REPURPOSING INTRACELLULAR BIOSENSORS
FOR DETECTION OF EXTRACELLULAR FLUX

A Masters Thesis Presented

By

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REPURPOSING INTRACELLULAR BIOSENSORS
FOR DETECTION OF EXTRACELLULAR FLUX

A Masters Thesis Presented
By
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Program in Neuroscience
March 7th, 2022
DEDICATION

I dedicate this thesis in loving memory of my grandparents: Alfredo Gutierrez & Margaret Navarro, and Gustav Niacaris & Norma Kilde. The commitment to each other and their family was unapparelled.
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First and foremost, I would like to thank Dr. Bill Kobertz, my thesis advisor. His guidance helped to develop strong problem-solving and trouble-shooting skills.

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Finally, I am indebted to my parents, Michael and Pamela, and to my brothers, David and Gabriel.
ABSTRACT

Investigation of the natural world relies on our ability to perceive it, and the conclusions we draw are limited by what we can detect. The development of new tools to probe and explore the cellular world require commitment, perseverance, and new approaches to research. This thesis focuses on the development of a new biochemical tool capable of attaching GFP-based fluorescent biosensors to the glycocalyces of cell surfaces to visualize the flux of ions and metabolites out of living cells.

Chapter I addresses the strengths and limitations of current methods to investigating large-pore hemichannels, including the approach developed here. Chapter II elucidates the design, development, synthesis, and characterization of a novel organic compound, Ni\textsuperscript{2+}-tris-NTA-PEG\textsubscript{4}-DBCO, that is capable of both covalently modifying the extracellular sugar coating of mammalian cells, and binding oligohistidine-tagged fluorescent biosensors. This unique approach to “biosensor repurposing” places ion and metabolite biosensors at the source of release. We demonstrate this approach to visualize glutamate and protons at the cell surface, and the numerous cell types capable of further investigation. Chapter III focuses on the first known attempt to visualize endogenous ATP release from large-pore hemichannels using fluorescent biosensors. Here, pannexin-1 and CALHM1 hemichannels were overexpressed in HEK293T and HeLa cells, respectively, leading to ATP release. We attempted to fluorescently visualize these release events in real-time at the cell surface. Chapter IV concludes with future directions.
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<thead>
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<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>$[_]_i$</td>
<td>Intracellular concentration</td>
</tr>
<tr>
<td>$[_]_o$</td>
<td>Extracellular concentration</td>
</tr>
<tr>
<td>%</td>
<td>Percent</td>
</tr>
<tr>
<td>∆</td>
<td>Delta (change)</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine 5′-diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine 5′-monophosphate</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>Asn</td>
<td>Asparagine</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5′-triphosphate</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid</td>
</tr>
<tr>
<td>BFP</td>
<td>Blue fluorescent protein</td>
</tr>
<tr>
<td>BRET</td>
<td>Bioluminescence resonance energy transfer</td>
</tr>
<tr>
<td>BzATP</td>
<td>2′(3′)-O-(4-Benzoylebenzoyl)adenosine 5′-triphosphate</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CALHM</td>
<td>Calcium homeostasis modulator</td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine 3′,5′-cyclic monophosphate</td>
</tr>
<tr>
<td>CH$_2$Cl$_2$</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>Cl$^-$</td>
<td>Chloride ion</td>
</tr>
<tr>
<td>cp</td>
<td>Circularly permuted</td>
</tr>
<tr>
<td>DBCO</td>
<td>Dibenzocyclooctyne</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>Dr</td>
<td><em>Danio rerio</em> (zebrafish)</td>
</tr>
<tr>
<td>EAAT</td>
<td>Excitatory amino acid transporter</td>
</tr>
<tr>
<td>EDIPA</td>
<td>Ethyldiisopropylamine</td>
</tr>
<tr>
<td>EDT</td>
<td>Ethane-1,2-dithiol</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid</td>
</tr>
<tr>
<td>FP</td>
<td>Fluorescent protein</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster (or Fluorescence) resonance energy transfer</td>
</tr>
<tr>
<td>Gd$^{3+}$</td>
<td>Gadolinium ion</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HIS</td>
<td>Histidine</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>Dihydrogen monoxide (water)</td>
</tr>
<tr>
<td>hrs</td>
<td>Hours</td>
</tr>
<tr>
<td>Hs</td>
<td><em>Homo sapiens</em> (humans)</td>
</tr>
<tr>
<td>Hyl</td>
<td>Hydroxylysine</td>
</tr>
<tr>
<td>Hyp</td>
<td>Hydroxyproline</td>
</tr>
<tr>
<td>IAM</td>
<td>Ions and metabolites</td>
</tr>
</tbody>
</table>
K⁺  Potassium ion  
Kₐ  Equilibrium dissociation constant  
kDa  Kilodalton  
kₐoff  Dissociation rate  
kₐon  Association rate  
LRRC  Leucine-rich repeat-containing  
MAC  Maxi-anion channel  
MCH  Multivalent chelator head  
MCT  Monocarboxylate transporter  
MeOH  Methanol  
Mg²⁺  Magnesium ion  
ms  Millisecond  
mV  Millivolt  
Na⁺  Sodium ion  
NHS  N-hydroxysuccinimide  
Ni²⁺SO₄  Nickel sulfate  
NTA  Nitrilotriacetic acid  
Oa  Ovis aries (sheep)  
Panx  Pannexin  
PBS  Phosphate-buffered saline  
PDB  Protein Databank  
Pd/C  Palladium/carbon  
PDGFR  Platelet-derived growth factor receptor  
PEG  Polyethylene glycol  
RR  Ruthenium red  
RT  Room temperature  
s.d.  Standard deviation  
s.e.m.  Standard error of the mean  
SER/Ser  Serine  
SLC  Solute carrying  
TBTU  2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate  
TFA  Trifluoroacetic acid  
TFB-TBOA  3S)-3-[[3-[[4-(Trifluoromethyl)benzoyl]amino]phenyl]methoxy]-L-aspartic acid  
Thr  Threonine  
TIS  Triisopropylsilane  
TMD  Transmembrane domain  
Tyr  Tyrosine  
UDP  Uridine 5’-diphosphate  
UTP  Uridine 5’-triphosphate  
VRAC  Volume-regulated anion channel  
VSD  Voltage sensing domain  
Zn²⁺  Zinc ion
PREFACE

All work in this thesis was accomplished by the author, save for:

Mei Zhang, who provided electrophysiology support (Figure 2-8c-g) for Chapter II. Analysis and figure presentation was provided by the author.

Karl Bellve assembled all microscopy equipment, including the FRET microscope utilized in Chapter III.
CHAPTER 1: Introduction and Literature Survey

Overview

Transmembrane channels and transporters regulate the bulk of ionic and metabolic flow between a cell’s internal milieu and the extracellular space, and allow passage of ions (potassium, sodium, calcium) and metabolites (ATP, lactate, glutamate) that influence a host of downstream effects, including initiating and sustaining neuronal action potentials (Catterall, 2000; Doyle et. al., 1998), inducing inflammatory responses (Makarenkova & Shestopalov, 2014; Zhou et. al., 2019; Begandt et. al., 2017), providing energetic support (Rae et. al., 2009; Pellerin & Magistretti, 1994), and many of which little is known regarding their individual contribution to the biochemical processes they influence. When mutations arise in any of these transmembrane regulators, disease can manifest, adversely impacting the lives of those individuals and populations affected.

However, the complex coordination of metabolic and ionic events makes it difficult to interpret the link between genetic changes and disease progression. A direct approach to providing more clarity is to see into the live cell and visualize cellular processes in real-time. With improved spatial and temporal resolution, we can identify not only direct changes to ionic and metabolic flow due to cellular activity, but new approaches to biosensor localization to the extracellular surface could potentially identify unknown substrates of channels and solute carrying (SLC) transporters whose contribution to many important cellular processes remain ambiguous.
Numerous approaches have been developed over the last ~80 years that have allowed researchers to gather a birds-eye-view of cellular behavior in response to varying stimuli. These include GFP-based fluorescent biosensors (Marvin et. al., 2018; Saito et. al., 2012; San Martin et. al., 2013), enzyme catalyzed, light-producing chemical reactions (Gandelman et. al., 1993; Lyman & DeVincenzo, 1967), organic fluorescent indicators (Baruah et. al., 2005; Aigner et. al., 2012; Parker et. al., 2005), physical/mechanical probes (Wen et. al., 2019; Canales et. al., 2015), electrophysiological investigation (Nambu & Llinas, 1994; Brokamp et. al., 2012; Sahu et. al., 2014), and more complex and global methods like BOLD fMRI (Hyder et. al., 2001; Ip et. al., 2017) and optogenetics (Boyden et. al., 2005). However, each approach has its individual benefits and drawbacks.

One of the most widely used methods to investigate intracellular changes of ions, metabolites, and voltage are the use of GFP-based biosensors. Originally identified in *Aequorea victoria* (jellyfish) (Shimomura et al., 1962), green fluorescent protein has been re-engineered to detect key analytes in the living cell, including protons (Gjetting et. al., 2012), ATP (Imamura et al., 2009; Lobas et al., 2019; Yaginuma et al., 2014), cAMP (Saito et. al., 2012; Krähling et al., 2013), citrate (Ewald et al., 2011), glucose (Deuschle et al., 2006; Veetil et al., 2010), glutamate (Hires & Tsien, 2008; Helassa et al., 2018), and lactate (San Martin et al., 2013). Among these biosensors are a wide range of colors that span the ultraviolet, visible, and infrared spectrum of light, and include single-wavelength (Shen et. al., 2014; Akerboom et. al., 2012), circularly permuted (cpGFP) (Marvin et. al., 2013), ratiometric (Urra et. al., 2008), Forster-resonance energy transfer
(FRET) (Takanaga & Frommer, 2008), and bioluminescence resonance energy transfer (BRET) (Min et. al., 2019) biosensors that increase or decrease in fluorescence intensity or fluorophore ratio due to analyte binding.

However, changes in intracellular fluorescence intensity can often be the result of analyte influx, consumption, conversion, or release from the cell, and deciphering fluorescence changes that are a result of egress can be challenging. To directly measure analyte release, several groups have localized intracellular biosensors for extracellular expression by genetically encoding the platelet-derived growth factor receptor (PDGFR) transmembrane domain (TMD) into the N- or C-terminus of a GFP-based biosensor, thereby anchoring it to the extracellular surface (Marvin et al., 2013). This strategy has proven effective for monitoring extracellular glutamate release during neurotransmission (Helassa et. al., 2018; Koch et. al, 2018) under several experimental paradigms. Unfortunately, only a small subset of transfected cells express the surface-localized biosensor in contexts where it is valuable to label all cells simultaneously and uniformly, thereby providing a global view of cellular activity in complex, mixed, and behaviorally distinct cellular environments.

In order to address the sparse availability of extracellularly anchored fluorescent biosensors to study analyte egress, this study focuses on the development of a novel approach to repurposing and localizing intracellular GFP-based biosensors to the extracellular surface to investigate the behavior of large-pore hemichannels.
**Large-pore hemichannels**

Nearly 1,400 genes that form transmembrane channels have been identified in humans, and of these, a subset translocalize and express at the cell surface, contributing to fundamental biological processes critical for life. Transmembrane channels play major roles in homeostatic cellular processes, long-range communication, inflammation, and autocrine/paracrine signaling. Large-pore channels are a particularly interesting group precisely because of their propensity to pass numerous substrates of unknown composition, exposing the intracellular environment to the extracellular space and vice versa. Among these are connexins, pannexins, calcium homeostasis modulators (CALHMs), volume-regulated anion channels (VRACs), and maxi-anion channels (MACs) (Figure 1-1).

**Connexin channels**

The connexin (Cx) family of genes in *Homo sapiens* contain about 20 members (Willecke et. al., 2002; Cruciani & Mikalsen, 2006; Beyer & Berthoud, 2009) and form gap-junctiions by trafficking to the extracellular surface and connecting the membranes of adjacent cells. As gap junctions, connexins mediate the exchange of small molecules and ions between cells, playing important roles in neurotransmission (Wang & Belousov, 2011; Hormuzdi et. al., 2001), muscle contraction (He et. al., 1999; Valiunas et. al., 2000), and cellular injury (Lin et. al., 1998). However, when connexins form hemichannels, that is, “half” of a (gap junction) channel, they expose the intracellular environment to the extracellular space, and is an important distinction compared to gap
junctio. Depending on the connexin subtype, open gap junctions have a pore diameter ~12-15 Å, allowing the fast and semi-selective flow of ions and metabolites through them, and hemichannel opening to the extracellular space would prove to be disastrous, resulting in the collapse of ionic gradients essential for maintaining resting potential and transport, including the loss of ATP, key metabolites, and second messengers. However, controlled opening of hemichannels could potentially provide a conduit for both release and uptake of ions and small molecules, and it has been proposed that Cx46 releases both glutamate and ATP from astrocytes, which could have major impacts on fundamental neuronal processes (Sáez et. al., 2003; Bennett et. al., 2003; Stout et. al., 2002; Ye et. al., 2003). To date, no study has systematically identified the collective substrates of connexin channels, primarily due to the limited availability of technologies or assays capable of distinguishing between analytes. Nearly all assays measure bulk accumulation of analytes as they are released into the extracellular space and are incapable of differentiating the cellular source of analyte release in mixed cell cultures or more physiologically relevant conditions. The large pore diameter of connexin hemichannels allows the theoretical passage of nearly all cations and metabolites small enough to traverse the charge-selective core.
Figure 1-1 Large-pore hemichannels can pass numerous and unidentified analytes

Electron microscopy-derived structures of HsLRRC8D (VRAC) (PDB: 6M04), HsPannexin-1 (PDB: 6V6D), OaConnexin-46 (PDB: 6MHQ), and DrCALHM1 (PDB: 6LYG). The large-pore nature of hemichannels allows the passage of analytes, yet are activated by diverse means, and leads to the theoretical release or uptake of numerous atomic ions and small (usually less than ~1 kDa) molecules. 3-dimensional models were downloaded from the Protein Databank (PDB). Molecular graphics were performed using UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco.
Pannexin channels

The pannexin family of hemichannels consists of Panx1, Panx2, and Panx3, and was first identified in the mammalian genome in the year 2000 (Panchina et. al., 2000). They possess limited sequence homology to the invertebrate gap junction proteins, innexins, but share similar topology consisting of four α-helical membrane domains, two extracellular and one intracellular loop, and N- and C-termini located in the cytoplasm (Yen et. al., 2007; Baranova et. al., 2004). Pannexin hemichannels have gained well-known recognition in recent years for having been identified for contributing to a wide range of cellular processes, including sustaining apoptosis through cleavage of its C-terminal tail (Chekeni et. al., 2012), releasing cytoplasmic ATP (Qu et. al., 2011; Imamura et. al., 2020), and ultimately leading to cell death (Zhou et. al., 2018), and has been shown to contribute to neuroinflammation by microglia in response to traumatic brain injury (Garg et. al., 2018). Pannexin1 is a heptameric hemichannel with a hydrated closed pore diameter of ~9 Å (Deng et. al., 2020), that due to its large pore size when open is theoretically capable of passing numerous analytes from the cytoplasm to the extracellular environment. It is also unique in that it is capable of activation by several means. Panx1 has been shown to release ATP due to mechanical stress (Bao et. al., 2004), cytoplasmic calcium increases (Bao et. al., 2004), membrane depolarization (Ma et. al., 2009), and P2X (Pelegrin & Surprenant, 2006) and P2Y (Locovei et. al., 2006) purinergic receptor activation. While pannexin1 is known to pass ATP upon channel opening, due to the unavailability of relevant technologies, it is challenging to ascertain unidentified substrates of pannexin1 that may influence the surrounding cellular
environment that could lead to important downstream physiological processes and responses.

**CALHM1 channels**

CALHM1 was first identified in 2008 for being associated with Alzheimer’s disease risk in older populations (Dreses-Werringloer et al., 2008). Since then, CALHM1 has been shown to regulate intracellular calcium levels (Moreno-Ortega et al., 2010), release ATP (Workman et al., 2017; Taruno et al., 2013), influence extracellular amyloid-β accumulation, and regulate taste perception (Ma et al., 2018; Taruno et al., 2013). CALHM1 channel opening is regulated by strong membrane depolarization and extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{o}\)) reduction, leading to increased channel open probability (Ma et al., 2012; Ma et al., 2016). Although the structure of human CALHM1 has yet to be elucidated, structural insights of *Danio rerio* (56% identical to human) CALHM1 reveals an octameric architecture, possessing four transmembrane domains, and a pore diameter (Ca\(^{2+}\)-free state) ~20 Å in its open state (Ren et al., 2020). Because of its unique properties, CALHM1, although known to pass ATP into the extracellular space upon extracellular calcium removal, most likely passes other key ions and metabolites upon opening, similar to other large-pore hemichannels, that are worth investigating.
Volume-Regulated Anion Channels

The Volume-Regulated Anion Channel (VRAC) is activated by cell swelling and plays a key role in cell volume regulation (Jentsch, 2016; Okada et. al., 2019; Pedersen et. al., 2016). Cell swelling leads to VRAC-mediated transport of chloride ions and small organic compounds, including taurine, glutamate, GABA, gluconate, or ATP, that are used to counteract changes in extracellular osmolyte concentration in order to maintain homeostatic balance. Five isoforms of leucine-rich repeat-containing (LRRC) proteins were identified as the pore-forming subunits of VRACs (Voss et. al., 2014). VRAC channels exhibit moderate outward rectification under hypotonic conditions yet are inactive at large positive membrane voltages. Although the electrophysiological characteristics of VRACs have been studied extensively, the molecular substrates of these mysterious channels have yet to be elucidated (Pedersen et. al., 2015).

With a plethora of channels available for investigation, particularly regarding their contribution to analyte uptake and release, identifying the strengths and limitations of current methods of measuring cellular flux will contribute to identifying any gaps in technology that could potentially address unmet needs.
Current methods for studying large-pore hemichannels

There are several experimental approaches for studying large-pore hemichannels. The following is a survey of currently available technologies and methods (Figure 1-2).

Organic dye uptake

Small molecule fluorescent dyes have been routinely used to identify under which experimental conditions large-pore channels open, their potential pore size, and/or their charge-specific selectivity. YO-PRO-1, TO-PRO-3, Lucifer yellow, and ethidium bromide dyes (Scemes et al., 2009; Chekeni et al., 2010; Dolmatova et al. 2012; Hansen et al. 2014) have been used extensively in experiments involving pannexins, connexins (Goliger et al., 1995; Sáez et al., 2020), CALHMs (Lohman et al., 2015), and VRACs (Ye et al., 2009; Li et al., 2020), and allow permeation of fluorescent dyes of masses up to 1.5 kDa. These experiments are predicated on the capacity of these small dyes to accumulate within the cytoplasm of cells as they traverse open pores, leading to fluorescence increases as they either bind DNA or collect in the cytosol. It was previously assumed that hemichannel opening allows the unrestricted flow of analytes through their pore, but these assays revealed dye uptake does not necessarily correlate with ion or metabolite fluxes and is often uncoupled with ionic current when cells expressing hemichannels are voltage-clamped, depolarized, and co-monitored for dye accumulation (Contreras et al., 2003, Nielsen et al., 2019; Gaete & Contreras, 2020). At the same time, while high permeability rates for ethidium uptake are observed at negative membrane potentials, ionic current is not detected (Hansen et al., 2014). While dye
uptake assays certainly provide valuable insights into the potential mechanisms of hemichannel structure and behavior, they are limited in their capacity to identify important ions and metabolites that traverse their large pore that ultimately lead to changes in intracellular and extracellular analyte composition.

**Electrophysiology**

Electrophysiology is a powerful and proven tool to investigate the behavior of large-pore, voltage-sensitive channels, and allows electrical control of the cell to study ionic current. For example, the most direct evidence of ATP permeability in pannexin-1 channels was acquired in excised inside-out patch membranes of *Xenopus* oocytes injected with human panx1 cRNA (Bao et. al. 2004). Under this experimental paradigm, single panx1 channels were loaded with a 10:1 outside-to-inside gradient of K$_2$ATP, resulting in a more negative (~+25 mV) reversal potential than the equilibrium potential of K$^+$ (~+60 mV) when unitary currents were applied and recorded, suggesting that ATP partly carried the currents, and exhibited at least five distinct open states that correlated to 5%, 25%, 30%, and 90% of maximal conductance.

Electrophysiologic experiments have also been conducted with CALHM1 in *Xenopus* oocytes (Ma et. al., 2018). Here, expression of CALHM1 or the single *C. elegans* homolog (Tanis et. al., 2013) generated voltage-gated currents that activated with slow kinetics, and when co-expressed with CALHM3, generated large currents with 50-fold faster activation kinetics ($\tau \sim 100$ ms) than those of CALHM1 ($\tau \sim 5,000$ ms) alone. At the same time, while a positive correlation between CALHM1 + CALHM3 membrane
currents and ATP release was observed using the luciferin/luciferase assay, it has been noted previously that ionic current cannot always be extrapolated to metabolite permeation and vice versa (Gaete & Contreras, 2020).

While these well-designed studies, and many more like it, clearly illustrate numerous conductance states of single and combinations of channels, electrophysiology cannot be used to observe macroscopic behavior of cell populations expressing large-pore channels in more biologically relevant contexts or directly observe metabolite release at the cell surface.

**Bioluminescent/colorimetric assays**

The most direct method of measuring metabolite release through large-pore channels are commercially available enzyme-mediated assays. These ultra-sensitive kits can provide absolute quantifications of lactate, glutamate, and ATP as they accumulate in the external solution, and offer limited temporal measurements but virtually no spatial resolution. For example, Wei and colleagues (2014) found within the same study that connexin-46- and pannexin-1-expressing astrocytes release both glutamate and ATP upon stimulation with ultrafine carbon black (CB) particles (< 2.5 µm), atmospheric particulate matter considered to be most related to human health with high biological activity and easy absorption of other toxic pollutants. Here, astrocytes were stimulated with CB followed by media extractions that were added to either the Amplex red glutamic acid/glutamate oxidase assay kit or the ATP bioluminescent luciferin/luciferase kit.
Available approaches to investigate the nature of large-pore channels

Several approaches are routinely used to study large-pore hemichannels, and include radiolabeled glucose metabolism, dye uptake and bioluminescent/colorimetric assays, GFP-based biosensors, and electrophysiology.
Standard curves of known ATP or glutamate concentrations are used to objectively quantify analyte release, the result of which is either a colorimetric change with specific wavelength absorbance or light (bioluminescent) emission. Such assays are used routinely in biological research, but as mentioned, lack spatial resolution.

**Fluorescent Biosensors**

First isolated as an accessory protein to Aequorin by Shimomura in 1962 (Shimomura et al., 1962), green fluorescent protein (GFP) has since been utilized and re-engineered for hundreds of bioimaging applications. An 11-stranded β-barrel protein with an autocyclized central chromophore consisting of -Ser$^{65}$-Tyr$^{66}$-Gly$^{67}$- running the barrel length, GFP is excitable at 395 nm and 475 nm and emits at 505 nm, and amino acid alterations along the β-barrel or the chromophore lead to changes in excitation and emission maximums. These unique properties of GFP have led to blue, green, yellow, orange, and red variants of the protein that have been used simultaneously in numerous biomedical applications due to non-spectral overlap.

The first genetically encoded GFP-based indicator was developed by Siegal and Isacoff in 1997 (Siegal & Isacoff 1997) to detect transmembrane voltage changes in single cells. Here, they fused a modified GFP into a voltage-sensitive K$^+$ channel so that voltage-dependent structural alterations in the K$^+$ channel would induce changes in fluorescence of GFP. This approach proved effective despite a maximal fluorescence change of ~5% but was comparable to other dye-dependent measuring modalities at the
time. This first attempt at utilizing GFP as a fluorescent readout of cellular activity informed more rational approaches to biosensor development and demonstrated the utility of structural rearrangements within the protein to induce fluorescence changes.

To date, hundreds of GFP-based indicators have been developed for intracellular detection of ions and metabolites. For example, over 30 GFP-based biosensors have been engineered to detect alterations in proton concentration alone which span the visible spectrum of light and possess a wide range of pKₐ values that consist of ratiometric, FRET, intensity, and excitation and emission ratio variants (Shen et al., 2014; Gjetting et al., 2012; Li & Tsien, 2012). Altogether, numerous intracellular biosensors have been engineered to detect key metabolites (ATP, cAMP, lactate, glutamate, citrate, glucose) and ions (Ca²⁺, Cu²⁺, and Zn²⁺).
Glycocalyx engineering and biosensor localization

The glycocalyx is a dense carbohydrate polymer coating covering every cell in the body. In humans, the glycocalyx is composed of glycoproteins, proteoglycans, and glycolipids that surround the cell membrane, with roughly 80% of all transmembrane proteins predicted to be glycosylated (Steentoft et. al., 2013). Composed of N- (Asn/Arg) and O-linked (Ser/Thr/Tyr/Hyl/Hyp) monomer sugars (glucose; N-acetylglucosamine; mannose; N-acetylgalactosamine; sialic acid), polymers can extend from 10 – 500 nm (Reitsma et. al., 2007; Möckl et. al., 2019) from the plasma membrane and play diverse roles in mechanotransduction (Pohl et. al., 1991; Hecker et. al., 1993; Florian et. al., 2003), inflammation (Mulivor et. al., 2004; Cancel et. al., 2016), viral infections (Becker et. al., 2015; Glasner et. al., 2017), and embryonic development (Henderson-Toth et. al, 2012; Huang et. al., 2014). Given the location of the glycocalyx relative to the plasma membrane, biochemical approaches have emerged that allow chemical modification of the glycocalyx that have elucidated both its composition and structure. Some of these approaches have been termed glycocalyx engineering.

Groups have previously utilized glycocalyx engineering to investigate the nanoscale organization of the glycocalyx (Möckl et. al., 2019), discover the glycocalyx-dependent mechanism of natural killer (NK) cell immunoevasion (Hudak et. al., 2014), and monitor proton efflux from cells in real-time (Zhang et. al., 2016), which employ unnatural sugar incorporation into the glycocalyx to covalently attach fluorescent dyes or small-molecule biosensors to monitor these events. On the other hand, nitritriolacetic acid (NTA) chemistries have been utilized to colocalize similar fluorescent dyes to the surface
of cells expressing oligohistidine-tagged transmembrane channels (Lata et. al., 2006) in order monitor protein-protein interactions at the cell surface. However, these two chemistries have not been combined previously. We hypothesized that synthesizing a single molecule to contain both an azide-reactive group (DBCO) and oligohistidine-binding (NTA) groups would allow covalent modification of the cell surface to cover the entirety of a cell with NTA. Once re-engineered, we could then non-covalently localize the hundreds of GFP-based fluorescent biosensors (or any protein) to the cell surface by oligohistidine-tagging and recombinantly producing them, placing them at the source of ion and metabolite release. Not only would this approach circumvent needing to genetically encode a particular biosensor that often results in low transfection efficiency, but it could also allow researchers to address unique biological questions that currently evade investigation due to limited technological approaches.

One such context where this approach would prove useful are those where among a mixture of different cell types, the cellular source of a particular ion or metabolite cannot be identified. For example, a hypothesis that has evaded researchers for decades, termed the astrocyte-neuron lactate shuttle hypothesis (ANLSH), states that during periods of prolonged neuronal firing, neurotransmitter-activated astrocytes utilize arterially derived glucose, convert it to lactate, and “shuttle” lactate to neurons where it is utilized to produce ATP (Pellerin & Magistretti, 1994). This hotly contested hypothesis has been investigated for decades but remains elusive because no group has been capable of localizing a lactate biosensor to the surface of cells in order to identify exactly which cell type releases lactate under stimulatory conditions. Although a lactate-sensitive FRET biosensor has been developed (San Martin et. al., 2013), it can only be expressed intracellularly (Mächler et. al.,
2016) where changes in FRET ratios are *interpreted* as being the result of release of lactate from astrocytes, and not the potential conversion or consumption of lactate by astrocytes. Placing a lactate biosensor on the surface of all cells could potentially identify precisely which cell type is releasing lactate under specific conditions. This hypothesis, and many like it, could be addressed by the placement of fluorescent biosensors directly adjacent to the channel or transporter responsible for their release.

Here, we developed a straightforward approach to labeling mammalian cell surfaces with recombinantly-produced intracellular biosensors using metabolic oligosaccharide engineering (Prescher et al., 2004). This approach exploits the inherent nature of mammalian cells to metabolize specific unnatural oligosaccharides that are incorporated into their glycocalyces. Cells can be chemically altered by metabolism of paracetylated ManNAz (Ac₄ManNAz) and strain-promoted cycloaddition, termed copper-free “click chemistry”, to allow covalent modification of the extracellular surface with azide-reactive reagents. We thus developed a novel chemical compound, Ni²⁺-tris-NTA-PEG₄-DBCO, capable of (1) reacting with glycocalyx-incorporated azide and (2) binding recombinantly produced polyhistidine-tagged fluorescent biosensors (Figure 1-3).

To this end, Chapters II will explore the design, development, synthesis, characterization, and utilization of our approach to visualize glutamate and proton accumulation at the cell surface. Chapters III will explore the first recorded attempt to fluorescently visualize endogenous ATP release from large-pore hemichannels at the cell surface, and Chapter IV describes future directions.
Figure 1-3 Glycocalyx engineering and biosensor localization

(i) Ac$_4$ManNAz is consumed by cells and (ii) shuttled through the enzymatically controlled 6-step sialic acid biosynthesis pathway before N-azidoacetyl sialic acid (SiaNAz) is (iii) delivered to glycoconjugate chains by sialyltransferase (iv) destined for the cell surface. (v) Ni$^{2+}$-tris-NTA-PEG$_4$-DBCO is added to oligosaccharide engineered cells, (vi) resulting in an azide-alkyne cycloaddition “click” reaction, (vii) before oligohistidine-tagged fluorescent biosensors are added, allowing the (viii) detection of local ion and metabolite fluctuations at the cell surface.
CHAPTER II: A novel approach to biosensor repurposing

Abstract

Cells release ions and metabolites to regulate their internal milieu, propagate action potentials, communicate with neighboring cells, and mount defenses against foreign pathogens and injury. Responsible for these release events are the hundreds of transmembrane channels and transporters that allow specific and non-specific passage of select ionic and metabolic substrates that serve to propagate a cascade of downstream actions. Developing a biochemical method of fluorescently visualizing these release events at the cell surface was the focus of our investigation. Here we show the development of a novel organic compound capable of localizing recombinantly produced oligohistidine-tagged fluorescent biosensors to the cell surface to visualize ion and metabolite release in real-time.
Introduction

The incredible capacity of individual cells to chemically respond to each other and their environment make them obvious targets for quantitative investigation. The primary unit of multicellular life, cells respond both rapidly and proportionally to the most minor perturbations in external composition that lead to gene expression and morphological changes, the release of neurotransmitters and growth factors, and the exchange of ions and metabolites (IAM) with the extracellular environment. Responsible for many of these phenomena are the hundreds of different ion channels and transmembrane transporters localized at the cell surface that allow passage of analytes to maintain intra- and extracellular homeostatic balance. When mutations arise in any of the protein channels and transporters responsible for maintaining cardiac rhythmicity (long QT syndrome, Brugada syndrome), neurotransmission (epilepsy, migraine, episodic ataxia), inflammatory responses (IBD), or endocrine function (diabetes mellitus, hypoglycemia), disease can manifest. Yet, given the importance of better understanding how and why these mysterious transmembrane regulators function, few biochemical tools exist that allow their study at the location where these events occur: at the extracellular surface.

One of the first labs to make use of fluorescent biosensors to visualize extracellular flux was that of Loren Looger (Marvin et al., 2012). A glutamate-sensing, intensity-based biosensor, iGluSnFR was constructed from E. coli GltI and circularly permuted (cp) GFP and is a bright and photostable sensor with a 4.5 ($\Delta F/F_0$)$_{\text{max}}$ and in
vitro (in solution) affinity of 107 ± 9 µM for glutamate. Although capable of reporting on intracellular glutamate changes, when genetically encoded and chimerically tethered to the platelet-derived growth factor receptor (PDGFR) transmembrane domain (TMD) and expressed in any number of cell types, plants, or animals, it is capable of sensing extracellular changes of glutamate derived from neurotransmitter release or flux. Since its development, surface-expressed iGluSnFR has been utilized in dozens of studies and has shed light on calcium-based plant defense responses (Toyota et al., 2018), neural-circuit-specialized astrocytes in the adult brain (Chai et al., 2017), and characterizing glutamate synapses in intact neural circuits (Borghuis et al., 2013).

Several major drawbacks to genetically encoding surface-expressed biosensors are worth noting, however. Depending on the cell type under question, genetic encoding typically requires the construction of viral vectors (AAV.hSynapsin.iGluSnFR, etc.) harboring the DNA sequence of the biosensor, which can be time consuming, expensive, and invasive to administer. The breeding of transgenic animals is also laborious in nature, and expression of the intended fluorescent indicator can be low, off target, and uncontrollable. The most obvious downsides to genetically encoding surface-expressed indicators is the diffuse distribution of expression due to low infection efficiency and the lack of expression level control. Developing a method of circumventing these issues was our focus.

Bertozzi and colleagues cleverly devised several bioorthogonal approaches to engineering the glycocalyces of living cells to allow covalent modification of the extracellular surface utilizing unnatural paracetylated sugars harboring specific functional
groups (Saxon & Bertozzi, 2000; Prescher et al., 2004). Under this paradigm, cells or animals are fed or injected with N-azidoacetylmannosamine-tetraacylated (Ac₄ManNAz) for several days. The sugar is taken up by cells, deacetylated, converted through six enzymatic steps to sialic acid (SiaNAz), and placed on the terminal sugar of glycoconjugates, making the azide available for copper-free click chemistry at the surface under physiological conditions (Baskin et al., 2007; Chang et al., 2010). With the extracellular surface poised for covalent modification, we designed a novel organic compound that reacts with azide groups and can bind any recombinant oligohistidine-tagged protein, and in this case, fluorescent biosensors (Figure 2-1). We envision our approach widely applicable under carefully designed experimental paradigms.
Figure 2-1 Approach to intracellular biosensor repurposing

Mammalian cells are fed a paracylated azido sugar (i) for a minimum of 48 hrs. Cells are then labeled with Ni^{2+}-tris-NTA-PEG₄-DBCO (ii) for 30 minutes, thereby coating the external cellular surface with Ni^{2+}-tris-NTA. Cells are then labeled with oligohistidine-tagged biosensors (iii), allowing the detection of surface-localized ionic and metabolic changes.
**Materials and Methods**

**Chemicals**

EDIPA (387649-100ML), DMF (227056-100ML), Pd/C (10%) (205699-10G), MeOH (34860-1L-R), TBTU (8.51008), CH$_2$Cl$_2$ (270997-1L), TFA (302031-100ML), EDT (02390-25ML), TIS (233781-10G), phenol (33517-100G), and Ni$^{2+}$SO$_4$ (227676-100G) were purchased from Sigma Aldrich.

**Chemical characterization**

Unless otherwise stated, all reactions were run under an inert environment of argon (Ar) from which water and oxygen were rigorously excluded. (S)-5-benzyl 1-tert-butyl 2-aminopentanedioate hydrochloride (1) was purchased from Ark Pharm (AK-44459). DBCO-PEG$_4$-NHS was purchased from BroadPharm (BP-22288). Deuterated solvents were purchased from CIL. Thin layer chromatography was used to monitor the progress of reactions with EM Science silica gel 60 F$_{254}$ plates or neutral aluminum oxide F$_{254}$ plates from EMD Chemicals. Flash chromatography was performed using silica gel 60 (40-63 μm) from BDH. The final compound was purified by HPLC using a Higgins Analytical PROTO 300 C-18 column (10 μm), 250 × 10 mm (RS-2510-W181) on a Hewlett Packard Agilent 1100 HPLC instrument equipped with G1315A DAD absorbance detector. NMR spectra were recorded in CDCl$_3$, CD$_3$OD on a Varian 400 MHz spectrometer; $^1$H NMR and $^{13}$C NMR signals are reported in chemical shift relative to the NMR solvent peak. High resolution mass spectra (HRMS) were obtained on a
Waters Q-TOF Premier Mass Spectrometer at the University of Massachusetts Medical School Proteomics and Mass Spectrometry Laboratory. Fluorescence spectroscopic measurements were performed on an F4500 (Hitachi).

Ni$^{2+}$-tris-NTA-PEG$_4$-DBCO synthesis

Synthesis of tris-NTA amino caproic acid 7 was described elsewhere (Lata, 2005) and procedurally followed without deviation. tris-NTA-PEG$_4$-DBCO (9) was synthesized by first dissolving 7 to a final concentration of 50mM in DMF (100µL). NHS-PEG$_4$-DBCO 8 was separately dissolved in DMF to a final concentration to 100mM (100µL). The two solutions were mixed in equal volumes followed by addition of EDIPA (100µL). The reaction mixture was stirred overnight at room temperature. Stirring was continued for 2 hours after the addition of 200µL water. Solvent was then removed under reduced pressure. The resulting oil was kept at -20ºC until HPLC purification. 9 was resuspended in water and purified by HPLC on a C18 reverse-phase column using a linear 100:0% to 0:100% water:acetonitrile gradient over 30 minutes while monitoring the 291 and 308nm absorbance of DBCO. Mass was verified using mass spectrometry (Figure A-2). The non-covalent addition of Ni$^{2+}$ was accomplished by overnight incubation of HPLC purified 9 with 3.2 molar equivalents of aq. Ni$^{2+}$SO$_4$ in 300uL of water at 4ºC with stirring. The entire incubation was loaded on to a 1.5 x 20cm Kimble flex-column loaded with Sephadex G10 in water. Elution fractions were monitored for DBCO absorbance at 291 or 308nm and free-Nickel (393nm). Ni$^{2+}$-tris-NTA-PEG$_4$-DBCO fractions were then
combined, lyophilized, and resuspended in 20 mM HEPES, 150 mM NaCl, 5% glycerol to 50mM and stored at -20°C until used.

**Plasmids and protein biosensors**

Plasmids containing human Hv-1 were gifts from David Chapham (Harvard Medical School). Plasmids containing pHuji (#61556) and iGluSnFR (#106175) were purchased from the Addgene vector database.

**Recombinant Protein Production and characterization**

All biosensors were cloned into a pNCS (Addgene #17362) vector (containing either an N- or C-terminal 6- or 10-oligohistidine tag) by PCR amplifying the gene of interest using forward and reverse primers possessing NdeI and HindIII restriction sites, then DNA sequenced. All plasmids were transformed into *E. coli* BL21 (DE3). A single colony was inoculated in 1 L of LB medium with 100 mg/mL ampicillin (without IPTG) and shaken in the dark for 72 h. Cells were collected by centrifugation at 5000 rpm (4°C) for 20 min and disrupted by sonication in 10 mL of 20 mM Tris-HCl buffer pH 8.0. A cell-free extract was obtained by centrifugation at 10,000 rpm (4°C) for 40 min. Proteins were purified using a Nickel resin (HisPur™ Ni-NTA from Thermo Fisher), dialyzed (3,000 MWC Thermo Fisher) in 20 mM Tris-HCl 150mM NaCl (pH 8.0) for 16 h (4°C), and concentrated using 10,000 NMWL Amicon Ultra – 15 centrifugal filters according to the manufacturer’s specifications. Protein purity was determined by SDS-PAGE. Protein concentration was determined using the BCA method. All purified proteins were stored at
-20ºC until used. *In vitro* protein characterization was performed in clear bottom 96-well plates (Corning) containing serial dilutions of pH solutions (pHuji) or amino acids (iGluSnFR) in 100 µL/well.

**Cell culture, transfections, and cell surface labeling**

Chinese Hamster Overy-K1 (CHO) cells were cultured in F-12K nutrient mixture (Invitrogen); HEK cells were cultured in high glucose DMEM medium. All media was supplemented with 10% fetal bovine serum (Hyclone) and 100 units/mL penicillin/streptomycin (Invitrogen). Cells were plated at 60-75% confluency in 35 mm glass bottom culture dishes (MatTek). After 24 h, cells were transiently transfected with 1 µg of ion channel or transporter DNA and 8 µL of Lipofectamine (Invitrogen) for CHO cells or 4 µL of Lipofectamine and 6 µL PLUSTM reagent (Invitrogen) for HEK cells in Opti-MEM (Invitrogen). After terminating the transfections, the cells were incubated in media containing 50 µM azido-sugar for 2 d, which was replenished after 24 h. On the day of experiments, media was removed from glass bottom dishes, rinsed with RT 1X PBS, and labeled with 50 µM Ni\(^{2+}\)-tris-NTA-PEG\(_4\)-DBCO in opti-MEM media for 30 minutes at 37ºC. Cells were then rinsed with RT 1X PBS and labeled with 800 nM oligohistidine-tagged fluorescent protein or biosensor in opti-MEM media at RT for 15 minutes in the dark. For imaging, cells were finally resuspended in external solution (150 mM NaCl, 2.5 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM glucose, 10 mM HEPES, pH 7.5).
Figure 2-2 Synthesis of Ni$^{2+}$-tris-NTA-PEG$_4$-DBCO

Reactions conditions for synthesis of 10 are as follows: (i) EDIPA, DMF, 55°C, 6 hr. (ii) Pd/C (10%), MeOH, RT, 6 hr. (iii) TBTU, EDIPA, CH$_2$Cl$_2$, RT, 12 hr. (iv) TBTU, EDIPA, CH$_2$Cl$_2$, RT, 12 hr. (v) TFA, EDT, TIS, Phenol. H$_2$O, RT, 2 hr. (vi) DMF, EDIPA. H$_2$O 12 hr. (vii) Ni$^{2+}$SO$_4$, 6 hr.
In situ titrations

Ni^{2+}-tris-NTA-PEG_{4}-DBCO and biosensor-labeled cells were resuspended in 2 mL of external solution on to poly-L-lysine coated glass-bottom 35 mm dishes and mounted on a Zeiss Axiovert 40 CFL microscope equipped with an Andor Zyla 4.2 megapixel sCMOS high-resolution camera and CoolLED pE-4000 multi-wavelength excitation light source. A gravity-fed perfusion system was adapted to allow 20 mL of fluid, per perfusion condition, to fill the 35 mm dish to a maximum volume of 2 mL over 1.5 minutes. Excess fluid was vacuumed during perfusion, allowing a volume of no greater than 2 mL to fill the dish. After each titration was perfused (lowest concentration to highest), 5-10 images were collected using 4x4 binning (reduces noise and improves signal-to-noise ratio) under 63x oil emersion as z-stacks (50 ms exposure, 100 ms collection rate) and averaged.

Hv-1 experiments

CHO cells were transiently transfected using Lipofectamine 2000 for 4 hours, followed by 50 µM azido sugar for 48 hours to allow both surface display of N_{3}- and expression of Hv-1. On the day of the experiments, cells were trypsinized and seeded on L-polylysine-coated glass bottom dishes for 1 hr, labeled with Ni^{2+}-tris-NTA-PEG_{4}-DBCO (50 µM), HIS_{10}-pHuji (800 nM) (Shen et al., 2014), then resuspended in external solution (pH 7.5). Cells were then patched in the whole cell configuration (pipette resistance 2.5-4.0 MΩ) using an internal recording solution of pH 6.0. In steps of +20 mV, cells were depolarized from -80 mV to 0 mV » +100mV. The patch clamp
(Axopatch 200B), light source, and camera were controlled with Clampex 10.5 (Molecular Devices); fluorescent images were collected (10 Hz) and processed using open-source software (micro-manager and ImageJ).

**Glial Cell Isolation and Labeling**

P0.5 pups were collected, euthanized, and decapitated prior to whole brain extraction. Brains were carefully separated from meninges, placed in ice cold 1X HBSS, disrupted using pipetting or trypsinization, and resuspended in advanced DMEM containing 10% FBS, 1% penicillin/streptomycin, and L-glutamate prior to seeding in 75cm cell culture flasks and placed in a 5% CO2 incubator at 37°C for 1-3 weeks. Media was replaced every 3 days. After 1-3 weeks, microglia were separated and separately cultured from astrocytes by shaking flasks 100 rps for 1hr at 37°C. Media and high purity microglial cells were collected without disrupting the astrocyte layer, concentrated, resuspended, and plated in DMEM, 10% FBS, and 1% penicillin/streptomycin. 48 hrs later, flask-attached microglia and astrocytes were plated on to 30 mm glass bottom dishes and were fed 50 μM azido sugar every other day for 5 days total, after which time they were labeled with Ni²⁺-tris-NTA-PEG₄-DBCO and recombinant HIS₁₀-GFP.

**Image analysis**

Collected images were analyzed using ImageJ software. Images were collected, background subtracted, photobleach corrected, and 10-15 regions of interest (ROIs) were
chosen (per dish) for analysis. Fluorescence intensity of ROIs were averaged and s.d. or s.e.m. was calculated.
**Results**

**Chemistry**

The Kobertz lab having shown previously the ability to attach small molecule biosensors (pH-DIBO) to the surface of cells utilizing a popular and widely used bioorthogonal copper-free click chemistry (Zhang et al., 2016), we hypothesized it was possible to engineer the cells glycocalyx to compose tris-nitrilotriacetic acid (tris-NTA) to bind oligohistidine-tagged recombinant fluorescent biosensors, thereby making use of the hundreds of ion and metabolite biosensors available (Greenwald et al., 2018). One obvious advantage to this approach is the already-present N- or C-terminal oligohistidine-tag used to purify recombinant proteins with NTA-agarose, thus, biosensors do not require additional chemical modification that could deleteriously alter their function or character.

Designing Ni$^{2+}$-tris-NTA-PEG$_4$-DBCO was straightforward. Lata and colleagues (Lata et al., 2005; Lata et al., 2006) aspired to create chemical recognition units which bind oligohistidine tags with high affinity and stability, as tools for selectively attaching spectroscopic probes and other functional elements to recombinant proteins. They designed several supramolecular entities containing 2-4 NTA moieties (bis-, tris-, tetrakis-NTA) and characterized their affinity, stability, and reversibility against HIS$_6$- and HIS$_{10}$-tagged proteins, compared to mono-NTA. Based on their data, there were obvious advantages to tris-NTA over mono-, bis-, and tetrakis-NTA. Equilibrium dissociation constants (K$_D$) determined by isothermal calorimetry (ITC) and time-
resolved fluorescence measurements showed nearly 750-fold higher affinity of tris-NTA for HIS$_6$ and HIS$_{10}$ proteins compared to mono-NTA, and 100-folder higher affinity compared to bis-NTA, but the addition of one more multivalent chelator head (MCH) of tetrakis-NTA did not increase binding affinity compared to tris-NTA.

Choosing tris-NTA as the optimal MCH, we synthesized 7 without modification. Briefly, $t$-butyl ester-protected nitrolotriacetic acid chelator heads were grafted in triplicate onto a tetraaza cyclam scaffold under stoichiometric control to yield 4, followed by the addition of a primary amine linker 5 to allow functionality after deprotection. There were several commercially available options that would allow the addition of an azide-reactive group on to the tris-NTA amino caproic acid MCH, one of which possessed a water-soluble polyethylene glycol linker (PEG$_4$) of sufficient length conjugated at one end to an amine-reactive N-hydroxysuccinimide (NHS) ester, and at the other end an azide-reactive dibenzocyclooctyne (DBCO). The 2:1 molar addition of 8 to 7 under inert conditions in DMF and EDIPA yielded 97% reaction efficiency. Once HPLC purified, 9 was metal ion loaded with excess aq. Ni$^{2+}$SO$_4$ and run down a size exclusion column yielding 10. (Figure A-1).
**Demonstrating surface labeling with HIS$_X$-FP**

Without a formal method of testing the functional characteristics of 10 to both covalently modify the glycocalyx of living cells and bind oligohistidine-tagged fluorescent proteins independently, we sought to demonstrate HIS$_X$-FP surface binding by culturing Chinese hamster ovary (CHO) cells for 48 hours in the presence of 50 µM N-azidoacetyl mannosamine (Ac$_4$ManNAz) to allow sufficient time for N$_3$-surface display, followed by the addition of 50 µM 10 for 30 min and 800 nM recombinant HIS$_6$-GFP for 15 min (Figure 2-3). Not surprisingly, FP surface localization was dependent on the presence of Ac$_4$ManNAz, nickelated-tris-NTA-PEG$_4$-DBCO, and HIS$_6$-GFP. Topographic analysis (Figure 2-4a) indicated that while some fluorescence is seen within the cell due to membrane endocytosis and lysosomal storage, the majority of signal originates from surface-localized FPs, as internalized FPs are naturally quenched due to the acidic environment. One unique feature of this approach is the ability to dual- or triple-label cells with different biosensors, enabling the detection of multiple parameters simultaneously, as seen by co-labeling HEK293T cells with HIS$_{10}$-iGluSnFR (glutamate) and HIS$_{10}$-pHuji (proton) (Figure 2-4b). And although fluorescence loss was due to internalization, fluorescence quenching, and loss into bulk solution, surface labeling in HEK293T cells remained relatively stable over a 4-hour period (Figure 2-4c).
Figure 2-3 Surface labeling of CHO cells

(a) DIC and fluorescence characterization of FP surface localization in CHO cells. Cells were cultured in 50 µM azido sugar for 48 hours before co-incubating with Ni²⁺-*tris*-NTA-PEG₄-DBCO and HIS₆-GFP. Variables include ± azido sugar, ± Ni²⁺, and ± GFP. Scale bar, 20 µm.
Figure 2-4 Topology, dual-labeling, and stability

(a) Topological analysis of surface labeling in CHO cells reveals strong extracellularly bound surface localization. Scale bar, 10 µm. (b) HEK293T cells co-labeled with glutamate- (HIS\textsubscript{10}-iGluSnFR) and proton-sensing (HIS\textsubscript{10}-pHuji) biosensors demonstrates the utility of this unique approach to potentially measure multiple parameters simultaneously. Scale bar, 30 µm. (c) Surface labeling of HEK293T cells with HIS\textsubscript{10}-GFP reveals stable fluorescence up to 4 hrs, despite morphological changes and FP internalization. Scale bar, 40 µm.
**Labeling optimization**

To optimize oligohistidine-FP surface retention, we tested the effect oligohistidine length would have on fluorescence over an extended period. Time-dependent loss of fluorescent signal between HIS$_6$- and HIS$_{10}$-GFP resulted in significant differences beginning at 30 minutes post-GFP labeling at 37°C, with a ~10% difference in fluorescence at 2 hours and a maximum fluorescence loss rate of HIS$_6$ = -0.3%/min vs. HIS$_{10}$ = -0.1%/min ($n = 3$ dishes; 10-15 cells/dish) between $t = 0$ and $t = 30$ min (Figure 2-5a). Next, CHO cells were labeled with a GFP-based H$^+$ biosensor (HIS$_{10}$-pHuji; pK$_a$ 7.58 ± 0.05) and perfused with 100 mM imidazole ($n = 3$ dishes; 10-15 cells/dish) (pH 7.5), and as a result, surface-localized fluorescence was nearly abolished, yet internalized fluorescence increased (Figure 2-5b). We concluded that imidazole thus plays two important roles with respect to fluorescent signal: 1) surface-attached biosensors are readily liberated from tris-NTA due to imidazole competition, and 2) membrane-permeant imidazole is internalized, increasing the pH of biosensor-quenched acidic lysosomes. To further optimize surface labeling using this assay, we altered the temperature that HIS$_{10}$-GFP was incubated and monitored surface fluorescence intensity and internalization over two hours ($n = 3$ dishes; 10-15 cells/dish) (Figure 2-4c). While surface fluorescence increased with temperature, biosensor internalization was only significantly higher when incubated at 37°C. Thus, the ratio of surface/internal was optimal when cells were FP-labeled at room temperature, so all future experiments were FP-labeled under these conditions (Figure 2-5d).
Figure 2-5 Oligohistidine length and labeling temperature affect surface stability

(a) CHO cells labeled with HIS$_6$- or HIS$_{10}$-GFP reveals significant differences of surface fluorescence retention beginning at 30 min post labeling. (b) CHO cells were labeled with HIS$_{10}$-pHuji and perfused (before and after) with 100 mM imidazole (pH 7.5), eliminating surface fluorescence and revealing internalized, acid quenched biosensors. Scale bar, 25 µm. (c-d) Temperature-dependent FP surface labeling and internalization of HIS$_{10}$-GFP labeled CHO cells at 4°C, room temperature, and 37°C. Optimized surface/internal ratio occurred when cells were labeled with HIS$_{10}$-GFP at RT. (One-way ANOVA. Students unpaired two-tailed t-test, $^*$$P$<0.05, $^{**}$$P$<0.01, $^{***}$$P$<0.001).
Effects of pH

We next sought to understand how extracellular pH effects the surface retention of fluorescence signal in labeled cells. The imidazole side chain of histidine has a pK$_a$ of approximately 6.0. Thus, at physiological pH (7.35-7.45), the positive charge is equally distributed between both nitrogens represented by two tautomeric states and it partially protonated. Under acidic conditions, both nitrogens are protonated and are unavailable for metal ion coordination by Ni$^{2+}$. First, in vitro characterization of HIS$_{10}$-GFP illustrated the relative stability of fluorescent signal near physiological pH ($n = 3$ dishes; 10-15 cells/dish) compared to HIS$_{10}$-pHuji (Figure 2-6a). CHO cells were thus labeled with HIS$_{10}$-GFP and perfused with decreasing pH solutions ($t = 2$ min – 7 min), revealing surface fluorescence loss after re-perfusion of physiological pH solution (7.45) at $t = 10$ min (Figure 2-6b). Cells held at pH 6.5 for 5 minutes lost $\sim 6.7\% \pm 2.8\%$ (s.e.m., $n = 3$ dishes; 10-15 cells/dish) of fluorescent signal due to oligohistidine protonation, and $\sim 35.7\% \pm 5.7\%$ when held at pH 5.5 for the same length of time (Figure 2-6c). The initial loss of fluorescence ($t = 2$ min – 7 min) is due to the inherent pH-sensitivity of all fluorescent proteins. When labeled cells were held at pH 5.5 for various periods, the fluorescence loss time constant $\tau$ was calculated to be $8.98 \pm 1.37$ min (s.e.m., $n = 3$ dishes; 10-15 cells/dish) ($\tau = \text{time @ 1 \over e} \approx 36.8\%$ fluorescence loss) (Figure 2-6d).
Figure 2-6 pH-dependent surface retention

(a) *In vitro* characterization of GFP and pHuji. Fluorescence of GFP is significantly more stable at and around pH 7.5. (b-c) GFP-labeled CHO cells were resuspended in 2 mL external solution (pH 7.5), then perfused with external solutions (20 mL) possessing varying pH, and re-perfused (20 mL) with external solution. Fluorescence loss was quantified as the remaining fluorescent signal at \( t = 10 \) min \((n = 3 \text{ dishes; 10-15 cells/dish})\). (d) CHO cells labeled with HIS10-GFP and held at pH 5.5 showed a 1 / e = 36.8% fluorescence loss at \( \tau = 8.98 \pm 1.37 \) min.
Glutamate sensing at the surface

Glutamate is an important molecule in cellular metabolism and neurotransmission and has been rigorously studied both intracellularly and extracellularly. By recombinantly producing a glutamate sensing GFP-based fluorescent biosensor (Martin et al., 2013) and localizing it to the plasma membrane using our approach, we were able to make several important observations.

First, HIS10-iGluSnFR responded nominally (expectedly) to glutamate and other key amino acids (aspartic acid, glutamine, asparagine, glycine) in situ and in vitro (Figure 2-7a), but perfusion of increasing glutamate concentrations in CHO cells (Figure 2-7b) revealed an inverse relationship between F₀ (baseline fluorescence) and K_D, suggesting increased surface density of biosensors increases their affinity for glutamate (Figure 2-7c,d).

Second, to fully appreciate this observation, cells were intentionally labeled with different concentrations of HIS10-iGluSnFR (800 nM, 400 nM, 40 nM) and perfused with increasing concentrations of glutamate, which resulted in decreasing K_D with increasing HIS10-iGluSnFR concentrations (Figure 2-7e). The Looger lab made a similar observation, where the K_D of genetically encoded iGluSnFR decreased 25-fold when localized to the plasma membrane: in vitro dissociation constant for glutamate was 107 ± 9 µM and in situ plasma membrane expression in HEK293 cells was 4 ± 1µM. Thus, the advantage of our approach is two-fold: 1) every cell is uniformly labeled with surface-localized biosensors, compared to biosensor transfection where only a subset of cells
express the construct, and 2) the surface density of biosensors can be altered, making it possible to finely tune the detection range of glutamate depending on one’s application.

While the inverse relationship between plasma membrane localization and $K_D$ has been demonstrated with iGluSnFR, it is not known whether this is a general property of protein-based fluorescent biosensors, or specific to iGluSnFR, thus more biosensors would need to be tested in order to validate these findings and develop a strong hypothesis to explain this phenomenon. One possible explanation for the apparent reduction in $K_D$ due to higher concentrations of biosensor could be the due to the increased probability of individual glutamate molecules rebinding to neighboring biosensors once unbound from a previous biosensor. In effect, the increased density of biosensors acts as a matrix for glutamate to continuously bind/unbind to neighboring biosensors and appears to lead to an increase in biosensor affinity, without the actual association ($k_{on}$) or dissociation ($k_{off}$) rates of the biosensor being influenced, termed *apparent* $K_D$. Modulating the biosensor density on the cell surface using a wider range of concentrations and different biosensors, a much more definitive relationship and hypothesis for this phenomenon could be established.
Figure 2-7 Glutamate sensing at the cell surface

(a) CHO cells labeled with HIS\textsubscript{10}-iGluSnFR showed nominal fluorescent responses to glutamate and other amino acids. $\Delta F/F_0$ of glutamate = 1.09 ± 0.19 (mean ± s.e.m.).

(inset) Genetically encoded iGluSnFR expressed in HEK293T and neurons shows reduced $K_D$ relative to \textit{in vitro}, and significantly reduced $\Delta F/F_0$ in neurons compared to HEK293T cells. (from Martin et al., 2013). (b) CHO cells showed a nearly uniform distribution of fluorescent signal when labeled with HIS\textsubscript{10}-iGluSnFR in the absence of glutamate. Regions between touching cells (arrows) showed higher fluorescence changes and reduced $K_D$ relative to other regions. Scale bar, 15 µm. (c-d) An individual cell with varying $F_0$ showed a linear relationship between baseline fluorescence and $K_D$. (inset) Individual surface regions selected and non-linearly fit using using the Hill equation in response to glutamate titrations. (e) CHO cells labeled with decreasing concentrations of HIS\textsubscript{10}-iGluSnFR and titrated with glutamate, including calculated $K_D$, showed significant differences in affinity for glutamate. (inset) Relative baseline fluorescence of labeled CHO cells with HIS\textsubscript{10}-iGluSnFR.
Visualizing Proton Efflux

To demonstrate proof-of-concept that our approach to biosensor repurposing can feasibly and reliably detect efflux, we chose to investigate proton release from CHO cells expressing a voltage-gated proton channel, human Hv-1 (Ramsey et al., 2006, Lee et al., 2009), labeled with HIS\textsubscript{10}-pHuiji (Shen et al., 2014). pHuiji is a red, proton sensitive, intensiometric biosensor with a pK\textsubscript{a} at roughly physiological pH that decreases in intensity upon H\textsuperscript{+} binding. When oligohistidine-tagged and bound to the surface of CHO cells with Ni\textsuperscript{2+}-tris-NTA-PEG\textsubscript{4}-DBCO, the biosensor responds to changes in pH nearly identically to the biosensor \textit{in vitro}, with a slight decrease in pK\textsubscript{a} \textit{in situ} 7.58 ± 0.05 (s.e.m., \textit{n} = 4 dishes; 10-15 cells/dish) \textit{(in vitro} 7.94 ± 0.05, s.e.m., \textit{n} = 4 wells/titration) (Figure 2-8a) and a (ΔF/F\textsubscript{o})\textsubscript{max} ≈ 1.5.

Possessing a putative voltage sensor domain (VSD), Hv-1 channels are activated by depolarizing voltages and increases in internal proton concentration (Lee et al., 2009; Ramsey et al., 2006), so we hypothesized that depolarization of CHO cells would open Hv-1 leading to detectable proton egress. To fluorescently visualize proton fluxes using our approach, we transiently transfected Chinese hamster ovary (CHO) cells with a GFP-tagged, human voltage-gated proton channel (Hv-1) and incubated with tetra-acetylated N-azidoacetyl-D-mannosamine (azidosugar) (Laughlin and Bertozzi, 2007; Saxon and Bertozzi, 2000). After 2 days, the cells were labeled with Ni\textsuperscript{2+}-tris-NTA-PEG\textsubscript{4}-DBCO for 30 min, then HIS\textsubscript{10}-pHuiji for 15 minutes, and the currents and fluorescence were measured using patch-clamp fluorometry in a bath solution with a low buffer capacity (0.1 mM) and pipette solution of pH 6.0. Fluorescence responses to depolarizing
membrane potentials were rapid and robust, and whose kinetics closely mimic those of the small molecule proton biosensor, pH-DIBO, found by Zhang et al. under identical conditions (Figure 2-8c, d, e). The average maximum fluorescence change of \( n = 20 \) cells at \(+100 \text{ mV} = -23\% \pm 2\% \) (s.e.m., \( n = 23 \) cells), indicating that surface pH was \( \approx 7.15 \) (max \( \Delta F/F_o = -41\% \approx \text{pH} \sim 6.7 \)) based on pHuj’s \textit{in situ} characterization prior and showed no appreciable loss of baseline fluorescence due to histidine protonation and biosensor loss (Figure 2-8f). These results were promising and illustrated the feasibility of our approach to visualize ionic fluxes at the cell surface.
Figure 2-8 Visualizing proton efflux through Hv-1

(a-b) *In vitro* and *in situ* titration of external solution with varying pH in CHO cells labeled with HIS$_{10}$-pHuji. (c) GFP-tagged Hv-1 transfected cells whole-cell patched and depolarized using a standard protocol. Cells were held at -80 mV and depolarized in steps of +20 mV from 0 to 100 mV for 4 sec. (d) Current trace of individual cell and corresponding fluorescence change (e). (f) Fluorescence responses of a single depolarized cell showed minimal signal loss due to oligohistidine protonation. (g) $n = 23$ cells.
**Alternate cell types for future investigation**

We have shown thus far several key aspects of our biosensor surface labeling approach. However, the limitations of this method are twofold: (1) the utility of this system is dependent on the extent to which a particular cell type is capable of metabolizing Ac$_4$ManNAz or any other azide-containing unnatural sugar and displaying the functional group at the plasma membrane for copper-free click chemistry, and (2) the changes in local ion and metabolite (IAM) concentrations at the cell surface under well-designed experimental paradigms must lie within the detectable range of the biosensor in question. While biosensor design, construction, and optimization are beyond the scope of this study, we demonstrated multiple cell types fed azido-sugar for 48 hrs and labeled with HIS$_{10}$-GFP (Figure A-6), and include primary murine microglia and astroglia cells, murine bone-forming osteocytes (OCY454), human neuroblastoma N2a cells, and human mammary carcinoma MCF-7 cells. Other studies have also demonstrated efficient azido sialic acid incorporation into sialoglycans in the heart, kidney, liver (Preacher et al., 2004), and brain (Xie et al., 2016). These data indicate a potentially large pool of studies capable of using our approach to probe fundamental biological processes previously beyond the capabilities of current investigative technologies under a wide variety of biological and experimental paradigms.
Discussion

Here we demonstrate a novel approach to localizing recombinantly produced oligohistidine-tagged fluorescent proteins and biosensors to the extracellular surface of mammalian cells using glycocalyx engineering and bioorthogonal chemistries. This unique methodology exploits the inherent capacity of numerous mammalian cell types to metabolize a plethora of non-toxic, unnatural, paracetylated sugars possessing a multitude of alternative bioorthogonal functional groups. Once displayed on the terminal sialic acid of membrane-embedded glycoconjugates, functional groups can be covalently modified under physiological conditions by “clicking” together compatible reactive groups, and in our case, resulting in an azide-alkyne cycloaddition. The addition of an original organic compound, Ni\(^{2+}\)-tris-NTA-PEG\(_4\)-DBCO, following the metabolism of Ac\(_4\)ManNAz, thus results in the uniform distribution of NTA moieties poised to non-covalently coordinate oligohistidine peptides. When oligohistidine tagged, GFP-based fluorescent biosensors are ultimately localized to the extracellular surface near the source of ion and metabolite release.

The approach we developed possesses several key advantages over previous methods. Compared to genetic engineering, our approach (1) leads to the relatively uniform distribution of biosensors over the cell surface, while (2) all cells are labeled simultaneously; (3) labeling occurs under physiological conditions; (4) multiple biosensors can be used concurrently to visualize more than one analyte concomitantly; (5) surface-labeling is stable over hour-long periods; and (6) biosensor labeling is fast.
and (7) controllable. In addition, although not demonstrated here, biosensor surface labeling could potentially be condensed into a single-step process, whereby Ni\textsuperscript{2+}-tris-NTA-PEG\textsubscript{4}-DBCO and HIS\textsubscript{10}-biosensor are pre-incubated, purified, and added to cells together. Such an approach could prove to be advantageous under certain experimental conditions, say, in the mouse or fly brain \textit{in vivo}. Here, previous groups have already demonstrated azide sugar metabolism and cell surface-labeling in the whole animal (Baskin et al., 2007; Xie et. al., 2016) using a liposome-assisted bioorthogonal strategy. Such an approach would not require the use of transgene-containing viruses or specific animal models. Labeling could occur in a 30-minute single-step process, allowing the visualization of cellular activity in real-time under more physiologically relevant contexts.

In addition, Zhang et. al. (2016) acutely demonstrated the ability to label CHO cells with pH-DIBO using glycocalyx engineering and monitoring proton efflux through Hv-1, as demonstrated here. While the approach to covalently modify the cell surfaces and activate (electrophysiology) proton efflux was identical, the localization of the proton biosensor was accomplished by different means. There are several differences to our approach compared to theirs. First, the proton biosensor we utilized in this study possessed a larger dynamic fluorescent range (ΔF/F\textsubscript{o}), and there are a vast array of available GFP-based proton biosensors that cover a wide variety of pK\textsubscript{a} values and emission wavelengths. Second, it is clear from image comparison that our approach to surface labeling led to higher surface vs. internalized biosensor ratios. It was unclear from their approach the proportion of internalized biosensor that could potentially
confound quantification, as their compound, pH-DIBO, could potentially cross the plasma membrane and diffuse into the cytosol, whereas Ni\textsuperscript{2+}-tris-NTA-PEG\textsubscript{4}-DBCO is most likely too large and acidic to readily diffuse into the cytoplasm. Third, our approach to biosensor localization is modular. Zhang illustrated their unique approach to visualize proton efflux using a single organic fluorescent indicator. Our approach on the other hand allows usage of virtually all pre-existing GFP-based biosensors available, granted the local concentration of ions or metabolites as they exit the cell falls within the detection range of the biosensor.

Lastly, although we demonstrate only a single example of ion release at high spatial and temporal resolution, the number of available GFP-based biosensors, cell types, channels, transporters, and experimental paradigms results in a potentially enormous number of possible combinations for further investigation. Thus, the method we developed could prove to be a highly valuable tool for several fields that wish to investigate more deeply the contribution of cellular phenomenon to ion and metabolite release at the cell surface, including currently unanswerable questions due to a lack of available technologies.
CHAPTER III: ATP egress through large-pore hemichannels

Abstract

Adenosine triphosphate (ATP) is one of the most evolutionarily conserved molecules found in biological life. From archaea to humans, ATP serves numerous critical functions within and outside of cells, as nearly all cells release ATP or other nucleotides in local autocrine and paracrine signaling. Recent advances to quantify ATP release more precisely from excitable and non-excitable cells have led to advances in novel technologies capable of measuring ATP release at its source: the extracellular membrane. ATP is released from cells by two primary mechanisms: (1) vesicular release, and (2) a large conductive pore. Here we demonstrate a novel method of localizing ATP-sensing fluorescent biosensors to the extracellular surface using glycocalyx engineering, copper-free click chemistry, and oligohistidine-tagged recombinant GFP-based biosensors. We attempt to stimulate ATP release through pannexin1 and CALHM1 hemichannels and visualize ATP accumulation at the surface. This study illustrates the first attempt at doing so.
Introduction

Cells can perform numerous complex functions. Depending on the cell type in question, they can relay electrical signals (neurons), mount inflammatory responses (microglia, macrophages), provide energetic support (astrocytes), morphologically contract (myocytes), swim (male gametes), and much more. Rightly considered the “energy currency” of biological life, virtually no complex cellular process can be achieved without adenosine 5’-triphosphosphate (ATP), as it serves a plethora of roles within and outside of cells. In the cytoplasm, ATP is produced both during glycolysis and oxidatively in the mitochondria, where it is ultimately utilized as a substrate for nucleotide synthesis, enzyme catalyzed reactions, maintaining electrochemical gradients, and intracellular signaling. However, ATP is often released into the extracellular space for a variety of critical intercellular functions. For example, the Na+/K+ pump is an ATP-dependent, electrogenic transmembrane ATPase and is required for the maintenance of osmotic equilibrium and membrane potentials, but ATP also acts as an extracellular neurotransmitter in the same cell type, that when released into the synaptic cleft via purinergic vesicle fusion with the presynaptic terminal, it binds post-synaptic purinergic receptors (P2X and P2Y) leading to fact-action neurotransmission in the thalamus, cerebral cortex, hippocampus, and brainstem.

But ATP has also been shown to traverse several large-pore, transmembrane hemichannels that open and release ATP into the extracellular space from a variety of intra- and extracellular stimuli. Five groups of channels are acknowledged as ATP-
releasing: pannexin1 (Panx1), volume-regulated anion channels (VRACs), connexin hemicannels, calcium homeostasis modulator 1 (CALHM1), and maxi-anion channels (MACs). Among these, Panx1 and CALHM1 have been investigated extensively in numerous biological contexts.

Panx1 has been implicated in many physiological and pathophysiological processes. It has been demonstrated to regulate blood pressure in vascular smooth muscle (Billaud et. al., 2011; Billaud et. al., 2015), mediate inflammation (Good et. al., 2018), and contributes to neurological disorders and pain (Thompson et. al., 2008; Gulbransen et. al., 2012; Weaver et. al., 2017). Panx1 can be opened by several sources of stimuli, either directly, or indirectly through the activation of other channels or receptors. Mechanically activated Panx1 induces ATP release from metastatic breast cancer cells (Furlow et. al., 2015) and urothelial cells upon bladder distension (Negoro et. al., 2014), whereas \([\text{Ca}^{2+}]_i\) increases through P2X and P2Y receptor activation lead to Panx1 opening and ATP release (Pelegrin & Surprenant, 2006; Locovei et. al., 2006). This flexible activation of Panx1 allows us to further investigate whether hemicannel-dependent ATP release can be visualized at the cell surface using glycocalyx-localized fluorescent biosensors with compatible stimulants, as doing so would be the first of its kind. So as not to confine our investigation to a single approach, additional hemicannels were considered.

First identified in a meta-analytic study surveying genes associated with late-onset Alzheimer’s disease (LOAD) (Dreses-Werringloer et. al., 2008), CALHM1 is a non-selective, \(\text{Ca}^{2+}\)-sensitive, \(\text{Ca}^{2+}\)- and ATP-permeable channel involved in purinergic
neurotransmission in taste cells and regulates neuronal excitability (Ma et. al., 2012; Taruno et. al., 2013). At physiological \([\text{Ca}^{2+}]_o\), CALHM1 assumes a closed state, and upon a reduction or removal of \([\text{Ca}^{2+}]_o\), CALHM1 opens, widening to a pore diameter > 25 Å, allowing the release of both intracellular Ca\(^{2+}\), ATP, and potentially other physiologically relevant analytes.

To date, no study has investigated ATP release through Panx1 or CALHM1 at the extracellular surface, as no currently available technology, without the use of expensive and highly specialized imaging equipment (Praetorius & Leipziger, 2009), is capable of monitoring ATP release at high spatiotemporal resolution. Here we demonstrate the first known attempt to fluorescently visualize ATP release at the extracellular surface through mPanx1 and hCALHM1 utilizing recombinantly produced ATP-sensitive fluorescent biosensors. Purified recombinant oligohistidine-tagged biosensors were localized to the extracellular surface using glycocalyx engineering and a novel organic compound, Ni\(^{2+}\)-tris-NTA-PEG\(_4\)-DBCO. Two ATP-sensitive GFP-based biosensors were chosen and characterized in vitro and in situ: the FRET-biosensor AT1.03\(^{YEMK}\) (Imamura, 2009), and the ratiometric biosensor, iATPSnFR (Lobas et. al., 2019).
Materials and Methods

Chemicals

EGTA (32-462-625GM), Zn$^{2+}$Cl$_2$ (208086-5G), Gd$^{3+}$Cl$_3$ hexahydrate (G7532-5G), Ruthenium Red (RR) (557450-250MG), ATP (A1852-1VL), BzATP (5057340001), ADP (01905-250MG-F), AMP (01930-5G), adenosine (A9251-5G), UTP (94370-250MG), UDP (94330-100MG), pyrophosphate (71501-100G), and cAMP (A9501-1G) were purchased from Sigma Aldrich. BAPTA (AAB2197106) and imidazole (AC122021000) were purchased from Thermo Fisher. Ac$_4$ManNAz was synthesized as described previously (Laughlin & Bertozzi, 2007). Synthesis and purification of Ni$^{2+}$-tris-NTA-PEG$_4$-DBCO was described in the Chapter II.

DNA constructs and design

Primers and synthetic constructs were manufactured by Integrated DNA Technologies (ITD). PCR products were amplified using NEBNext High-Fidelity 2X PCR Master Mix. Restriction enzymes were purchased from NEB. PCR reactions were purified using QIAGEN’s MinElute PCR Purification Kit. Gel extracted PCR products were purified using QIAGEN’s QIAquick Gel Extraction Kit. Ligation was accomplished using T4 ligase (NEB) and buffer. All purified DNA and PCR products were sequenced by GeneWiz Corp (South Plainfield, NJ).
AT1.03\textsuperscript{YEMK}

pDR-GW AT1.03\textsuperscript{YEMK} (plasmid #28004) was purchased from Addgene and several derivative constructs were designed, synthesized, and recombinantly produced. Because the monomeric super-enhanced cyan fluorescent protein (mseCFP) and monomeric Venus (mVenus) sequences of the biosensor are 98% identical, the biosensor was PCR amplified in a two-step process and ligated into a pNCS backbone. First, mseCFP was PCR amplified using forward primers containing \textit{NdeI}-\textit{HIS}\textsubscript{10}-\textit{XhoI} sequences (5’-ATATA\textit{CATATG}CATCATCATCATCATCATCATCATGCCCC\textit{CGAGTATG}ATGGTGA GCAAGGGCGAGGAGCTGTTCACCGGG-3’), and reverse primers overlapping the \textit{EcoRV} restriction sequence of ε subunit of \textit{F}_{0}\textit{F}_{1}-\textit{ATP synthase}, with the addition of a \textit{HindIII} (5’-CCATCTCG\textit{CATATC}AGCGTCGTAGACTGAAGCTTGCCCA-3’; 809 bp product) for pNCS vector ligation. mVenus was similarly amplified using forward primers overlapping the ε subunit (5’-CTACGACGCT\textit{CATATC}AGATGGTGTCCG AGGGCC-3’) and reverse primers including \textit{HindIII} restriction sites (5’- GGATC\textit{AAGCCTT}ACTCGATGTTCTGGC CGGATCTTTGAAGTTGG-3’; 1109 bp product). Each PCR product was run separately on a 1% agarose gel, extracted, and purified. pNCS vector and mseCFP PCR product were separately digested using \textit{NdeI} and \textit{HindIII} enzymes, purified, and ligated according to standard procedures. The mVenus PCR product was then digested and ligated into pNCS-\textit{NdeI-HIS}\textsubscript{10}-\textit{XhoI}- mseCFP-ε subunit-\textit{EcoRV-HindIII} using \textit{EcoRV} and \textit{HindIII} restriction sites and
sequenced. The addition of \((\text{TM}+\text{T7})_n\) and all \(\text{SER}_n\) \((n = \text{number of tandem repeats})\) linkers between \(\text{HIS}_{10}\) and \(\text{AT1.03}^{\text{YEMK}}\) was accomplished by PCR amplification of synthetically produced sequences (IDT) possessing unique 5’ and 3’ ends for efficient amplification using forward and reverse primers containing \(XhoI\) restriction sites in each. The resulting FRET constructs synthesized are:

1. pNCS-HIS\(_{10}\)-AT1.03\(_{\text{YEMK}}\)
2. pNCS-HIS\(_{10}\)-(T7-TM)\(_1\)-AT1.03\(_{\text{YEMK}}\)
3. pNCS-HIS\(_{10}\)-(T7-TM)\(_2\) -AT1.03\(_{\text{YEMK}}\)
4. pNCS-HIS\(_{10}\)-SER\(_{20}\)-AT1.03\(_{\text{YEMK}}\)
5. pNCS-HIS\(_{10}\)-SER\(_{40}\)-AT1.03\(_{\text{YEMK}}\)
6. pNCS-HIS\(_{10}\)-SER\(_{60}\)-AT1.03\(_{\text{YEMK}}\)

\textit{Ruby-iATPSnFR}

The Ruby-iATPSnFR plasmid was kindly gifted by the Looger Lab. The biosensor DNA sequence was PCR amplified using a forward primer containing \(XhoI\) and reverse primer containing \(HindIII\) restriction sites. The product was digested and ligated into pNCS-HIS\(_{10}\)-\(XhoI\)-\(HindIII\) and sequenced.
**mP2Y2/4-BFP**

The protein coding region of the mRNA sequences of murine P2Y$_2$ (NCBI Reference Sequence: NM_001302346.1) and P2Y$_4$ (NCBI Reference Sequence: NM_020621.4) were synthetically produced as ssDNA sequences by IDT. The P2Y$_2$ DNA construct was PCR amplified with primers (forward: 5’-ATCCGCTAGCATGGCAGCAGACCTGGAACCTGGGAATAGC-3’; reverse: 5’-GTCGACTGCAAGATTCCTAGCCGAATGTCCCTTAGTCTCACTTCCA-3’) containing NheI and EcoRI restriction sites, cloned into a pEBFP2-N1 (Addgene plasmid #54595) vector, and sequenced. The P2Y$_4$ DNA construct was PCR amplified using primers (forward: 5’-TAAAGCTAGCATGACCAGTGCGAGACTCCTTGGCTATTCACATCACT-3’; reverse: 5’-GCGAAGCTTTAATCGGTCAACCCTCATAAGCAGGGAAGATGC-3’) containing NheI and HindIII restriction sites, cloned into the pEBFP2-N1 vector, and sequenced.

**mPannexin1-mCherry (mPanx1)**

The protein coding region the mRNA sequence for murine Panx1 (NCBI Reference Sequence: NM_019482.2) was synthetically produced as a ssDNA sequence by IDT. The DNA sequence was PCR amplified using primers (forward: 5’-AGCTCAAGCTTTAGGCCATCGCCCACTTGCCACCGA-3’; reverse: 5’-CAGAATTCCGCAGGGATCATTCAGAAGCTCCTGCG-3’) containing HindIII and EcoRI restriction sites, cloned into mCherry2-N1 (Addgene plasmid #54517), and sequenced.
**hCALHM1-mCherry and hCALHM3-BFP**

hCALHM1 was kindly gifted by Jorge E. Contreras, Ph.D. (UC Davis). The protein coding sequence was PCR amplified using primers (forward: 5’-AGATCCGCTAGCATGGGACAAGTTCCGGATGATC-3’; reverse: 5’-GACTGCAAATTGCACTTTGCTGAAGTAGGTGGCC-3’) containing *NheI* and *EcoRI* restriction sites, cloned into mCherry2-N1 (Addgene plasmid #54517), and sequenced.

hCALHM3 (NCBI Reference Sequence: NM_001129742.2) was synthetically produced by IDT, PCR amplified using primers (forward: 5’-AGATCCGCTAGCATGGGATAAGTTGGATGCTGGTCCAACACT-3’; reverse: 5’-TGCAGAATTGCAGACATCAGTGTGCTGTGAGAGTCTTGTCCC-3’) containing *NheI* and *EcoRI* restriction sites, cloned into pEBFP2-N1, and sequenced.

pCMV-GCaMP5G (Plasmid #31788) was purchased from Addgene.

**Protein expression and purification**

Protein production of pNCS-HIS10-AT1.03YEMK, and its derivatives, and pNCS-HIS10-Ruby-iATPSnFR was identical. Briefly, plasmids were transformed into *E. coli* BL21 (DE3). A single colony was inoculated in 1 L of LB medium with 100 mg/mL ampicillin (without IPTG) and shaken in the dark for 72 h. Cells were collected by centrifugation at 5000 rpm (4°C) for 20 min and disrupted by sonication in 10 mL of 20
mM Tris-HCl buffer pH 8.0. A cell-free extract was obtained by centrifugation of sonicated product at 10,000 rpm (4°C) for 40 min. Proteins were purified using a Nickel resin (HisPur™ Ni-NTA from Thermo Fisher), dialyzed (3,000 MWC Thermo Fisher) in 20 mM Tris-HCl 150mM NaCl (pH 8.0) for 16 h (4°C), and concentrated using 10,000 NMWL Amicon Ultra – 15 centrifugal filters according to the manufacturer’s specifications. Protein purity was determined by SDS-PAGE. Protein concentration was determined using the BCA method. All purified proteins were stored in 20 µL aliquots at -20°C until used.

**In vitro nucleotide titrations**

Stock concentrations of nucleotide (ATP, ADP, AMP, UTP, etc.) were made in external solution, pH adjusted (7.5), and diluted ½ or 1/3 using external solution (pH 7.5), resulting in concentrations ranging ~0.001 µM – 200 µM. 50 µL of purified biosensor was resuspended in 950 µL external solution. 75 µL of serially diluted nucleotide was added in triplicate to white walled clear-bottom 96-well plates (Corning), followed by the addition of 25 µL of 1:20 diluted purified biosensor (100 µL final volume per well). Final nucleotide concentrations were calculated as ¾ serially diluted concentration. Scanned and single wavelength emission profiles were read on a Tecan Safire spectrophotometer using 3 nm wavelength steps, 5 nm excitation bandwidth, 5 nm emission bandwidth, gain = 100, number of flashes = 10, and 500 µs integration time.
Cell culture, transfections, and cell surface labeling

Chinese Hamster Overy-K1 (CHO) cells were cultured in F-12K nutrient mixture (Invitrogen); HEK cells were cultured in high glucose DMEM medium. All media was supplemented with 10% fetal bovine serum (Hyclone) and 100 units/mL penicillin/streptomycin (Invitrogen). Cells were plated at 60-75% confluency in 35 mm glass bottom culture dishes (MatTek). After 24 h, cells were transiently transfected with 1 µg of ion channel or transporter DNA and 8 µL of Lipofectamine (Invitrogen) for CHO cells or 4 µL of Lipofectamine and 6 µL PLUSTM reagent (Invitrogen) for HEK cells in Opti-MEM (Invitrogen). After terminating the transfections, the cells were incubated in media containing 50 µM azido-sugar for 2 d, which was replenished after 24 h. On the day of experiments, media was removed from glass bottom dishes, rinsed with RT 1x PBS, and labeled with 50 µM Ni²⁺-tris-NTA-PEG₄-DBCO in opti-MEM media for 30 minutes at 37°C. Cells were then rinsed with RT 1X PBS and labeled with 800 nM oligohistidine-tagged fluorescent protein or biosensor in opti-MEM media at RT for 15 minutes in the dark. Cells were finally resuspended in external solution (150 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPEs, pH 7.5) just prior to imaging.

Calcium imaging (pCMV-GCaMP5G)

\[ mP2Y_{2,4}-BFP + mPanx1-mCherry \]

HEK293T cells were seeded (20,000 cells) and cultured on to 35 mm glass bottom dishes until 70-80% confluent before being transiently transfected with a total of
4 µg of DNA and 10 µL Lipofectamine 2000 for 4-6 hours. Media was replaced and the cells were cultured for 24 hrs. Just prior to calcium imaging, cells were rinsed with RT 1x PBS and resuspended in 2 mL of external solution. BFP, GFP, and mCherry co-transfected cells were identified under 63x magnification using separate excitation and emission wavelengths and filter sets specific to each fluorophore. 20 mL of 10 µM UTP was perfused starting at 1 min and calcium (GFP) responses recorded. GCaMP5G was imaged at 1 Hz, 50 ms exposure, and 460 nm excitation.

\textit{hCALHM1-mCherry}

Identical culturing and transfection conditions were followed as described previously. Briefly, HeLa cells were co-transfected with pCMV-GCaMP5G and hCALHM1-mCherry for 24 hrs prior to experiments. Cells were perfused with 20 mL of external solution (in mM) (150 NaCl, 2.5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, 10 D-glucose, pH 7.5), Ca$^{2+}$-free (150 NaCl, 2.5 KCl, 1 MgCl$_2$, 10 HEPES, 10 D-glucose, pH 7.5), Ca$^{2+}$/Mg$^{2+}$-free (150 NaCl, 2.5 KCl, 10 HEPES, 10 D-glucose, pH 7.5), or 5 mM EGTA (150 NaCl, 2.5 KCl, 5 mM EGTA, 10 HEPES, 10 D-glucose, pH 7.5). Co-transfected cells were identified separately using GFP and mCherry excitation and emission wavelengths and filter sets. GFP images were collected every 5 seconds (460 nm excitation, 50 ms exposure).
In situ nucleotide titrations

Ni\textsuperscript{2+}-tris-NTA-PEG\textsubscript{4}-DBCO and biosensor-labeled cells were resuspended in 2 mL of external solution in glass-bottom 35 mm dishes and mounted on to either a Zeiss Axiovert 40 CFL or a Zeiss 200M microscope (MatTek). A gravity-fed perfusion system was adapted to each microscope to allow 20 mL of fluid, per nucleotide concentration, to fill the 35 mm dish to a total volume of 2 mL over 1.5 minutes. Excess fluid was vacuumed during perfusion, allowing a volume of no greater than 2 mL to fill the dish. After each nucleotide concentration was perfused (lowest concentration to highest), 5-10 images were collected as a z-stack (50 ms exposure, 100 ms collection rate) and averaged.

Bioluminescence ATP Assay

Extracellular ATP levels were measured using the luciferin-luciferase assay (FL-AAM, Sigma Aldrich). HeLa or HEK293 cells were seeded onto white or clear-bottomed 96-well microplates (Corning) at a cell density of 20,000 per well one day prior to transfection. For mP2Y\textsubscript{2/4}-BFP, mPanx1-mCherry experiments, cells in each well were transfected with 0.2 µg total DNA (0.1 µg purinergic receptor + 0.1 µg channel) or mock vector (pEBFP2-N1) using 0.4 µL Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. For experiments involving hCALHM1-mCherry and hCALHM3-BFP, cells in each well were transfected for 4-6 hours using 0.2 µg total DNA or empty vector, with Lipofectamine 2000. After 4-6 hours, media was removed and cells were incubated overnight in cell-appropriate media. 1 h prior to the start of the
experiment, media was removed and cells were rinsed with RT 1x PBS (containing 2 mM Ca\textsuperscript{2+}) and resuspended in 100 µL external solution (150 mM NaCl, 2.5 mM KCl, 2 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 10 mM glucose, 10 mM HEPEs, pH 7.5) at 37ºC. If the experiment required the addition of drug or metal inhibitor (Zn\textsuperscript{2+}, Gd\textsuperscript{3+}, etc.), it was added during the 1 hr incubation period. For experiments with mP2Y\textsubscript{2/4}-BFP and mPanx1-mCherry stimulated with 10 µM UTP, just prior to the start of the experiment, 75 µL of external solution was removed, followed by the addition of 75 µL of 13.3 µM UTP (in external solution). For experiments involving hCALHM1/3, just prior to the start of the experiment, 75 µL of external solution was removed, followed by the addition of chelator (5 mM EGTA, 5 mM BAPTA) ± inhibitor. The plate was then immediately placed in a microplate luminometer (EnVision 2104 Multilabel reader, PerkinElmer) and 10 µL of ATP assay solution was dispensed into each well. ATP release was measured every 2 minutes. ATP concentrations were calculated from a standard curve created in each plate containing identical cell-treated conditions, including experiments involving drugs or inhibitors.

**Cell imaging of Ruby-iATPSnFR**

Biosensor-labeled cells (see *Cell culture, transfections, and cell surface labeling*) were imaged on an Axiovert 40 CFL microscope equipped with an Andor Zyla 4.2 megapixel sCMOS high-resolution camera and CoolLED pE-4000 multi-wavelength excitation light source. The microscope was equipped with separate filter sets: EBFP (31041, Chroma); GFP (49002, Chroma); ECFP/EYFP/mCherry (69008, Chroma). For
experiments involving mP2Y\textsubscript{2/4}-BFP and mPannexin1-mCherry, receptor and channel co-expression was first identified using excitation wavelengths for each using the appropriate filter set. For experiments involving hCALHM1-mCherry, transfected cells were identified by excitation at 580 nm and the RFP filter set. Ruby-iATPSnFR was imaged under 63x magnification using the GFP filter set and excited using 470 nm. Images were captured using 50 ms light exposure at 0.5 Hz and 4x4 binning (ImageJ/\textmu Manager). hCALHM1-mCherry experiments were imaged every 5 seconds. Collected images were analyzed using ImageJ software.

**Cell imaging of AT1.03\textsuperscript{YEMK}**

Biosensor-labeled cells (see *Cell culture, transfections, and cell surface labeling*) were imaged under 63x magnification on an Axiovert 200M microscope equipped with a CFP/YFP filter cube (59217, Chroma) and excited using a CoolLED pE-4000 multi-wavelength excitation light source at 435 nm. Emission profiles of CFP and YFP were beam split and collected separately on two heat sunked Chameleon3 USB CMOS imaging sensors. Collected images were analyzed using ImageJ software (see Figure 3-3 for analysis process).

**Statistics**

All statistics were performed using one-way ANOVA and students one- or two-tailed t-tests with GraphPad Prism Version 9.2.0.
3-D protein models

3-dimensional models were downloaded from the Protein Databank (PDB).

Molecular graphics and analyses were generated using UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311.
Results

Characterization of ATeam FRET biosensor

Of the available GFP-based ATP-sensing biosensors, two were chosen to further investigate the function of at the cell surface. The ATeam series of biosensors (Imamura, 2009) are fluorescence resonance energy transfer (FRET)-based biosensors that utilize the ε subunit of the bacterial F₀F₁-ATP synthase. Several advantages to utilizing the ε subunit is its ability to bind ATP while not hydrolyzing it, and its specificity for ATP over other nucleotides. The ε subunit is one of the smallest ATP-binding proteins and is composed of one N-terminal β-barrel domain and two C-terminal α-helices. The resulting large conformational change upon ATP binding make it a more-than-ideal substrate for the construction of a FRET biosensor, thus, when chimerically joined at the N-terminus to monomeric super enhanced cyan fluorescent protein (mseCFP), and C-terminally to monomeric Venus (mVenus) (Figure 3-1a), the maximum ratiometric change over baseline between 527 nm (mVenus) and 475 nm (mseCFP) in the presence of ATP is more > 4 and a K_D = 273 ± 15 µM. We hypothesized that by recombinantly producing an oligohistidine-tagged form of the ATP-sensing FRET biosensor (HIS₁₀-AT1.03_YEMK) and localizing it to the extracellular surface using glycocalyx engineering and Ni²⁺-tris-NTA-PEG₄-DBCO, we would be capable of sensing extracellular ATP and visualize its release through large-pore hemichannels in mammalian cells in real-time.
Figure 3-1 Cloning strategy for FRET biosensors

(a) Cartoon schematic of AT1.03YEMK. FRET induction occurs upon association/dissociation of ATP with the ε subunit of *Bacillus subtilis* F₀F₁-ATP synthase (PDB: 2E5Y). The conformational change due to ATP binding of the chimeric protein results in the acceptor fluorophore (mVenus) localizing within the Förster radius.
(typically 3-6 nm) of the donor (mseCFP) fluorophore, leading to enhanced acceptor fluorescence. ATP concentrations are determined as the ratio of mVenus:mseCFP. (b) AT1.03\textsuperscript{YEMK} was cloned into a pNCS vector backbone in a two-step process, making use of the \textit{EcoRV} restriction site within the ATP-sensing \( \varepsilon \) subunit. Derivative constructs containing \((\text{TM+T7})_n\) and \(\text{SER}_n\) (\(n = \# \) of SER repeats) domains were cloned utilizing the \textit{XhoI} restriction site. All constructs were sequenced to verify insert directionality. All constructs were recombinantly produced in \textit{E. coli} (DE3/BL21) and purified.
AT1.03\textsuperscript{YEMK} was acquired through the Addgene vector database and cloned into a pNCS vector backbone possessing an N-terminal HIS\textsubscript{10} tag by utilizing a two-step cloning strategy (Figure 3-1b). The purified recombinantly produced protein (see Materials and Methods) was first characterized \textit{in vitro} using serial dilutions of ATP ranging from 0.01 µM – 5 mM. In solution, the biosensor responded nominally to ATP (Figure 3-2a), exhibiting robust FRET induction upon nucleotide binding. While the focus of our investigation was not to fully characterize the biosensor in response to various nucleotides as previously described by Imamura, we did however seek to determine whether the biosensor, while localized to the extracellular surface using our unique approach, behaved \textit{in situ} similarly to that seen \textit{in vitro}. To investigate this further, Figure 3-2b shows the theoretical fluorescence profile of mseCFP and mVenus, through their respective emission filters, in the presence and absence of 1 mM ATP. The CFP emission filter collects light between 461 to 486 nm, while the YFP filter collects light between 526 to 552 nm, thus by using a 3 nm binning width, multiplied by the relative fluorescence intensity at each wavelength, and comparing the ratios of mVenus:mseCFP (R) in the presence and absence of ATP, the calculated \( \Delta R_{\text{ATP}}/R_{\text{noATP}} = 3.22 \) (Figure 3-2c). By fully integrating the collected light we find a \( \Delta R_{\text{ATP}}/R_{\text{noATP}} = 3.25 \). These theoretically determined changes in mVenus:mseCFP ratios are the expected results as collected through a FRET microscope while HIS\textsubscript{10}-AT1.03\textsuperscript{YEMK} is cell-surface attached.
Figure 3-2 FRET biosensor response in vitro

(a) Recombinantly produced HIS$_{10}$-AT1.03$^\text{YEMK}$ behaved nominally in solution; $K_D = 278.5 \pm 15.3$ µM (mean ± s.e.m.). (b) Predicted emission profile of mseCFP and mVenus in the presence and absence of ATP as emitted, filtered, split, and collected on separate sCMOS cameras. (c) Estimation of ratiometric change between mVenus:mseCFP of emission intensity at each wavelength. Estimated $\Delta R/R_0 = 3.22$. (d) Estimation of ratiometric change between mVenus:mseCFP ± ATP by integration. $\Delta R/R_0 = 3.25$. 
We thus sought to experimentally validate these predictions. We did so by culturing Chinese hamster ovary cells on glass-bottomed 35 mm dishes for 48 hours in the presence of 50 µM Ac₄ManNAz. After 48 hours, cells were labeled with 50 µM Ni²⁺-tris-NTA-PEG₄-DBCO, 800 nM HIS₁₀-AT1.03⁷EMK (see Materials and Methods for detailed labeling procedure) and viewed under a FRET-equipped microscope. Image stacks were collected after each perfusion of ATP (lowest concentration to highest). Figure 3-3 illustrates the process of image collection and analysis. Briefly, image stacks corresponding to each ATP concentration were separately collected, split, and averaged. Cellular regions of interest (ROIs) were automatically detected using intensity thresholding and overlayed on CFP and Venus. The resulting ratiometric changes (mVenus:mseCFP) were based on the fluorescence intensity of each fluorophore with increasing ATP concentration. Figure 3-4b shows the results and comparison between in vitro and in situ ATP response of HIS₁₀-AT1.03⁷EMK. In vitro, the biosensors’ 527/475 ratio in the presence of 1 mM ATP = 4.5, but no ratiometric changes occurred when the biosensor was cell-attached. However, when HIS₁₀-AT1.03⁷EMK was resuspended in external solution and viewed under a FRET microscope in the presence and absence of ATP (3-4b inset), the predicted ratiometric change $R_{\text{no ATP}} \approx 1$ and $R_{\text{ATP}} \approx 3.3$ occurred, concluding that the newly-built FRET microscope operated as expected. We hypothesized that this observation could be explained by the inhibition of the ε subunit “closing” upon ATP binding due to steric effects while cell-attached, e.g. biosensors in close proximity to neighboring glycocalyx sugars inhibit conformational changes. To circumvent this issue, we hypothesized that by increasing the length between the HIS₁₀-
tag and the FRET biosensor with long and/or flexible polypeptide linker sequences, the biosensors’ dynamic movement would mirror that found *in vitro* (in fluid).
Following perfusion of 20 mL of each ATP concentration, 5-10 images were collected (a), channel split (b), and averaged (c). (d) Cells are computationally identified by thresholding relative to background. (e) Due to a minor misalignment of our camera setup, x,y translation was performed for perfect alignment. Particles were automatically detected (f) and overlayed (g) onto averaged fluorescent images and quantified. Image size = 8,500 µm².
Several derivative constructs possessing peptide linkers between the HIS\textsubscript{10} and AT1.03\textsuperscript{YEMK} domains were synthesized and recombinantly produced. Linkers ranged from 26 to 66 amino acids and varied in composition, flexibility, and serine content. Two of the five constructs contained either a single or duplicate copy of tandem T7+TM tags (Figure 3-1b), as is utilized is surface displayed (pDisplay) iGluSnFR (Marvin et. al., 2013). Three additional constructs possessing increasing serine composition were also produced. Van Rosmalen et. al. (2017) convincingly demonstrated that when two FRET pairs (CFP and YFP) are tethered at either end of long and flexible polyserine linkers sequences, their FRET efficiencies $\langle E \rangle$ are proportional to both their “persistence length” ($l_p$) and serine composition percent (%). Thus, $\langle E \rangle$ decreases with increasing linker length and serine composition.

Based on this finding, DNA sequences of HIS\textsubscript{10}-SER\textsubscript{20}-AT1.03\textsuperscript{YEMK}, HIS\textsubscript{10}-SER\textsubscript{40}-AT1.03\textsuperscript{YEMK}, HIS\textsubscript{10}-SER\textsubscript{60}-AT1.03\textsuperscript{YEMK} were cloned (Figure 3-1b). Recombinant expression of these constructs in \textit{E. coli} yielded only two of the three, as HIS\textsubscript{10}-SER\textsubscript{60}-AT1.03\textsuperscript{YEMK} was incapable of being purified, likely due to bacterial protease cleavage of the long SER\textsubscript{60} linker during the purification process, despite the addition of protease inhibitors (Figure 3-4a). Nevertheless, although the constructs capable of being purified performed nominally \textit{in vitro} (Figure 3-4b), when each construct possessing (TM+T7)$_n$ or SER$_n$ linkers were bound to the cell surface, no FRET induction occurred in the presence of ATP (data not shown), and mimicked identically the behavior of HIS\textsubscript{10}-AT1.03\textsuperscript{YEMK}. The reasons for this phenomenon are still not well understood and requires further investigation. To date, no FRET biosensor, ATP-sensing or otherwise, has been
successfully localized to the extracellular surface. Our novel attempt at doing so was the first of its kind but yielded nebulous results. Although our attempts to fluorescently visualize ATP at the cell surface using a FRET-based biosensor was unsuccessful, other ATP-sensing, GFP-based biosensors are available.
Figure 3-4 *In vitro vs. in situ* response of AT1.03\textsuperscript{YEMK} constructs

(a) SDS-PAGE of NTA-agarose purified (1) HIS\textsubscript{10}-AT1.03\textsuperscript{YEMK}, (2) HIS\textsubscript{10}-SER\textsubscript{20}-AT1.03\textsuperscript{YEMK}, (3) HIS\textsubscript{10}-SER\textsubscript{40}-AT1.03\textsuperscript{YEMK}, (4) HIS\textsubscript{10}-SER\textsubscript{60}-AT1.03\textsuperscript{YEMK}. (b) *In vitro* ATP titration of HIS\textsubscript{10}-AT1.03\textsuperscript{YEMK} and derivative constructs. In solution, ratiometric changes were calculated as the change in fluorescence intensity between 527 nm and 475 nm. When cell-attached and imaged on a FRET-equipped microscope, no ratiometric change was observed with increasing concentration of ATP in any FRET protein (HIS\textsubscript{10}-ST1.03\textsuperscript{YEMK} shown). (inset) HIS\textsubscript{10}-AT1.03\textsuperscript{YEMK} was resuspended in 500 µL external solution in a 35 mm glass-bottom dish and viewed under a FRET microscope in the presence and absence of ATP. Ratiometric changes between mVenus and mseCFP occurred as predicted.
Characterization of mRuby-iATPSnFR in vitro and in situ

mRuby-iATPSnFR is an intensio- and ratio-metric GFP-based biosensor that utilizes the same microbial $F_0F_1$-ATP synthase $\varepsilon$ subunit found in the ATeam series of FRET biosensors (Lobas et. al., 2019). It is constructed from circularly permuted superfolder GFP (cpSFGFP) inserted between the two $\alpha$-helices of the $\varepsilon$ subunit, with mRuby fused to the N-terminus of the $\beta$-barrel domain of $\varepsilon$ subunit (Figure 3-5a). In vitro, the GFP fluorescence intensity of recombinantly produced HIS$_{10}$-mRuby-iATPSnFR increased with increasing ATP concentrations (Figure 3-5b), while the red fluorescence of mRuby did not (Figure 3-5c-d). To determine whether HIS$_{10}$-mRuby-iATPSnFR would be able to detect ATP changes at the cell surface, CHO cells were seeded onto 35 mm glass-bottom dishes and cultured for 48 hours in Ac$_4$ManNAz. Cells were then labeled with 50 $\mu$M Ni$^{2+}$-tris-NTA-PEG$_4$-DBCO and 800 nM HIS$_{10}$-mRuby-iATPSnFR and viewed under a fluorescence microscope. By monitoring the GFP emission profile, clear increases in fluorescence occurred in the presence of ATP: in vitro $K_D = 1.58 \pm 0.52$ $\mu$M; in situ $K_D = 0.53 \pm 0.06$ $\mu$M (Figure 3-5e). The 3-fold reduction in $K_D$ when cell-attached is a phenomenon like that found when comparing HIS$_{10}$-iGluSnFR in vitro and in situ, but less pronounced. When cell attached, HIS$_{10}$-mRuby-iATPSnFR also responded to BzATP, and ADP, but not other nucleotides, including UTP, UDP, pyrophosphate, and cAMP (Figure 3-5f). These results indicated that cell-attached HIS$_{10}$-mRuby-iATPSnFR could potentially sense ATP egress from an appropriate experimental paradigm, given that the local concentration of ATP at the cell surface falls within the detection range of the biosensor. We thus chose to demonstrate the utility of our surface-
labeling approach using two well-known mechanisms of ATP release: purinergic receptor (P2Y)-stimulated, UTP-induced ATP release through pannexin1 (Panx1) channels, and ATP egress through Ca\(^{2+}\)-sensitive human calcium homeostasis modulator 1 (hCALHM1) channels.
Figure 3-5 HIS\textsubscript{10}-Ruby-iATPSnFR characterization

(a) Cartoon schematic of mRuby-iATPSnFR. (b) *In vitro* response to ATP. GFP emission profile of Ruby-iATPSnFR and (c) stable RFP emission in response to ATP titrations. (d) Calculated fold-change of GFP and RFP emission in response to ATP. ($n = 4$ wells/titration). (e) Comparison between *in situ* and *in vitro* ATP response. When cell attached, the effective $K_D$ HIS\textsubscript{10}-mRuby-iATPSnFR is nearly 3x lower ($n = 3$ dishes; 10-15 cells/dish). (f) Relative fold-change of cell-attached HIS\textsubscript{10}-AT1.03\textsuperscript{YEMK} in response to different nucleotides ($n = 3$ dishes/nucleotide; 10-15 cells/dish).
**ATP egress through mPannexin1**

Pannexins are a family of large-pore forming, integral membrane hemichannels that release ATP due to mechanical stress (Bao et. al., 2004), cytoplasmic calcium increases (Bao et. al., 2004), membrane depolarization (Ma et. al., 2009), C-terminal cleavage via caspase (Gulbransen et. al., 2012), and P2X (Pelegrin & Surprenant, 2006) and P2Y (Locovei et. al., 2006) purinergic receptor activation. Pannexin1 (Panx1) is the most widely studied member of the pannexin family and is ubiquitously expressed throughout the body, particularly within the central nervous system (CNS), and plays crucial roles in physiological processes such as apoptosis progression (Chekeni et. al., 2010), neuropathic pain (Bravo et. al., 2014; Weaver et. al., 2017), neuroinflammation (Garg et. al., 2018; Wu et. al., 2017), and blood pressure regulation (Billaud et. al., 2011).

To determine whether our approach to biosensor surface labeling could appreciably detect Panx1-dependent ATP release events, a carefully chosen experimental paradigm was developed. One of the most rigorously investigated mechanisms of Panx1-dependent ATP release is P2X$_7$ purinergic receptor activation, a major consequence of which is the influx of Ca$^{2+}$ through the non-selective channel (North et. al., 2013; Kaczmarek-Hájek et. al., 2012; Liang et. al., 2015). However, the P2X family of ionotropic receptors are activated by ATP ($EC_{50}$ ~ 5.5 mM) and the potent prototypic agonist 2′(3′)-O-(4-benzoylbenzoyl) adenosine-5′-triphosphate (BzATP) (Xu et. al., 2012), both of which stimulate mRuby-iATPSnFR at physiologically relevant concentrations. As an alternative, the rhodopsin-like G protein-coupled family of P2Y receptors can be activated by a myriad of other agonists, including UTP and UDP, neither
of which influence the fluorescence intensity of surface localized HIS$_{10}$-mRuby-iATPSnFR. Of the eight characterized P2Y receptors, P2Y$_2$ and P2Y$_4$ endogenously respond to UTP, and activate Panx1 hemichannels following intracellular Ca$^{2+}$ increases in osteocytes (Kringelbach et. al., 2014), carotid body type II (Zhang et. al., 2012), HeLa (Okuda et. al., 2003), and HEK293 cells (Ma et. al., 2009). We hypothesized that co-expression of P2Y receptors and Panx1 channels would lead to intracellular calcium increases and ultimately ATP release. Other GPCR-mediated mechanisms of Panx1 activation were not considered because no evidence in the literature was found to suggest that such approaches would lead to ATP release, and it was clear from the characterization of mRuby-iATPSnFR that UTP would not adversely affect the biosensor, suggesting the approach we chose was inherently compatible. Cellular transfection of constructs was also the preferred approach due to its simplicity and minimal adverse effects on cells. Here, cells are transiently transfected with an inexpensive, commercially available reagent, and 24-48 hours later, receptor and channel expressing cells are clearly identified due to their BFP or mCherry fluorescence. More complex methods such as piggybac (Wilson et. al., 2007) or lentiviral transduction (Elegheert et. al., 2018) combined with FACS was not necessary to identify cells co-expressing our genes of interest.

To this end, murine P2Y$_2$ and P2Y$_4$ were cloned and C-terminally tagged with enhanced blue fluorescent protein (EBFP) to allow their visualization. Murine Panx1 was also cloned but C-terminally tagged with mCherry, and all constructs were expressed in HEK293T cells. As a result, receptors and hemichannels displayed predicted surface
expression (Figure 3-6a). To determine whether receptor activation by UTP leads to previously observed intracellular Ca\(^{2+}\) increases, HEK293T cells were co-transfected with GCaMP5G, a green, fast-acting, GFP-based intracellular calcium indicator developed specifically for neuronal imaging (Akerboom et. al., 2012). Combinations of mP2Y\(_2\)-EBFP, mP2Y\(_4\)-EBFP, mPanx1-mCherry, and GCaMP5G were expressed in HEK293T cells and perfused with 10 µM UTP (Figure 3-6b), leading to significant cytoplasmic Ca\(^{2+}\) increases, and are either the result of intracellular Ca\(^{2+}\) store release, extracellular influx through Panx1, or both. However, when any receptor or hemichannel was expressed alone with GCaMP5G, cytoplasmic Ca\(^{2+}\) increases did not occur or were undetectable (Figure 3-6c). To determine whether this phenomenon is correlated with ATP release, the luciferin-luciferase assay was used.
Figure 3-6 P2Y and Panx1-dependent Ca^{2+} response to UTP

(a) mP2Y_2-BFP, mP2Y_4-BFP, and mPanx1-mCherry expression in HEK293T cells. Scale bar, 25 µm. (b) Cells co-transfected with purinergic receptor and Panx1 with pCMV-GCaMP5G showed rapid increases in intracellular calcium levels (Ca^{2+})_i levels in response to 10 µM UTP within seconds. (c) Quantification of intracellular calcium increases in response to 10 µM UTP. n = 4 dishes/condition (7-10 cells/dish) (Mean ± s.e.m. One-way ANOVA. Students unpaired two-tailed t-test, *P<0.05, **P<0.01, ***P<0.001).
The luciferin-luciferase assay is a common method for measuring extracellular ATP accumulation from cells. The conversion of D-luciferin to light-producing oxyluciferin by firefly luciferase is an O₂, Mg²⁺, and more importantly, ATP-dependent mechanism, and light production (bioluminescence) is directly correlated with ATP concentrations (Figure A-11). To understand how UTP stimulation affects extracellular ATP accumulation using this assay, HEK293T cells were seeded at a density of 20,000 cells per well in white walled, clear bottom, 96-well plates for 24 hours. The next day, cells were transiently transfected with combinations of mP2Y₂-EBFP, mP2Y₄-EBFP, mPanx1-mCherry, or mock (eBFP) for 4-6 hours (n = 8 wells/transfection condition). After 16-24 hours, cells were stimulated with ± 10 µM UTP immediately prior to the addition of 10 µL bioluminescent assay reagent (110 µL total volume) and read on a luminometer every 2 min for 38 min total. The time from UTP addition to light measurement is < 2 min. Figures 3-7a-d illustrates the results. The addition of 10 µM UTP led to immediate and measurable extracellular ATP release from HEK293T cells under all transfection conditions, with peak accumulation occurring at ~ 7 min averaging an increase of 745 ± 47 nM ATP (mean ± s.e.m.). However, no difference in ATP release between mock, Panx1 alone, or P2Y₂/Panx1 transfected cells was observed, with only a small increase in ATP release in P2Y₂ transfected cells. Similar results were observed in P2Y₄/Panx1 transfected cells. However, P2Y₄ and P2Y₄/Panx1 showed significant increases in ATP release compared to mock and Panx1 alone. Previous studies show that HEK293T cells endogenously express P2Y₂ receptors (Schachter et. al., 1997; Fischer et. al., 2005), including purinergic-activated, ATP-passing connexin channels (Tachikawa et.
al., 2020). These results indicate that endogenous mechanisms of ATP release in response to UTP are present in HEK293 cells and could potentially be observed via the attachment of surface-localized ATP biosensors in real-time at the extracellular membrane.
Figure 3-7 ATP release from HEK293T cells

(a) ATP release time-course of mP2Y<sub>2</sub>-eBFP and mPanx1-mCherry over 38 minutes. 10 µM UTP added -2 min. (b) Summary of results. (c) ATP release time-course of mP2Y<sub>4</sub>-eBFP and mPanx1-mCherry over 38 minutes. 10 µM UTP added -2 min. (d) Summary of results. (One-way ANOVA. Students unpaired two-tailed t-test, *P<0.05, **P<0.01, ***P<0.001).
To test the hypothesis that surface-attached biosensors could fluorescently visualize these ATP release events, HEK293T cells were seeded onto 35 mm glass bottom dishes, cultured in 50 µM Ac4ManNAz for 48 hours, transfected with combinations of mP2Y2-EBFP, mP2Y4-EBFP, mPanx1-mCherry, or EBFP (mock), surface labeled with 50 µM Ni²⁺-tris-NTA-PEG₄-DBCO and 800 nM HIS₁₀-mRuby-iATPSnFR, and perfused with 10 µM UTP (Figure 3-8). As a result, no fluorescent changes were observed in response to UTP stimulation. The results indicate that while the luciferin-luciferase assay can measure extracellular ATP accumulation from > 20,000 cells, surface-localized ATP-sensing biosensors with an affective K_D ~ 0.5 µM are incapable of detecting such release events from individual cells. This was the first known attempt to fluorescently visualize endogenous ATP release at the cell surface, but the results are clear, and suggests that the local extracellular concentration of ATP released under these conditions is below 100 nM during the maximum release rate (0 < t < 2 min). Although we were unable to detect ATP release under the experimental paradigm described, other large-pore hemichannels known to release ATP were worth investigating.
Figure 3-8 Cell-attached HIS\textsubscript{10}-Ruby-iATPSnFR is unable to detect ATP release from HEK293T cells

(a) HEK293T cells were co-transfected with combinations of purinergic receptor and Panx1, labeled with Ni\textsuperscript{2+}-tris-NTA-PEG\textsubscript{4}-DBCO and HIS\textsubscript{10}-Ruby-iATPSnFR, fluorescently visualized under 63x magnification, and perfused with 10 µM UTP at 2 minutes. (b) Quantification of maximum fluorescence change in response to 10 µM UTP. $n = \#$ of cells. (Mean ± s.e.m. Statistical differences were calculated by one-way ANOVA and students unpaired two-tailed t-test. *$P<0.05$, **$P<0.01$, ***$P<0.001$).
**CALHM1**

The human calcium homeostasis modulator 1 (hCALHM1) hemichannel is a unique transmembrane, calcium-sensitive, large-pore channel that was first identified in genetic linkage studies that recognized a nonsynonymous single-nucleotide polymorphism (SNP) rs2986017, which results in a proline-to-leucine substitution at codon 86, that was significantly increased in Alzheimer’s disease cases in five independent cohorts (Dreses-Werringloer et. al., 2008) and is highly expressed in the hippocampus. CALHM1 was since been found to also act as a conduit to G protein-coupled receptor-dependent ATP neurotransmitter release in taste bud cells (Ma et. al., 2018; Taruno et. al., 2013), mediates extracellular Ca\(^{2+}\) regulation of neuronal excitability (Ma et. al., 2012), and shares structural similarities to both pannexins and invertebrate innexins (Siebert et. al., 2013). Ma and colleagues (2012) found that when hCALHM1 was C-terminally tagged with GFP, overexpressed in HeLa cells, and activated by extracellular calcium removal with EGTA, significant ATP release occurred over 30 minutes (~15 nM). They also found that the combination of hCALHM1 + hCALHM3 led to increased ATP release (~35 nM) over the same period. For these reasons, we chose to test the hypothesis that localization of an ATP-sensitive biosensors could appreciably detect these ATP-release events. CALHM1 is an ideal candidate for such investigation due to the high level of ATP release and simple activation mechanism (calcium chelation).
**hCALHM1-dependent ATP release**

Human CALHM1 and CALHM3 were first cloned into mCherry2-N1 and pEBFP2-N1 vectors, respectively, and fluorescently visualized in transfected HeLa to validate hemichannel expression. The luciferin-luciferase assay was immediately used to determine if overexpression of either channel, or a combination thereof, led to ATP release. HeLa cells were thus seeded (20,000 cells per well) onto white-walled, clear-bottom 96-well plates and cultured for 24 hrs. The following day, hCALHM1-mCherry, hCALHM3-EBFP, hCALHM1-mCherry + hCALHM3-EBFP, and mock (pEBFP-N1) vectors were transiently transfected for 4-6 hrs and then cultured in the appropriate media. The following day, 1 hr prior to the start of the experiment, media was removed, and transfected cells were resuspended in 100 µL of external solution (see Materials and Methods) containing 2 mM Ca^{2+} and 1 mM Mg^{2+} and incubated at 37ºC. 75 µL of external solution was then removed from all wells (25 µL remained as to not stimulate hemichannel opening) and 75 µL of 5 mM EGTA was added immediately prior to the addition of 10 µL luciferin-luciferase assay reagent. The 96-well plate was then added to the luminometer, and bioluminescence readings were recorded every 2 minutes for 38 minutes total. Figure 3-9 illustrates the results. hCALHM1-mCherry alone and its co-expression with hCALHM3-EBFP showed significant ATP release (~165 ± 45 nM; mean ± s.e.m.) over 38 minutes, but did not significantly differ from one another. Why co-expression of CALHM1 with CALHM3 did not lead to increased ATP release over CALHM1 alone, as seen previously (Ma et. al., 2018), is not clear. It is also not apparent why, in our study, nearly 165 nM of ATP is released compared to only 15 nM in other
studies. This quantitative difference could potentially be explained by altered transfection efficiencies, as more transfected cells expressing CALHM1 release more ATP. Because no difference between CALHM1 and its co-expression with CALHM3 could be observed, all future experiments described here focus solely on hCALHM1-mCherry transfected HeLa. The addition of 75 µL of 5 mM EGTA also led to significantly more ATP release compared to 1 mM and 0.5 mM EGTA (Figure 3-9b inset), suggesting that the addition of 5 mM EGTA would lead to both the greatest rate and total amount of ATP release, and thus most likely to be fluorescently visualized at the cell surface compared to other conditions.

To determine whether ATP release could be optimized using alternative methods of extracellular Ca\(^{2+}\) removal in hCALHM1-mCherry transfected cells, two additional conditions were tested: 5 mM BAPTA (a Ca\(^{2+}\)-specific chelator with a \(~2000\)-fold higher on rate (\(K_{on}\)) and \(~140\)-fold slower off rate (\(K_{off}\)) for Ca\(^{2+}\) compared to EGTA) (Naraghi, 1997) and no Ca\(^{2+}\). Interestingly, the addition of 5 mM BAPTA had no appreciable effect on ATP release compared to external solution alone (Figure 3-10a) and resulted in less ATP release compared to external solution without Ca\(^{2+}\), and both conditions led to significantly less extracellular ATP accumulation in hCALHM1-mCherry transfected HeLa compared to 5 mM EGTA. ATP release was also significantly inhibited in the presence of 20 µM Zn\(^{2+}\), but not ruthenium red (RR) or Gd\(^{3+}\) (Figure 3-10b).
Figure 3-9 hCALHM1-mCherry shows robust ATP release in response to 5 mM EGTA in HeLa cells

(a) Time-course of extracellular ATP accumulation in hCALHM1-mCherry and hCALHM3-BFP transfected HeLa cells in response to 5 mM EGTA using the luciferin-luciferase assay. \( n = 8 \) replicates/condition. (b) Summary of ATP release at 40 minutes in hCALHM and mock transfected cells. (inset) CALHM1-mCherry transfected HeLa were treated with 5, 1, and 0.5 mM EGTA. All statical data is shown as mean ± s.e.m. Statistical differences were calculated by one-way ANOVA using student’s t-tests. *\( P<0.05 \), **\( P<0.01 \), ***\( P<0.001 \).
(a) Summary of extracellular ATP release at 40 minutes in hCALHM1-mCherry transfected HeLa in response to external solution, 5 mM EGTA, 5 mM BAPTA, and Ca\(^{2+}\)-free solutions. \(n = 8\) for each condition. (b) Summary of extracellular ATP release at 40 minutes in response to various inhibitors. All conditions consist of HeLa transfected with hCALHM1-mCherry and treated with 5 mM EGTA, with or without inhibitor. \(n = 6\) for each condition. (c) Summary of extracellular ATP release at 40 minutes in response to 5 mM EGTA in hCALHM1-mCherry transfected HeLa labeled with Ni\(^{2+}\)-tris-NTA-PEG\(_4\)-DBCO and HIS\(_{10}\)-Ruby-iATPSnFR. \(n = 6\) for each condition. All statical data is shown as mean ± s.e.m. Statistical differences were calculated by one-way ANOVA using student’s t-tests. *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\).
**Effects of surface labeling on ATP release through hCALHM1-mCherry**

Because our goal was to fluorescently visualize ATP release events at the cell surface using glycocalyx engineering and fluorescent biosensor repurposing, we tested whether the consumption of Ac$_4$ManNAz by HeLa, or the addition of our labeling reagents, would appreciably influence ATP release in hCALHM1-mCherry transfected cells. To this end, hCALHM1-mCherry transfected HeLa were cultured in ± 50 µM azido sugar, labeled ± 50 µM Ni$^{2+}$-tris-NTA-PEG$_4$-DBCO and ± 800 nM HIS$_{10}$-mRuby-iATPSnFR, and chelated with 5 mM EGTA (Figure 3-10c). As a result, no significant increase or decrease in ATP release from cells was observed using our labeling approach.

**EGTAs effect on [Ca$^{2+}$]$_i$ in hCALHM1-mCherry HeLa**

To better understand how extracellular Ca$^{2+}$ chelation influences intracellular Ca$^{2+}$ levels and ultimately ATP release, “addback” experiments were conducted. These experiments are predicated on the understanding that CALHM1 is sensitive to extracellular Ca$^{2+}$ levels, opens upon extracellular Ca$^{2+}$ removal, and allows the release of ions (Ca$^{2+}$, Na$^+$, K$^+$, Cl$^-$) and metabolites (ATP, glutamate, etc.) through its large, non-selective pore. It is also hypothesized that upon the removal of extracellular Ca$^{2+}$, cytoplasmic Ca$^{2+}$ is released through the pore, leading to extracellular Ca$^{2+}$ accumulation and channel closing (self-regulating). HeLa cells were thus co-transfected with hCALHM1-mCherry and an intracellular Ca$^{2+}$ indicator, GCaMP5G, and perfused with varying concentrations of divalent cations (Figure 3-11a). Upon perfusion of external solutions containing either 0 mM Ca$^{2+}$ or 0 mM Ca$^{2+}$/Mg$^{2+}$, small fluctuations in
cytoplasmic Ca$^{2+}$ levels can be observed but may be the result of cellular transients or an artifact of the liquid perfusion process. However, when 5 mM EGTA was perfused over the surface, significant decreases in cytoplasmic Ca$^{2+}$ levels were noted, which returned upon perfusion of external solution containing 2 mM Ca$^{2+}$. This loss of intracellular Ca$^{2+}$ in the presence of 5 mM EGTA was also blocked by 20 µM Zn$^{2+}$, which was previously found to inhibit ATP release. EGTA most likely functions as an extracellular buffer of Ca$^{2+}$, pulling intracellular Ca$^{2+}$ through the non-selective pore down its concentration gradient, bringing cytoplasmic ATP with it (Figure 3.11c). These results, combined with our ATP release findings, suggests that extracellular ATP accumulation is dependent on the continuous chelation of extracellular Ca$^{2+}$ by EGTA, as the mere removal of extracellular Ca$^{2+}$ leads to the hypothesized self-regulated closing of CALHM1 by cytoplasmic Ca$^{2+}$, and thus significantly reduced ATP release.
Figure 3-11 Intracellular calcium loss due to hCALHM1-mCherry channel opening

(a) hCALHM1-mCherry transfected HeLa were co-transfected with a fast-acting calcium indicator pCMV-GCaMP5G that reports on intracellular calcium (Ca\(^{2+}\)) levels in real-time. Cells were perfused with Ca\(^{2+}\)-free, Ca\(^{2+}\)/Mg\(^{2+}\)-free, 5 mM EGTA, or external solution. (b) Summary of (Ca\(^{2+}\)) fluorescence loss ± hCALHM1-mCherry and ± 20 µM Zn\(^{2+}\) (n = 5 dishes/condition; 7-12 cells/dish). (c) Graphical representation of hCALHM1 activation with the addition of EGTA, resulting in channel opening, extracellular calcium chelation, intracellular calcium loss through hCALHM1, and extracellular ATP accumulation. (Statistical data is shown as mean ± s.e.m. Statistical differences were calculated by one-way ANOVA and unpaired two-tailed students t-test. *P<0.05, **P<0.01, ***P<0.001).
5 mM EGTA adversely affects surface-bound biosensors

With the conditions for ATP release optimized, we evaluated how these conditions influence surface-bound HIS_{10}-mRuby-iATPSnFR. HeLa were subsequently seeded on to 35 mm glass-bottom dishes, cultured in 50 µM Ac_{4}ManNAz for 48 hours, labeled with 50 µM Ni^{2+}-tris-NTA-PEG_{4}-DBCO and 800 nM HIS_{10}-mRuby-iATPSnFR, titrated with increasing concentrations of ATP, and monitored via fluorescence microscopy. Although surface bound biosensors were capable of reporting on changes in extracellular ATP concentrations in the presence of 5 mM EGTA, the dynamic range (B_{max}) was 20% of that when titrated with ATP in external solution (Figure 3-12a). To determine whether this drastic loss in B_{max} was due to the loss of surface-bound biosensors from Ni^{2+} chelation by EGTA, we first established a linear relationship between the baseline fluorescence of surface-attached biosensors and ∆F in response to 30 µM ATP (Figure 3-12b). We found that when cells were labeled with reduced concentrations of biosensor, the dynamic range (∆F_{max}) was proportionally reduced (Figure 3-12c). This relationship suggests that the reduction in B_{max} in the presence of 5 mM EGTA could be attributed to biosensor loss. To resolve this issue, we hypothesized that the perfusion of 5 mM EGTA during the second of three perfusion steps, the first and last of which contained external solution, ∆F_{max} of the third would be greatly reduced in the presence of 30 µM ATP if the biosensor was liberated during the second perfusion. Figure 3-12d illustrates that when 30 µM ATP is sequentially perfused in the presence of external solution alone, the ratio of the third perfusion to the first (C/A) results in 0.555 ± 0.006. The reduction in dynamic range is likely attributed to incomplete washout of ATP.
Nevertheless, when 30 µM ATP is perfused in the presence of 5 mM EGTA during the second of three perfusion steps, the previously observed reduction in ∆F occurs (B), but with no loss in the C/A ratio (0.74 ± 0.02) (Figure 3-12e-f). By comparison, when the second perfusion step contains 50 mM imidazole (outcompetes the oligohistidine-tag), the biosensors response to 30 µM ATP is virtually lost during the third perfusion (C/A = 0.07 ± 0.02). These results suggest that the reduction in dynamic range of the biosensor to ATP in the presence of 5 mM EGTA is not due to biosensor loss, but EGTA’s effect on the biosensor itself. Interestingly, the C/A ratio is clearly increased in EGTA perfused cells. The reason for this phenomenon will be explained next.
Figure 3-12 EGTA reduces $B_{\text{max}}$ of in situ Ruby-iATPSnFR, but does not liberate biosensors

(a) Cell-attached HIS$_{10}$-Ruby-iATPSnFR’s overall dynamic range is reduced in the presence of 5 mM EGTA ($B_{\text{max}} = 0.09$) in response to ATP by over half, compared to external solution ($B_{\text{max}} = 0.5$). (b) HeLa surface-labeled with varying concentrations of HIS$_{10}$-Ruby-iATPSnFR show a strong linear relationship between biosensor surface concentration, and hence baseline fluorescence, and $\Delta F_{\text{max}}$ (c). (d) Fluorescence response of HeLa cells surface-labeled with 800 nM HIS$_{10}$-Ruby-iATPSnFR and perfused with 30 µM ATP. (e) Fluorescence response of HeLa cells surface-labeled with 800 nM HIS$_{10}$-Ruby-iATPSnFR and perfused with 30 µM ATP in the presence of 5 mM EGTA during the second perfusion. (f) Quantification of $\Delta$ratio (C/A) between the 3rd:1st perfusion. No loss in fluorescence due to EGTA chelation of Ni$^{2+}$ led to a reduction in the 3rd perfusion, as seen by cells perfused with imidazole. Statical data is shown as mean ± s.e.m. Statistical differences were calculated by one-way ANOVA and students unpaired two-tailed t-test, *$P<0.05$, **$P<0.01$, ***$P<0.001$. 
Fluorescently visualizing ATP release from hCALHM1-Cherry

Despite establishing the adverse effect EGTA has on surface-bound HIS_{10}-mRuby-iATPSnFR, we attempted to fluorescently visualize ATP release from hCALHM1-mCherry transfected HeLa. Cells were thus transfected with hemichannel and labeled with 50 µM Ni^{2+}-tris-NTA-PEG_{4}-DBCO and 800 nM HIS_{10}-mRuby-iATPSnFR as described previously. Cells were perfused with 5 mM EGTA at 2 min and fluorescently monitored over 30 min (Figure 3-13a). As a result, fluorescence intensity increased in both mock and hCALHM1-mCherry-transfected cells, and no significant differences were found between the two conditions, either in their change of maximum fluorescence post-perfusion (Figure 3-13b), or the time to maximum increase (figure 3-13c). While the results were initially perplexing, figure 3-13d shows that the fluorescence intensity of mock (no ATP release, as determined by the luciferin-luciferase assay) and hCALHM1-mCherry-transfected cells increased in every cell. It was clearly observed that upon 5 mM EGTA perfusion, cellular morphology was also drastically altered (figure 3-13e) and led to the detachment of cells from the underlying extracellular matrix (ECM) and poly-D-lysine coated glass, presumably through the dissociation of Ca^{2+}-dependent integrins (Leavesley et. al., 1993), cadherins (Hirano et. al., 1987; Kim et. al., 2011), and other cell adhesion molecules. The withdrawal of filopodia from the ECM likely resulted in the condensation of cellular membrane, and thus surface-attached biosensors, leading to an observable increase in fluorescence, as we’ve shown previously that biosensor fluorescence increases with biosensor concentration. The compound effects of EGTA made it virtually impossible to distinguish between fluorescence
increases due to local ATP accumulation, and mere alterations to cellular morphology and biosensor sensitivity to EGTA.
Figure 3-13 EGTA increases biosensor fluorescence and alters cellular morphology

(a) hCALHM1-mCherry transfected HeLa cells were surface labeled with Ni\(^{2+}\)-tris-NTA-PEG\(_4\)-DBCO and HIS\(_{10}\)-Ruby-iATPSnFR and perfused with 5 mM EGTA beginning at 2 min. One cell from CALHM1 transfected HeLa, and 1 mock cell (different dish) shown for comparison. A summary of both the maximum fluorescence change after EGTA perfusion (b) and time to maximum fluorescence change (rate) between mock and hCALHM1-mCherry transfected HeLa. (d) All cells increased in fluorescence intensity in the presence of 5 mM EGTA. GFP channel colored to illustrate fluorescence intensity changes. Scale bar, 20 µm. (e) HeLa cells labeled with HIS\(_{10}\)-Ruby-iATPSnFR readily detached from the extracellular matrix (ECM) in the presence of 5 mM EGTA compared to external solution, altering cellular morphology and condensing surface-attached biosensors, thereby confounding quantification of ATP efflux. Scale bar, 25 µm. Statical data is shown as mean ± s.e.m. Statistical differences were calculated by one-way ANOVA and student’s two-tailed t-test. *P<0.05, **P<0.01, ***P<0.001.
Discussion

Here, we show the first known attempt to visualize endogenous ATP release through large-pore hemichannels at the cell surface utilizing surface-bound, ATP-sensitive, GFP-based biosensors. We found that localizing FRET-based biosensors to the extracellular surface using glyocalyx engineering and Ni^{2+}-tris-NTA-PEG₄-DBCO inhibited the inherent function of the biosensor to induce Förster resonance energy transfer from one fluorophore to the other. One common genetic approach to displaying proteins or biosensors to the extracellular surface is using the platelet derived growth factor receptor (PDGFR) domain, but FRET biosensors chimerically incorporated with this domain are easily inhibited from trafficking to the extracellular surface through the secretory pathway. Although our unique approach circumvented this issue, the biosensor was still incapable of detecting ATP while surface bound. The reasons for this are still not well understood. We attempted to mimic *in vitro* conditions by incorporating linkers between the oligohistidine-tag and biosensor, and thus distance between the Ni^{2+}-tris-NTA moiety and FRET pair, but no resonance energy transfer was observed. Other unexplored linker types may be more suitable for further investigation, however.

The intensiometric ATP biosensor mRuby-iATPSnFR was fortunately capable of functioning expectedly while surface attached. We showed strong evidence for it to detect extracellular ATP concentrations above ~100 nM, and its selectivity for ATP over other nucleotides, including a 3-fold reduction in $K_D$ *in situ* vs. *in vitro*. iATPSnFR was however incapable of detecting ATP release from HEK293T cells stimulated with 10 µM
UTP. The luciferin-luciferase assay convincingly demonstrated ATP release from > 20,000 cells in mock, P2Y$_2$, P2Y$_4$, and Panx1 transfected conditions. UTP-dependent ATP release is a common mechanism found in numerous cell types, including bone-forming osteocytes, osteoclasts (Kringelbach et. al., 2012; Orriss et. al., 2017), and carotid body type II cells (Zhang et. al., 2012), but our hypothesis that the local concentration of ATP when released exceeds the minimum detection limit of a surface attached biosensor is false however, as the local ATP concentration depends on both the quantity and rate of release per cell. Addressing the inherent sensitivity of biosensors is a question other labs routinely investigate, but beyond the scope of this current study. Here, both biosensors utilized the ε subunit from bacterial F$_0$F$_1$-ATP synthase, but more favorable ATP-binding protein domains may be more suited, and more highly sensitive to ATP, yielding promising results.

Other potential reasons our approach was unsuccessful could be due to our surface labeling approach inhibiting the release of ATP. Covalent modification of transmembrane glycoprotein channels (connexin or pannexins) could have adversely affected their function, thereby rendering them incapable of passing ATP. Another possible reason, besides the relatively low amount of ATP released per cell, could be the propensity of ATP to merely bypass binding to biosensors as it traverses through the glycocalyx into the bulk extracellular solution. By comparison, studies where genetically encoded, extracellularly expressed iGluSnFR was able to detect extracellular glutamate release at the synaptic terminal (Hefendehl et. al., 2016; Borghuis et. al., 2013), glutamate was able to accumulate in cell-dense regions in vivo. In the present study, cell density is
far lower compared to in situ levels and does not allow analyte accumulation at the surface.

Our second attempt to fluorescently visualize ATP release from CALHM1 also proved unsuccessful. Here, the stimulus used to elicit channel opening caused deleterious effects to both the cells’ morphology and biosensor. We demonstrated strong evidence for CALHM1-dependent ATP release over a ~ 40 min period, with a maximum ATP release rate of 7.1 nM · min⁻¹ at 20 min. However, the chelation of extracellular Ca²⁺ detached cells from the underlying substrate and drastically reduced the dynamic range of the biosensor in the presence of ATP. The combined effects of EGTA on fluorescence quantification rendered these experiments incapable of distinguishing between ATP release and mere artifact. There was however no means of circumventing the use of EGTA to elicit ATP release from CALHM1, as ATP release is dependent on continuous chelation of extracellular calcium through the duration of measurement.

Although this study successfully demonstrated the novel use of glycocalyx engineering to localize ATP-sensing fluorescent biosensors to the apical surface and detect extracellular concentration changes, the compatibility between cell type, channel, chemical stimulus, biosensor, and analyte release rate must favorably coincide. Future studies involving alternative cell types expressing ATP-releasing channels or transporters are certainly worth investigating.
Chapter IV: Discussion and Future Directions

Endogenous physiological responses of cells to insult, injury, or standard environmental stimuli, require cells to regulate internal and external ions and metabolites (IAMs) using transmembrane channels and transporters, which were the focus of our investigation. There are several large-pored channels associated with disease progression or endogenous physiological responses whose substrates are either known, implicated, or unidentified. Developing a robust biochemical method to investigate IAM release in real-time at the cell surface will allow researchers to identify cellular sources of IAMs of mixed cell cultures, provide quantitative concentrations of local IAMs near the surface, and precisely ascertain previously unidentified IAMs that pass through large-pored channels that potentially contribute to downstream physiological processes. To isolate the behavior of transmembrane channels and transporters, it is common to overexpress them in non-endogenous cell types that allow the identification of their contribution to IAM kinetics, dynamics, location, temporal release, diffusion, downstream effect, and cellular source. At the same time, there are nearly 100 protein-based fluorescent biosensors that were originally designed for intracellular detection of IAMs, all of which can be recombinantly produced and localized to the extracellular surface to detect IAM release.

In Chapter II, we demonstrated the design and synthesis of a novel chemical compound that covalently modifies a cell’s glycocalyx to allow binding of oligohistidine-tagged biosensors that results in their localization to the cellular surface, and in theory, allowing the detection of IAM release in real-time. This *sui generis* approach to biosensor
repurposing opens the door to new and unanswered questions in the fields of neurobiology, biophysics, and others.

In Chapter III, we focused primarily on attempting to visualize ATP release from non-excitable cells. However, due to technical limitations we were unable to successfully visualize ATP release for several reasons. To visualize IAM release from non-excitable cells using our novel fluorescent biosensor cell surface-labeling approach, we attempted dozens of combinations of biosensors, cell types, chemical and non-chemical stimuli, molecular triggers (receptors), channels, and experimental paradigms (Table 4-1). Of the dozens of combinations we attempted, one combination yielded successful results: Chinese hamster ovary (CHO) cells transiently transfected with Hv-1 (a voltage-sensitive proton channel) surface-labeled with pHuji, a proton-sensitive biosensor. Cells were patched in the whole-cell configuration and depolarized using a standard protocol. Under these conditions, we were able to successfully visualize proton release via fluorescence changes of the surface-bound biosensor. This combination of cell type, trigger (membrane depolarization and VSD activation), channel, and biosensor, were compatible for several reasons:
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<td>mRuby-iATPSnFR</td>
<td>HEK293T</td>
<td>Panx1-mCherry</td>
<td>P2Y7-BFP</td>
<td>UTP</td>
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<td>?</td>
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<td>CHO</td>
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<td>—</td>
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<td>hypotonic soln.</td>
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<td>Panx1</td>
<td>P2X-D</td>
<td>UTP</td>
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</table>
Nearly four dozen separate attempts to visualize ions and metabolites at the cell surface were conducted. Among the repeated reasons for unsuccessful visualization are (1) FRET biosensors did not function properly while surface-bound, (2) the chemical stimulus used to elicit ion or metabolite release adversely affected the biosensors or cells, (3) no fluorescence response was detected, (4) cells were incapable of metabolizing the azido sugar, or (5) biosensors were incapable of detecting their intended analyte.

<table>
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<tr>
<th>#</th>
<th>metabolite</th>
<th>biosensor</th>
<th>cell type</th>
<th>channel</th>
<th>trigger</th>
<th>stimulus</th>
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<td>—</td>
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<td>CHO</td>
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<td>—</td>
<td>—</td>
<td>does not FRET on cell surface</td>
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<tr>
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<td>—</td>
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<td>44</td>
<td>—</td>
<td>HIS10-GFP</td>
<td>primary neurons</td>
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<td>—</td>
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<tr>
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<td>HIS10-p-fuji</td>
<td>CHO</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>—</td>
<td>depolarization</td>
<td>successfully visualized proton efflux</td>
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</table>

Table 4-1 Attempts to visualize ions and metabolites at the cell surface
(1) Glycocalyx engineering: CHO cells can undergo glycocalyx re-engineering using a membrane permeant precursor of sialic acid (N-acetylmannosamine) harboring a small azide chemical handle (ManNAz), display azide on their surface, and undergo covalent modification with Ni\(^{2+}\)-tris-NTA-PEG\(_4\)-DBCO and polyhistidine-tagged biosensors. Cells incapable of metabolizing Ac\(_4\)ManNAz we are unable to use, despite a particular cell type being optimal for ion or metabolite release (e.g. neurons, cardiomyocytes, etc.). This technical limitation restricts the use of cells that usually do not endogenously express the channels, transporters, or receptors at high enough levels to release sufficient quantities of ions or metabolites detectable by surface-localized biosensors.

(2) Stimuli: Depolarization of CHO cells using the whole-cell configuration does not interfere with or adversely affect externally bound biosensors. By contrast, most of the experimental approaches we attempted consisted of using chemical agonists to activate membrane receptors or to open transmembrane channels to induce the release of metabolites. The issue we faced numerous times however is the effect a particular chemical stimulus has on externally bound biosensors. Many of the chemicals we used either led to fluorescence increases or decreases of the biosensor or reduced their dynamic range (max \(\Delta F/F_o\)), confounding quantification or rendering the biosensors ineffective. In Chapter III, we demonstrated the efficient use of EGTA to stimulate CALHM1 channel-opening in HeLa cells to release ATP. 5 mM EGTA led to ATP release that we were able to quantify using the D-luciferin/luciferase assay. However, we were unable to fluorescently visualize these release events using our surface-labeling
approach because EGTA (a) significantly reduced the biosensors dynamic fluorescence rage, and (b) detached cells from the poly-L-lysine coated dish, presumably via Ca$^{2+}$-dependent cell adhesion molecules (CAMS, integrins, cadherins, etc.). These experiments acutely illustrated the necessary compatibility between a particular stimulus and biosensor.

(3) *Measuring release*: Upon membrane depolarization, Hv-1’s voltage-sensitive domain undergoes conformational changes that lead to channel opening. In combination with a pipette solution of sufficiently low pH and low extracellular buffer capacity, protons are released and accumulate near the surface. As a result of this process, protons interact with surface-bound biosensors leading to observable fluorescence changes. It is worth noting that only through the *over-expression* of Hv-1 are we able to observe proton release, suggesting that endogenous levels of channels or transporters may be insufficient to yield local ion or metabolite concentrations detectable by a biosensor, and overexpression may be and is likely necessary. For example, using the highly sensitive (400 pM – 2 µM) ATP D-luciferin/luciferase assay, we showed that untransfected HEK293T cells release ATP in the presence of 10 µM UTP (UTP does not affect the ATP biosensor) within seconds, but an ATP fluorescent biosensor with a $K_D \approx 0.5 \mu\text{M}$ and a fluorescence range of 0.2 µM ($\Delta F/F_o \sim 5\%$) – 100 µM ($\Delta F/F_o \sim 55\%$) attached to the surface is incapable of detecting these release events. Although the D-luciferin/luciferase assay demonstrated ATP release of > 20,000 cells, an individual cell’s contribution is miniscule and nearly 1000-fold below the detection limit of the biosensor.
Among the requirements responsible for successful use of our novel approach is the compatibility of individual molecular, cellular, and/or chemical components. Despite perfect overlap of individual constituents, the biosensor in question must possess a $K_D$ near or below the local concentration of analytes.

Figure 4-1 The necessary compatibility of components
For the reasons mentioned, testing multiple experimental components is necessary to find compatible combinations (Figure 4-1). The most efficient approach is a large and thorough screen. Of utmost importance is the compatibility between biosensor and chemical stimulus. As it applies to ATP release, a screen would consist of testing different cell types with combinations of chemical stimuli in the D-luciferin/luciferase assay. For other metabolites (e.g. glutamate), a robust assay would be needed. Immediately upon discovering a suitable chemical/cell combination that leads to robust ATP release, we would test the effect the chemical stimulus has on the biosensor’s fluorescence and overall detection of ATP. In addition, upon finding that the cell type in question is unable to metabolize the azido sugar, the approach would be abandoned. Listed below are four individual approaches for potential future investigation:

1. Mechanical stimulation of astrocytes, HeLa, or HEK293T cells via pannexin1: evidence suggests that pannexin1, a large-pored, ATP releasing, transmembrane channel can be stimulated and opened upon mechanical perturbation. This phenomenon has been most acutely demonstrated in primary astrocytes but may be recapitulated in HeLa or HEK293T cells overexpressing pannexin1. The downside to this approach is the absence of a robust assay capable of measuring ATP release. Physical deformation of cells also makes cell boundary tracking challenging, while simultaneously quantifying fluorescence changes (Wang et. al., 2000; Newman, 2001; Arcuino et. al., 2002).

2. HUVEC stimulated with compound 48/80 via unknown G protein: Gruenhagen, et al. demonstrated in 2004 the release of ATP from human umbilical vein
endothelial cells (HUVEC) using compound 48/80, a polymer produced by the condensation of N-methyl-p-methoxyphenethylamine with formaldehyde. Although the study did not identify the G protein in question, or the channel through which ATP was released, it nevertheless demonstrated ATP release following initial intracellular \( \text{Ca}^{2+} \) increases by optically imaging luminescence from the conversion of D-luciferin to oxyluciferin by luciferase in the bath solution. Repeating this study under identical conditions would be necessary. After demonstrating ATP release using their conditions, cells would be labeled with the ATP biosensor using our approach and repeat the experiment. It would also be necessary to determine the effect compound 48/80 has on the biosensor’s overall response in the presence of increasing concentrations of ATP and whether HUVEC can metabolize azido sugar.

3. Whole-cell patch CALHM1 in HeLa cells: CALHM1 is a wide-pored transmembrane channel that regulates intracellular and extracellular calcium levels and is sensitive to calcium. In the absence of extracellular calcium, CALHM1 opens and releases both intracellular calcium, ATP, and potentially other ions and metabolites. We demonstrated that chelation of extracellular calcium using 5 mM EGTA, although it leads to ATP release, adversely influences surface-bound biosensors, as mentioned previously. In addition to being calcium-sensitive, CALHM1 also possesses a putative voltage-sensitive N-terminal domain, that when expressed in HeLa and N2a cells and depolarized, open and may lead to ATP release (Ma et. al., 2018; Ma et. al., 2012).

4. P2Y\textsubscript{2/4} + pannexin1 + UTP: We previously demonstrated that HEK293T and HeLa cells, when stimulated with 10 \( \mu \text{M} \) UTP, release ATP within minutes. However,
upon surface-labeling with using our method, we was unable to fluorescently visualize these events, precisely because the local concentration of ATP surrounding a cell’s glycocalyx upon release is below the detection limit of the biosensor. Other cell types may prove more amenable to measurable ATP concentrations. Zhang et al. (2014) demonstrated that carotid body type II cells, and not type I, release ATP through pannexin channels upon UTP stimulation through activation of P2Y2 receptors. Validating whether this cell type is capable of metabolizing azido sugar would need to be demonstrated first, followed by recapitulation of their experiments.

As illustrated, the experimental space is large yet worth investigating in the future. Although we were unable to successfully visualize metabolite release through large-pore hemichannels, certainly other systems not tested here will prove more fruitful.

Conclusion

This thesis contributes to the growing body of both biotechnological tools and knowledge. Our approach, while useful for localizing biosensors to the cell surface, could be used to attach any recombinantly produced protein to the apical surface for reasons we have not explored or elucidated here. And while we demonstrated ATP release using a highly-sensitive luciferin/luciferase assay, biosensors were incapable of detecting these release events due the small individual contribution per cell and the insensitivity of biosensors to their substrate, pointing to the need for more robust biosensor design. Lastly, it is my hope future scientists will continue developing new and groundbreaking technologies that aim to uncover the beauty of our hidden reality, as attempted here.
APPENDICES
Figure A-1 Ni\textsuperscript{2+}-tris-NTA-PEG\textsubscript{4}-DBCO elution from 14 cm Sephadex G10 column

*Tris*-NTA-PEG\textsubscript{4}-DBCO was co-incubated with excess Ni\textsuperscript{2+}SO\textsubscript{4} and run down a 14 cm column containing Sephadex G10. The 291 and 308 nm absorbance of DBCO was co-monitored with free Ni\textsuperscript{2+} (393 nm). ~30 µL elution droplets were collected into 1.5 mL microfuge tubes (10 droplets per tube). Fractions were separately and individually monitored for DBCO and Ni\textsuperscript{2+} absorbance on a NanoDrop 1000 (Thermo Fisher Scientific). Collected fractions containing nickelated-*tris*-NTA-PEG\textsubscript{4}-DBCO were combined, lyophilized, and weighed until reconstituted to 50 mM.
Figure A.2: High-resolution mass spectrometry of compound 9 (Chapter II)
Figure A-3 High-resolution mass spectrometry of compound 10 (Chapter II)
Figure A-4 SDS-PAGE of recombinantly produced fluorescent proteins and biosensors

All DNA sequences coding for fluorescent proteins (FP), single FP biosensors, and FRET biosensors were cloned into HIS-tagged pNCS vectors, transformed into BL21 (DE3) *E. coli* (NEB), grown for 72 hours at 20ºC, harvested, sonicated, purified using Ni²⁺-NTA agarose (HisPur™ Ni-NTA from Thermo Fisher), dialyzed, concentrated, run on NuPAGE Bis-Tris gels (Thermo Fisher NP0301BOX) under reducing conditions, and stained using a standard Coomassie Blue stain/destain.
Figure A-5 Surface localization of HIS$_{10}$-GFP relative to cell boundary

CHO cells were fed 50 µM azido sugar for 48 hrs, labeled with 50 µM Ni$^{2+}$-tris-NTA-PEG$_4$-DBCO for 30 minutes, 800 nM recombinant HIS$_{10}$-GFP, and viewed under 63X. DIC, GFP, and merged imaged shown, demonstrating fluorescent signal relative to cell boundary. Scale bar, 10 µm.
Figure A-6 Surface-attached HIS_{10}-iGluSnFR is sensitive to nucleotides

(a) In order to determine how nucleotides affect surface-attached glutamate biosensors, CHO cells were fed 50 µM azido sugar for 48 hrs, labeled with 50 µM Ni^{2+}-tris-NTA-PEG_{4}-DBCO for 30 min, 800 nM recombinant HIS_{10}-iGluSnFR for 15 min, resuspended in external solution (pH 7.5), viewed under 63X, and perfused with external solution or 1 mM nucleotide at 1 min. Individual traces of 10-15 cells from single dishes shown. (b) Summary data of \( n = 4 \) dishes/condition (10-15 cells/dish). TFB-TBOA (TOCRIS Cat.)
No. 2532) is a high affinity EAAT1 and EAAT2 blocker. MRS2179 (TOCRIS Cat. No. 0900) is a selective P2Y\textsubscript{1} antagonist. Fluorescence quenching occurs due to the presence of nucleotide by an unknown mechanism, particularly due to ADP. These results indicate that the fluorescence detection of surface-attached HIS\textsubscript{10}-iGluSnFR for glutamate could be influenced by nucleotides co-released through large-pore hemichannels. (One-way ANOVA. Students unpaired two-tailed t-test, \textasteriskcentered \textless 0.05, \textasteriskcentered\textasteriskcentered \textless 0.01, \textasteriskcentered\textasteriskcentered\textasteriskcentered \textless 0.001).
Primary microglia were cultured with (a) and without (c) azide sugar, labeled with Ni²⁺-tris-NTA-PEG₄-DBCO and HIS₆-GFP, and immunostained against Iba-1 (b & d). Scale bar, 15 µm. Primary astrocytes were cultured with (e) and without (f) azide sugar and then labeled with Ni²⁺-tris-NTA-DBCO and HIS₆-GFP. Scale bar, 10 µm. (g-i) Murine osteocytes, neuroblastoma, and human MCF-7 cells were also labeled using our approach.
Figure A-8 Identifying tagged channels and receptors relative to surface-attached biosensors

(a) HEK293T cells were transfected with P2Y2-BFP, fed 50 µM azido sugar for 48 hrs, labeled with 50 µM Ni2+-tris-NTA-PEG4-DBCO for 30 min, 800 nM recombinant HIS10-mRuby-iATPSnFR for 15 min, resuspended in external solution (pH 7.5), viewed under 63X. BFP was excited at 388 nm and viewed under the EBFP filter set (31041, Chroma). mRuby-iATPSnFR was excited at 460 nm and viewed under the GFP filter set (49002, Chroma). In experiments involving purinergic receptors and pannexin channels (Chapter III), co-transfected cells were first identified and chosen for quantification, before visualizing fluorescence changes of mRuby-iATPSnFR in response to 10 µM UTP in the same cells. Scale bar, 15 µm.

(b) The same procedure was followed, but cells were transfected with Panx1-mCherry. Panx1-mCherry was excited at 580 nm and viewed using the appropriate filter set (69008, Chroma).
HEK293T cells were co-transfected with P2Y$_2$-BFP, Panx1-mCherry, and GCaMP5G. Cells were excited at 460 nm and visualized using the GFP filter set under 63X. At 1 min, cells were perfused with 10 µM UTP. Images were collected at 1 Hz. Images at the beginning of each minute shown. Images were background subtracted and false-colorized. Data corresponds to Figure 3-6b-c.
Figure A-10 N2a cells show surface expression of hCALHM1-mCherry

Neuro-2a cells (ATCC #CCL-131) were transfected with hCALHM1-mCherry, displaying similar expression patterns found in Ma et. al., 2018. hCALHM1-mCherry was later shown to result in ATP release from HeLa cells in Chapter III. Scale bar, 15 µm.
Figure A-11 Luciferin-luciferase standard curve and luminescence conversion

(a) Titrations of ATP are made prior to the start of the experiment. 100 µL of standard curve are loaded into each well just prior to the addition of 10 µL assay reagent and read on a luminometer. (b) A standard curve must be generated for each condition tested, as experimental conditions (e.g. ± EGTA, ± blockers, etc.) can affect the conversion of D-luciferin to oxyluciferin by luciferase. (c-e) Converting from light emission to [ATP] requires fitting a non-linear standard curve and solving for concentration, allowing the absolute quantification of ATP over a given period.
Figure A-12 iGluSnFR-labeled Cx32 HeLa did not release measurable quantities of glutamate

(a) CHO cell surface-attached HIS10-iGluSnFR response to glutamate titrations. (b) HIS10-iGluSnFR labeled, doxycycline-induced Cx32-expressing HeLa cells were perfused with 10 µM UTP at 1 min. Fluorescent visualization of iGluSnFR (GFP) was monitored for fluorescence intensity changes (∆F/F₀). Fluorescent traces of cells from single dishes shown. (c) Comparison of maximum fluorescence change in response to perfusion of 10 µM UTP in ± doxycycline induced Cx32 HeLa. n = 4 dishes/condition (8-12 cells/dish). (d-e) Minimum and maximum fluorescence intensity changes of UTP perfused, doxycycline induced cells. n = 4 dishes/condition (8-12 cells/dish). Although significant maximum intensity changes between ± UTP perfused cells is shown, these differences are likely artifacts or noise. (Students unpaired two-tailed t-test, *P<0.05, **P<0.01, ***P<0.001). Doxycycline inducible Cx32 HeLa were a gift from Jorge Contreras, PhD (UC Davis).
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