure A-2: 122 amu channel from IMC analysis of tumour tissue from the hydralazine-treated mouse model. This channel corresponds to $^{122}$Telox 2. Lighter colours represent more intense signal. Scale bar = 100 µm.
Figure A-3: 168 amu channel from IMC analysis of tumour tissue from the hydralazine-treated mouse model. This channel corresponds to the $^{168}$Er-labelled antibody against Ki-67. Lighter colours represent more intense signal. Scale bar = 100 µm.
Figure A-4: 193 amu channel from IMC analysis of tumour tissue from the hydralazine-treated mouse model. This channel corresponds to the $^{193}$Ir-containing nucleic acid intercalator (nuclear stain). Lighter colours represent more intense signal. Scale bar = 100 µm.
Figure A-5: 162 (red), 156 (green), and 193 (blue) amu mass channels overlayed for IMC data collected from the hydralazine-treated mouse model. Red corresponds to the signal from the $^{162}$Dy-labelled anti-pan keratin antibody. Green corresponds to the signal from the $^{156}$Gd-labelled anti-vimentin antibody. Blue corresponds to the $^{193}$Ir-containing nucleic acid intercalator (nuclear stain). Scale bar = 100 µm.
Figure A-6: Fluorescence microscopy image of a tumour tissue section from the hydralazine-treated mouse model. This section was adjacent to that presented in Figure S-14. The channel for the Cy5.5-labelled anti-EF5 antibody is presented in pink and FITC labelled anti-CD31 in green. DAPI stain (nuclear) is presented in blue.
Figure A-7: 125 amu channel from IMC analysis of tumour tissue from the carbogen (95% O₂)-treated mouse model. This channel corresponds to $^{125}$Telox 2. Lighter colours represent more intense signal. Scale bar = 100 μm.
Figure A-8: 122 amu channel from IMC analysis of tumour tissue from the carbogen (95% O$_2$)-treated mouse model. This channel corresponds to $^{122}$Telox 2. Lighter colours represent more intense signal. Scale bar = 100 µm.
**Figure A-9:** 168 amu channel from IMC analysis of tumour tissue from the carbogen (95% O₂)-treated mouse model. This channel corresponds to the $^{168}$Er-labelled antibody against Ki-67. Lighter colours represent more intense signal. Scale bar = 100 µm.
Figure A-10: 193 amu channel from IMC analysis of tumour tissue from the carbogen (95% O$_2$)-treated mouse model. This channel corresponds to the $^{193}$Ir-containing nucleic acid intercalator (nuclear stain). Lighter colours represent more intense signal. Scale bar = 100 µm.
Figure A-11: 162 (red), 156 (green), and 193 (blue) amu mass channels overlayed for IMC data collected from the carbogen (95% O₂)-treated mouse model. Red corresponds to the signal from the $^{162}$Dy-labelled anti-pan keratin antibody. Green corresponds to the signal from the $^{156}$Gd-labelled anti-vimentin antibody. Blue corresponds to the $^{193}$Ir-containing nucleic acid intercalator (nuclear stain). Scale bar = 100 µm.
**Figure A-12:** Fluorescence microscopy image of a tumour tissue section from the carbogen (95% O$_2$)-treated mouse model. This section was adjacent to that presented in Figure S-23. The channel for the Cy5.5-labellel anti-EF5 antibody is presented in pink and FITC labelled anti-CD31 in green. DAPI stain (nuclear) is presented in blue.
Figure A-13: 125 amu channel from IMC analysis of tumour tissue from the normoxia (21% O₂)-breathing mouse model. This channel corresponds to $^{125}$Telox 2. Lighter colours represent more intense signal. Scale bar = 100 μm.
Figure A-14: $^{122}$ amu channel from IMC analysis of tumour tissue from the normoxia (21% O$_2$)-breathing mouse model. This channel corresponds to $^{122}$Telox 2. Lighter colours represent more intense signal. Scale bar = 100 µm.
Figure A-15: 168 amu channel from IMC analysis of tumour tissue from the normoxia (21% O_2)-breathing mouse model. This channel corresponds to the ^{168}Er-labelled antibody against Ki-67. Lighter colours represent more intense signal. Scale bar = 100 µm.
Figure A-16: 193 amu channel from IMC analysis of tumour tissue from the normoxia (21% O₂)-breathing mouse model. This channel corresponds to the \(^{193}\)Ir-containing nucleic acid intercalator (nuclear stain). Lighter colours represent more intense signal. Scale bar = 100 µm.
**Figure A-17:** 162 (red), 156 (green), and 193 (blue) amu mass channels overlayed for IMC data collected from the normoxia (21% O₂)-breathing mouse model. Red corresponds to the signal from the $^{162}$Dy-labelled anti-pan keratin antibody. Green corresponds to the signal from the $^{156}$Gd-labelled anti-vimentin antibody. Blue corresponds to the $^{193}$Ir-containing nucleic acid intercalator (nuclear stain). Scale bar = 100 µm.
Figure A-18: Fluorescence microscopy image of a tumour tissue section from the normoxia (95% O₂)-breathing mouse model. This section was adjacent to that presented in Figure S-32. The channel for the Cy5.5-labelled anti-EF5 antibody is presented in pink and FITC labelled anti-CD31 in green. DAPI stain (nuclear) is presented in blue.
**Figure A-19:** 125 amu channel from IMC analysis of tumour tissue from a patient-derived tumour (OCIP-51) xenograft mouse model breathing normoxic atmosphere (21% O₂). This channel corresponds to $^{125}$Telox 2. Lighter colours represent more intense signal. Scale bar = 100 µm.
Figure A-20: 122 amu channel from IMC analysis of tumour tissue from a patient-derived tumour (OCIP-51) xenograft mouse model breathing normoxic atmosphere (21% O₂). This channel corresponds to 122Telox 2. Lighter colours represent more intense signal. Scale bar = 100 µm.
Figure A-21: 168 amu channel from IMC analysis of tumour tissue from a patient-derived tumour (OCIP-51) xenograft mouse model breathing normoxic atmosphere (21% O₂). This channel corresponds to the 168Er-labelled antibody against Ki-67. Lighter colours represent more intense signal. Scale bar = 100 μm.
Figure A-22: 193 amu channel from IMC analysis of tumour tissue from a patient-derived tumour (OCIP-51) xenograft mouse model breathing normoxic atmosphere (21% O₂). This channel corresponds to the ¹⁹³Ir-containing nucleic acid intercalator (nuclear stain). Lighter colours represent more intense signal. Scale bar = 100 µm.
Figure A-23: 162 (red), 156 (green), and 193 (blue) amu mass channels overlayed for IMC data collected from tumour tissue from a patient-derived tumour (OCIP-51) xenograft mouse model breathing normoxic atmosphere (21% O₂). Red corresponds to the signal from the $^{162}$Dy-labelled anti-pan keratin antibody. Green corresponds to the signal from the $^{156}$Gd-labelled anti-vimentin antibody. Blue corresponds to the $^{193}$Ir-containing nucleic acid intercalator (nuclear stain). Scale bar = 100 µm.
Figure A-24: Fluorescence microscopy image of a tumour tissue section from the patient-derived tumour (OCIP-51) xenograft mouse model breathing normoxic atmosphere (21% O₂). This section was adjacent to that presented in Figure S-41. The channels for the Cy5.5-labelled anti-EF5 antibody is presented in pink and FITC labelled anti-CD31 in green. DAPI stain (nuclear) is presented in blue.
**Figure A-25:** 125 amu channel from IMC analysis of tumour tissue from the hypoxia (7% O₂)-treated mouse model. This channel corresponds to $^{125}$Telox 2. Lighter colours represent more intense signal. Scale bar = 100 µm.
Figure A-26: 122 amu channel from IMC analysis of tumour tissue from the hypoxia (7% O₂)-treated mouse model. This channel corresponds to $^{122}$Telox 2. Lighter colours represent more intense signal. Scale bar = 100 μm.
Figure A-27: 168 amu channel from IMC analysis of tumour tissue from the hypoxia (7% O₂)-treated mouse model. This channel corresponds to the $^{168}\text{Er}$-labelled antibody against Ki-67. Lighter colours represent more intense signal. Scale bar = 100 μm.
Figure A-28: 193 amu channel from IMC analysis of tumour tissue from the hypoxia (7% O₂)-treated mouse model. This channel corresponds to the $^{193}$Ir-containing nucleic acid intercalator (nuclear stain). Lighter colours represent more intense signal. Scale bar = 100 µm.
Figure A-29: Fluorescence microscopy image of a tumour tissue section from the hypoxia (7\% O_2)-treated mouse model. This section was adjacent to that presented in Figure S-49. The channel for the Cy5.5-labelled anti-EF5 antibody is presented in pink and FITC labelled anti-CD31 in green. DAPI stain (nuclear) is presented in blue.
Appendix II: NMR Spectra

\[ \text{H NMR 500 MHz (CDCl}_3, 25^\circ \text{C)} \]

\[
\text{Te} \quad \text{OH} \\
\begin{array}{c}
\text{CH}_2
\end{array}
\]
$^{13}C$ NMR 125 MHz (CDCl$_3$, 25°C)
157

H

Te

2

H

157 129 NMR 126 MHz (CDCl3, 25℃)
$^{1}H$ NMR 400 MHz (CDCl$_3$, 25 °C)
$\text{TE NMR 189 MHz (CDCl}_3, 25^\circ\text{C)}$
$\text{H NMR 500 MHz (MeOD, 25°C)}$

\begin{align*}
\delta (\text{ppm}) & = 0.85, 2.04, 2.84, 3.29, 3.31, 2.73, 2.76, 5.17, 7.44, 7.45
\end{align*}
$^{13}C\text{ NMR } \delta_{\text{MeOD, } 25^\circ \text{C}}$
Telox (6)

\[
\begin{align*}
\text{NMR} & \quad 150 \text{ MHz (MeOD, 25 °C)}
\end{align*}
\]
Telox (6)

Tel NMR 126 MHz (MeOD, 25°C)
$^1$H NMR 500 MHz (CDCl$_3$, 25 °C)
$^{13}$C NMR, 125 MHz (CDCl$_3$, 25 °C)

- 77.16 CDCl$_3$
- 62.59
- 8.40
- -22.41
H NMR 500 MHz (CDCl₃, 25 °C)
$^{1}H$ NMR 500 MHz (CDCl$_3$, 25 °C)
1H NMR 500 MHz (CDCl₃, 25 °C)
$^{13}$C NMR 125 MHz (CDCl$_3$, 25 °C)
\[
\text{MeThO}
\]
H NMR 400 MHz (DMSO-d$_6$, 25 °C)
$^{1}H_{\text{NMR}}$ 400 MHz (DMSO-$d_{6}$, 25 °C)
$^1$H NMR 400 MHz (CDCl$_3$, 25°C)
$^{13}C$ NMR at 125.8 MHz (CDCl$_3$, 25°C)

Figure showing a NMR spectrum with various chemical shifts indicated. The spectrum includes peaks at 137.52, 132.19, 128.19, 126.38, 52.83, and 37.31 ppm.
$^1$H NMR 600 MHz (CDCl$_3$, 25 °C)
$\text{TeNMR} \ 189.3 \text{ MHz (CDCl}_3, 25^\circ \text{C)}$
$^1$HNMR 500 MHz (CDCl$_3$, 25°C)
References


