Fluorescent Visualization of Cellular Proton Fluxes

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FLUORESCENT VISUALIZATION OF CELLULAR PROTON FLUXES

A Dissertation Presented

By

Lejie Zhang

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 6, 2018

BIOCHEMISTRY AND MOLECULAR PHARMACOLOGY
To my son, Ruichen (Ryan)

whose smile makes me like a proton and stay positive.
ACKNOWLEDGEMENTS

First and foremost, I want to thank my advisor, Bill Kobertz who has always shown patience and gave encouragement and invaluable advice on science, English writing and speaking, and life to his #8 graduate student. I would also like to thank my committee members, Tony Carruthers, Steve Miller, Mike Francis and Gang Han for their insightful feedback and suggestions throughout my thesis research and their permission to have my defense in 2018. Many thanks to Scott Ramsey for serving as my external examiner.

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The last word of acknowledge is for my wife, Libo Chen who sacrificed her career and has been with me all these years. Without her companion and support, I could not imagine how I could finish my 6-years PhD study.
ABSTRACT

Proton fluxes through plasma membranes are essential for regulating intracellular and extracellular pH and mediating co-transport of metabolites and ions. Although conventional electrical measurements are highly sensitive and precise for proton current detection, they provide limited specificity and spatial information. My thesis focuses on developing optical approaches to visualize proton fluxes from ion channels and transporters.

It has been demonstrated that channel-mediated acid extrusion causes proton depletion at the inner surface of the plasma membrane. Yet, proton dynamics at the extracellular microenvironment are still unclear. In Chapter II, we developed an optical approach to directly measure pH change in this nanodomain by covalently attaching small-molecule, fluorescent proton sensors to the cell’s glycocalyx using glyco-engineering and copper free ‘click’ chemistry. The extracellularly facing sensors enable real-time detection of proton accumulation and depletion at the plasma membrane, providing an indirect readout of channel and transporter activity that correlated with whole-cell proton current. Moreover, the proton wavefront emanating from one cell was readily visible as it crossed over nearby cells.

The transport of monocarboxylates, such as lactate and pyruvate is critical for energy metabolism and is mainly mediated by proton-coupled monocarboxylate transporters (MCT1-MCT4). Although pH electrodes and intracellular fluorescent pH sensors have been widely used for measuring the transport of proton-coupled MCTs, they are unable to monitor the subcellular activities and may underestimate the transport rate
due to cell’s volume and intracellular buffering. In Chapter III, we used the Chapter II approach to visualize proton-coupled transport by MCT1-transfected HEK293T cells and observed proton depletion followed by a recovery upon extracellular perfusion of L-lactate or pyruvate. In addition, we identified a putative MCT, CG11665/Hrm that is essential for autophagy during cell death in *Drosophila*. The results demonstrate that Hrm is a bona fide proton-coupled monocarboxylate transporter that transports pyruvate faster than lactate.

Although the approach developed in Chapter II enables visualization of proton fluxes from ion channels and transporters, it’s not applicable in some cell types which cannot incorporate unnatural sialic acid precursors into their glycocalyx, such as INS-1 cells and cardiomyocytes. To address this, in Chapter IV we developed a pH-sensitive, fluorescent WGA conjugate, WGA-pHRho that binds to endogenous glycocalyx. Compared to the results in Chapter II and III, cell surface-attached WGA-pHRho has similar fluorescent signals in response to proton fluxes from proton channel H,1, omega mutant Shaker-IR R362H and MCT1. With WGA-pHRho, we were able to label the plasma membrane of INS-cells and cardiomyocytes and visualized the transport activity of MCT1 in these cells.

Taken together, these findings provide new insights into proton dynamics at the extracellular environment and provide new optical tools to visualize proton fluxes from ion channels and transporters. Moreover, the modularity of the approaches makes them adaptable to study any transport events at the plasma membrane in cells, tissues, and organisms.
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<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>H$_v$</td>
<td>Voltage-gated proton channel</td>
</tr>
<tr>
<td>hH$_v$</td>
<td>Human voltage-gated proton channel</td>
</tr>
<tr>
<td>Na$_v$</td>
<td>Voltage-gated sodium channel</td>
</tr>
<tr>
<td>K$_v$</td>
<td>Voltage-gated potassium channel</td>
</tr>
<tr>
<td>K$_v$AP</td>
<td>Voltage-gated potassium channel from <em>Aeropyrum pernix</em></td>
</tr>
<tr>
<td>Ca$_v$</td>
<td>Voltage-gated calcium channel</td>
</tr>
<tr>
<td>VSD</td>
<td>Voltage-sensing-domain</td>
</tr>
<tr>
<td>PD</td>
<td>Pore domain</td>
</tr>
<tr>
<td>S1-S6</td>
<td>Transmembrane segments denoted from N- to C-terminus</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>R1-R4</td>
<td>Arginine residues denoted from extracellular to cytoplasmic side</td>
</tr>
<tr>
<td>G-V Curve</td>
<td>Conductance-voltage curve</td>
</tr>
<tr>
<td>I-V Curve</td>
<td>Current-voltage curve</td>
</tr>
<tr>
<td>ClC</td>
<td>Chloride channel or transporter</td>
</tr>
<tr>
<td>MCT</td>
<td>Monocarboxylate transporter</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute carrier</td>
</tr>
<tr>
<td>NHE</td>
<td>Sodium-proton exchanger</td>
</tr>
<tr>
<td>NOX</td>
<td>NADPH oxidase</td>
</tr>
<tr>
<td>KO</td>
<td>Knock out</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>Zinc ion</td>
</tr>
</tbody>
</table>
H⁺  Proton
Cl⁻  Chloride ion
K⁺  Potassium ion
Ca²⁺  Calcium ion
Cs⁺  Cesium ion
Li⁺  Lithium ion
pH₀  Extracellular pH
pHᵢ  Intracellular pH
B₀  Extracellular buffer capacity
Bᵢ  Intracellular buffer capacity
[Cl⁻]₀  Extracellular chloride concentration
[Cl⁻]ᵢ  Intracellular chloride concentration
Eᵢ  Reversal potential
CHC  2-Cyano-3-(4-hydroxyphenyl)-2-propenoic acid
Kᵢ  Michaelis constant
Kᵢ  Inhibitor constant
Kᵢ  Equilibrium dissociation constant
pKᵢ  Logarithmic form of equilibrium association constant
pH₁/₂  pH at which half of the overall pH-dependent signal is observed
BCECF-AM  2',7'-Bis-(2-carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester
FITC  Fluorescein isothiocyanate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PET</td>
<td>Photoinduced electron transfer</td>
</tr>
<tr>
<td>WGA</td>
<td>Wheat germ agglutinin</td>
</tr>
<tr>
<td>DIBO</td>
<td>Dibenzocyclooctyne</td>
</tr>
<tr>
<td>Boc</td>
<td>tert-Butyloxycarbonyl protecting group</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>INS-1</td>
<td>Insulin secreting beta cell derived line</td>
</tr>
<tr>
<td>K</td>
<td>Equilibrium constant</td>
</tr>
<tr>
<td>USL</td>
<td>Unstirred layer</td>
</tr>
<tr>
<td>$K_B$</td>
<td>Proton equilibrium constant between bulk and shell</td>
</tr>
<tr>
<td>I</td>
<td>Current</td>
</tr>
<tr>
<td>$IC_{50}$</td>
<td>Concentration of an inhibitor where the response is reduced</td>
</tr>
<tr>
<td>$\Delta F$</td>
<td>The change in fluorescence intensity</td>
</tr>
<tr>
<td>$F_0$</td>
<td>Baseline level of fluorescence</td>
</tr>
<tr>
<td>&lt;</td>
<td>Less than</td>
</tr>
<tr>
<td>&gt;</td>
<td>Greater than</td>
</tr>
<tr>
<td>±</td>
<td>Plus-minus sign</td>
</tr>
<tr>
<td>~</td>
<td>Approximately</td>
</tr>
<tr>
<td>$V_{0.5}$</td>
<td>Potential of half-maximal activation</td>
</tr>
<tr>
<td>DCC</td>
<td>N,N’-Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>hr/hrs</td>
<td>Hour/hours</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>d</td>
<td>Diameter</td>
</tr>
<tr>
<td>wt%</td>
<td>Weight percentage</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HypoPP</td>
<td>Hypokalemic periodic paralysis</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>

Standard one letter or three letter code was used to abbreviate amino acids. Mutated residues were abbreviated by position number and one letter amino acid codes.
PREFACE

The experimental work in Chapter II has been published in a peer-reviewed journal. Reference to publication that represent the work contained within this chapter:


The experimental work in Chapter III and IV has been written up as manuscripts for publication at the time of this thesis publication. I synthesized azido-sugar, pH-DIBO and pH-Rho-COOH, and cloned all genes into pcDNA3.1 and performed the perfusion experiments in HEK293T and INS-1 cells. Dr. Mei Zhang made all WGA conjugates used in Chapter IV, performed patch clamp in CHO cells and perfusion experiments in cardiomyocytes. She is also credited in isolation and culture of cardiomyocytes. Genes of human GFP-Hv1, ClC-5, Shaker-IR omega mutants and MCT1 were gifts from David Clapham (Harvard Medical School), Michael Pusch (Istituto di Biofisica), Baron Chanda (University of Wisconsin-Madison) and Dr. Sebastián Brauchi (Universidad Austral de Chile), respectively. Panagiotis D. Velentzas (Baehrecke Lab, UMassMed) provided the gene of Hrm.
CHAPTER I. INTRODUCTION

Rapid proton transport through voltage-gated proton channels (Hv1) and proton-coupled membrane transporters is essential for maintaining intracellular pH, driving substrate transport, regulating gastric and airway mucosal acidity and providing electrogenic shunts for phagocytic and endocytic processes. Gain-of-function mutations that create unregulated proton pores in voltage-gated cation channels have been implicated in myotonias, periodic paralysis, and some forms of long QT syndrome. Given their varied physiological and pathophysiological roles, proton transport proteins are important therapeutic targets for treating human diseases. However, conventional electrical measurements do not directly reveal the identity of the transported ion(s) or the subcellular location(s) of activity, nor do they report on non-electrogenic transport events. Thus, an approach for spatiotemporally detecting proton fluxes at the plasma membrane remains an important research methodology to develop.

Voltage-gated Proton Channels (Hv1)

The existence of voltage-gated proton channels was first suggested in bioluminescent dinoflagellates in 1972 by Margaret Fogel and J.W. Hastings [1]. Ten years later, voltage-activated proton currents were first recorded in snail neurons [2] and subsequently observed in a number of different cells (e.g. airway epithelium [3], sperm [4] and microglia [5]). It was not until 2006 that a voltage-gated proton channel (Hv1) was identified by two groups independently [6,7].
Hv1 is distributed in diverse species and has wide physiological and
pathophysiological roles [8]. For example, protons extruded by Hv1 in phagocytes
facilitate the production of reactive oxygen species (ROS) to kill pathogens [9–15]. In
Hv1 KO mice, ROS production in neutrophils is reduced by 65-75% [12], 30% [11] or 74%
[13], in B lymphocytes by 65% [14] and in microglia by 50% [15]. Hv1 also plays a key role in the capacitation and motility of human sperm [8]. It is proposed that a high
collection of ambient Zn$^{2+}$ in seminal fluid prevents Hv1 from opening [16], and that
low intracellular pH keeps sperm quiescent. After entering the female, the environment
becomes more alkaline with a lower Zn$^{2+}$ concentration and sperm extrudes protons
through Hv1, leading to stimulation of the metabolic activity and motility [17].
Nevertheless, Hv1 is also detrimental in some respects. Recent studies raise concerns
about Hv1 involvement in breast cancer metastasis. Wang et al. found high levels of
hHv1 mRNA in the metastatic cell line MB-231 and low levels in a weakly metastatic
line MCG7 [18]. Further analysis performed by the same group suggests that there is a
significant correlation between hHv1 expression and tumor size, tumor classification and
clinical stage [19]. Moreover, Hv1 in microglia is required for NOX-dependent ROS
production that induces neuronal cell death after ischemic stroke [15].

Canonical voltage-gated ion channels, such as Na$_v$, Kv, and Ca$_v$ channels are
composed of four subunits each of which has six transmembrane segments (S1-S6),
including a voltage-sensing-domain (VSD, S1-S4) and a pore domain (PD, S5-S6)
(Figure I-1A) [20]. In contrast, Hv1 has a VSD, but lacks a PD (Figure I-1B) [6,7]. It
forms a dimer on the biological membranes, and each monomer has its own proton
permeation pathway [21–23] and gates cooperatively [24]. The fourth transmembrane segment (S4) has three positively charged residues (Arg) at every third position. Upon depolarization, S4 moves upward relative to other helices [24], as it does in other voltage-gated ion channels, opening the proton pathway. Asp112 in S1 is highly conserved and is critical for the selectivity filter of Hv1. Replacing Asp112 with a neutral amino acid causes a loss of proton specificity and makes Hv1 anion-selective or non-conductive [25]. Phe150 in S2 is located close to the proton permeation pathway and is required for the intracellular binding of a guanidine compound, 2-guanidinobenzimidazole (2GBI) that acts as an open-channel blocker [26]. Zinc ion (Zn\(^{2+}\)) is the most potent channel inhibitor for Hv1 (micromolar level) [27]. The inhibition is thought to be the result of four residues (His136, His189, Glu115 and Asp119) that may contribute to the Zn\(^{2+}\) ion coordination in Hv1 according to the crystal structure of Hv1 in the resting state obtained in 2014 [28] (Figure I-1C).
Voltage and pH sensing by the voltage-gated proton channel, HV1

Biophysical properties of the voltage-gated proton channel HV1

**Figure I-1. Topology schematics and representative currents of voltage-gated K^+ channels and Hv1 channels.** (A) In voltage-gated K^+ channels, the first four transmembrane domains (S1-S4, colored green) comprise the voltage sensing domain (VSD); four Arg residues are on S4 (denoted as R1 to R4 from extracellular to cytoplasmic side); the fifth and sixth transmembrane domains (S5-S6, colored orange) form the pore domain (PD); representative currents are recorded from *Xenopus* oocytes expressing *Shaker*-IR channels [29] and are elicited from a holding potential of –90 mV by activating pulses of –60 to 50 mV with intervals of 10 mV (scale bars: 20 ms, 1 µA). (B) Hv1 channels only have S1-S4 and three Arg residues on S4 (R1 to R3); representative currents are recorded from HEK293 cells expressing hHv1 channels [6] and are elicited from a holding potential of –40 mV by activating pulses of –60 to 120 mV with intervals of 20 mV (scale bar: 200 ms, 1 nA). (C) Zn^2+ binding site in Hv1 in resting state. Zn^2+ is drawn in blue mesh and views from different directions are shown in (a) and (b) [28].
One of the most surprising characteristics of Hv1 is its incredibly high selectivity for protons. The estimate of the selectivity factor for protons over other cations is more than $10^7$. Therefore, it is proposed that protons are conducted by a hydrogen bond chain in Hv1 using a Grotthuss mechanism whereby protons diffuse through the hydrogen bond network of water molecules and/or other hydron-bonded residues [30]. Interestingly, mutations in the VSD of other voltage-gated ion channels can also induce a leaky proton current called omega current through the VSD [31–33], implying that they might have a similar proton conduction mechanism to Hv1. The details of this leaky proton current will be discussed later in this chapter.

In addition, Hv1 is voltage-gated, meaning that it opens when the membrane potential is depolarized. The activating current is curved in a sigmoid manner and deactivating current is exponential (Figure I-1A) [34]. However, at least in mammals, Hv1 gating is much slower (> 5 seconds in phagocytes) than Kv and Na_v channels, which open within a millisecond (Figure I-1B) [34,35]. Consequently, it is suggested that the two monomers must undergo conformational changes upon depolarization before either one can conduct protons [24]. Several studies have demonstrated that Hv1 functions as a monomer and exhibits a 5-7 times faster gating when the C-terminus is truncated [21,36,37].

Hv1 is also sensitive to the intracellular (pH_i) and extracellular pH (pH_o). Increasing pH_o or decreasing pH_i shifts the G-V curve negatively by 40 mV per unit change in pH [38]. This sensitivity makes it difficult to determine the maximal proton current experimentally, because proton efflux can cause a local proton depletion in the
cytoplasmic side and thus increase local pH, even with 100-200 mM buffer in the pipette solution [3,38]. Direct evidence for this local proton depletion was provided by De-la-Rosa et al. who labeled Hv1 with a genetically-encoded pH sensor at the cytoplasmic domain [39]. Proton dynamics at the extracellular space upon Hv1 opening are still unclear.

Buffer capacity on both sides (B_o and B_i) also affects Hv1 activity. At a constant pH_i of 5.5, increasing B_i from 5 to 120 mM increases the maximum current density from 8.7 to 27.3 pA/pF [3]. A follow up study by the same group shows that decreasing B_o from 100 to 1 mM does not alter the G-V curve but reduces the current density by 10-30% [40]. In contrast, the same decrease in B_i alters the current density more distinctly while subtly changing the kinetics [40]. All these experimental results suggest that lower B_o causes more proton accumulation at the extracellular side and thus reduces the proton gradient, leading to a smaller proton current. It is also supported by a mathematical model that estimates changes in pH_o at different B_o [41]. However, to date, there is no direct experimental evidence to support this hypothesis.

Omega Currents

Canonical voltage-gated cation channels (Na^+, K^+ and Ca^{2+} channels) are widely distributed and are crucial for the propagation of electrical signals in neurons. Their VSDs are formed by the first four transmembrane segments and share a similar structure to Hv1 but have more than four positively charged residues (Arg or Lys) in S4 (denoted as R1, R2, R3, R4…from extracellular to cytoplasmic side). The PDs constituted by S5-6
form central pores (also known as alpha pores), allowing ions to travel through when channels open (Figure I-1A). Unlike Hv1, the outward movement of S4 in these channels does not open a proton permeable pathway in VSD, but opens the central pore via the force of conformation changes [20,42,43].

Mutations of the charged residues in S4 in these channels may cause a leaky ion flux through the VSDs called omega current. It was first described in a voltage-gated potassium channel, *Shaker*. Replacing the first arginine R1(362) with histidine (R1H) in *Shaker* generates an inward proton current upon hyperpolarization (Figure I-2A) [32]. This omega proton current is voltage dependent and becomes larger when the proton reversal potential (E_H) is more positive. It can be blocked by addition of nickel, which binds to histidine, but is not affected by the alpha pore blocker agitoxin II. These data indicate that the omega current travels through the VSD with the involvement of the replaced histidine. Additionally, substitution of R1 by other residues with shorter side chains (e.g. cysteine, serine and alanine) generates non-proton omega currents carried by alkali metal cations [44], such as Cs+, K+ and Li+. In comparison with R1H, *Shaker* mutant R4(371)H produces an outward proton current upon depolarization (Figure I-2C) [45]. Meanwhile, R2(365)H and R3(368)H mutants behave as voltage-gated proton transporters (Figure I-2B) [31].

Other voltage-gated cation channels also have similar gain of function mutations which can result in channelopathies [46]. For instance, hypokalemic periodic paralysis (HypoPP) is a heritable muscle disorder that has been linked to mutations located in the S4 arginine residues in skeletal muscle channels Na_v1.4 and Ca_v1.1. Similar with *Shaker*
R1H, both Na\(_{v}1.4\) R1(669)H and Ca\(_{v}1.1\) R1(528)H mutants produce inward omega proton currents in the resting state [33,47,48]. Although the omega current is only 1% of the alpha current, this sustained proton leak may contribute to the abnormally depolarized muscle membrane and the failure of muscle action potential firing in HypoPP. Omega currents also have been implicated in other channelopathies, such as benign familial neonatal seizures (BFNS) [49], long QT syndrome (LQT) [50], and familial hemiplegic migraine (FHM) [51], among others.

The production of omega currents is explained by a voltage-dependent salt-bridge switching mechanism [52]. In the resting state, the first S4 arginine (R1) with an acidic residue (S2 E283 in KvAP) forms a salt-bridge that functions as a gate segregating the extracellular fluid from the cytoplasmic fluid (Figure I-2A, top). Shortening or neutralizing the arginine residue breaks the gate and opens the omega current pathway (Figure I-2A, middle). With S4 moving outwardly upon depolarization, one of the other deeper arginine residues occupies the R1 position and forms a new salt-bridge (Figure I-2B and C, top). Therefore, mutation in R2, R3 or R4 only opens the omega pore at certain membrane potentials. In some cases, mutations on two adjacent arginine residues are required to generate omega currents suggesting that the size and shape of the omega gate is variable in different voltage-gated ion channels [53,54].

To study omega mutations, the central pore must be blocked as omega currents are small with respect to the alpha current. However, blocking the central pore either with chemical blockers like tetrodotoxin [50] or using a non-conducting mutation (W434F in *Shaker*) [32] does not completely inhibit the alpha current. Moreover, both methods may
change the conformation of VSD within which the omega current pathway is located.

Hence, a new technique is desirable to detect omega currents without blocking the central pore.
Figure I-2. The ‘salt-bridge switching’ model of S4 movement. Plus signs (red) represent R1-R4 on S4; minus signs (blue) represent acidic residues on S1-S3. (A) Mutation R1H induces inward proton current in the resting state. (B) R2H or R3H mutant behaves as proton transporter. (C) mutation R4H induces outward proton current in the active state. I-V curves of R1H, R3H and R4H (from left to right) under different proton equilibrium potentials (E_H) are shown at bottom [32,45].
Proton-coupled Transporters

Protons represent one major driving force for secondary active transporters. The electrochemical gradient of protons provides energy to transport another ion or metabolite against its electrochemical gradient. A few examples of proton-coupled transporters are shown in Figure I-3A. In the following section, I will focus on the transporters ClC-5 and MCT1, which are two classical exemplars of proton-coupled antiporters and symporter, respectively.

ClC-5, a Voltage-gated H+/Cl⁻ Antiporter

Cl⁻ is the most abundant anion in both extra- and intra-cellular fluid and is essential for many critical physiological functions such as neuronal and muscle excitability, osmotic homeostasis and endosome-lysosome acidification [55]. ClC proteins represent a large family of Cl⁻ transporters in cells. Since the first ClC protein, ClC-0 was discovered [56] and was fully characterized [57] from Torpedo marmorata, the roles of Cl⁻ flux have attracted research interest resulting in the identification of nine mammalian ClC proteins (ClC-1 to ClC-7, ClC-Ka and ClC-Kb). These ClC proteins are expressed as dimers with each monomer exhibiting independent ion conductance. Although they have similar structures, four of them function as Cl⁻ channels at the plasma membrane (ClC-1, -2, -Ka, -Kb), and the other five are organelle H⁺/Cl⁻ antiporters (ClC-3 to ClC-7) [55].

ClC-5 is highly localized in endosomes of renal proximal tubule cells [58]. It was first identified as a protein defective in Dent’s disease [59], a chronic kidney disorder that
has dysfunction of tubular reabsorption for low molecular weight proteins and Ca\(^{2+}\). Mice lacking ClC-5 exhibits several characteristic symptoms of Dent’s disease, such as loss of low molecular weight proteins and defective endocytosis [60]. In addition, ClC-5 is colocalized with V-type H\(^+\)-ATPase in endosomal membranes which actively pumps H\(^+\) into endosomes [61]. Therefore, it was thought that ClC-5 was a Cl\(^-\) channel, an electrical shunt for maintaining ATP-induced acidification in endosomes. This supposition was accepted until 2005 when ClC-5 was determined to be a H\(^+\)/Cl\(^-\) antiporter instead of a Cl\(^-\) channel, as the proton flux from this antiporter acts in opposition to the activity of the H\(^+\)-ATPase [62,63]. Later on, it was found that the point mutation E211A blocks the proton pore and converts ClC-5 into a pure Cl- conductor [64]. Renal endosomal acidification is normal in mice carrying this mutation but is reduced in ClC-5 KO mice. Surprisingly, these two types of mice both show impaired endocytosis. All these results indicate that H\(^+\)/Cl\(^-\) exchange is crucial for endocytosis. However, the exact role of ClC-5 is still poorly understood.

ClC-5 is the most well-studied human ClC transporter largely due to the fact that it can be robustly expressed at the plasma membrane in heterologous expression systems, like HEK293 cells and Xenopus oocytes [58,65]. According to electrophysiological studies, ClC-5 is voltage dependent and is active only when the membrane potential is more than 0 mV [62,66]. It only conducts outward currents, corresponding to Cl\(^-\) influx and H\(^+\) efflux [66]. In general, changes in intra- and extra-cellular pH and Cl\(^-\) ([Cl\(^-\)]\(_i\) and [Cl\(^-\)]\(_o\)) have a small effect on current density and activation of ClC-5. Decreasing pH\(_o\) from 7.4 to 5.4 reduces the current density and shifts activation to more positive
potentials. Changes in intracellular pH have a similar effect on voltage-activation but have little effect on current density. Meanwhile, changing \([Cl^{-}]_o\) causes a small change in current density, but does not affect voltage-activation. Increasing \([Cl^{-}]_i\) from 10 to 140 mM shifts both G-V and I-V curves to more positive potentials \([66]\). Despite the shifts in the G-V and I-V curves, no inward current is observed. Such an extreme outward rectification makes it infeasible to determine ClC-5 stoichiometry accurately using electrical measurements. Consequently, an optical method has been developed to specifically detect changes in the extracellular proton concentration close to the surface of *Xenopus* oocytes by means of a pH sensitive dye, BCECF and demonstrated a 2 Cl/1 H\(^+\) stoichiometry for ClC-5 \([67]\). Unfortunately, this method is not adaptable in mammalian cells as the detectable surface area is negligible.

A hypothetical model for the mechanism of Cl\(^-\) and H\(^+\) transport is proposed based on mutagenesis studies and the crystal structure of bacterial ClC-ec1 that has a similar molecular architecture to mammalian CIC proteins \([68,69]\). There are three anion binding sites: the external (S\(_{ext}\)) , central (S\(_{cen}\) ) and internal (S\(_{in}\) ) binding sites in the ion permeation pathway (Figure I-3B). A negatively charged residue, referred to as the “gating glutamate”, E\(_{gat}\) (E211 in ClC-5) is a central element for Cl\(^-\) and H\(^+\) transport. When E\(_{gat}\) is in an unprotonated form, its side chain swings from extracellular environment driving 2 chloride ions to the intracellular solution (Figure I-3B, b and c). Subsequently, E\(_{gat}\) accepts a proton from the intracellular proton pathway followed by a conformation change that delivers the proton to the extracellular solution (Figure I-3B, d
and e). However, the exact mechanism of this transport has not been fully experimentally verified yet.
Figure I-3. Proton-coupled transporters. (A) Five examples of proton-coupled transporters are shown. R-COO\(^-\) represents monocarboxylates such as lactate and pyruvate. FOL\(^-\) represents folate. (B) A schematic model of the ClC-5 transport cycle: the side chain of E211 is deprotonated (a) and swing to S\(_{\text{ext}}\) (b) to S\(_{\text{cen}}\) (c) and two Cl\(^-\) ions move intracellularly; in state (d), intracellular proton binds to E211 and move outwardly (e). (C) A schematic model of the MCT1 transport cycle: K38 is protonated (b) followed by R-COO\(^-\) binding; in state (d), proton and R-COO\(^-\) are translocated to D302 and R306, respectively; conformation changes after translocation and relax back after proton and R-COO\(^-\) being released intracellularly (e).
Monocarboxylates, such as pyruvate, lactate and ketone bodies are often products of carbohydrate, fat and amino acid metabolism and must be released across the plasma membrane rapidly for prevention from excessive acidosis. In some tissues (brain, heart, red skeletal muscle), they are also utilized as respiratory fuels and need to be taken up by cells [70–75]. Both types of transport are mainly mediated by proton-coupled monocarboxylic acid transporters (MCTs), including MCT1-MCT4 encoded by a SLC16 family of genes in mammals.

According to theoretical predictions and biochemical assays, these four MCTs have a similar topology comprised of 12 transmembrane (TM) domains with cytoplasmic N- and C- termini, one large intracellular loop between TM6 and TM7, and two highly conserved sequences in TM1 and TM5 [75]. Localization of MCTs to the plasma membrane requires association with an ancillary protein: basigin (CD147) or embigin (gp70) which has a single transmembrane domain, an immunoglobulin-like extracellular segment and a short cytoplasmic tail. It has been shown that MCT1, 3 and 4 associate with basigin [76,77], while MCT2 associates with embigin [78].

The structure of MCT1 derived from molecular modeling [73] and mutagenesis studies [79–82] reveals that K38 provides a binding site for the monocarboxylate anion, such as lactate after protonation (Figure I-3C, b and c). This binding event induces a conformational change which drives a translocation of lactic acid to the D302/R306 site (Figure I-3C, d). Meanwhile, K38 is deprotonated and the conformation relaxes, resulting in D302/R306 exposure to the intracellular environment and subsequent lactic acid
release (Figure I-3C, e) [79,80,82]. F360, which closes to D302/R306, is critical for the substrate selectivity. An F360C mutation enables MCT1 to transport mevalonate, a larger monocarboxylate [83].

MCT1 was first identified in Chinese hamster ovary (CHO) cells [83] and is the most well-studied MCT. Despite its ubiquitous tissue distribution, MCT1 exists in specific locations of cell membranes. For example, in isolated rat cardiomyocytes, MCT1 is more expressed in intercalated disc and T-tubule regions [84]. There is a broad range of substrates for MCT1, such as lactate, pyruvate, hydroxybutyrate, acetoacetate, acetate and butyrate. Although acetate and butyrate can be transported by MCT1, they mainly go across the plasma membrane by passive diffusion in undissociated forms because of their high pKa values [73]. Lactate is the most important substrate for MCT1. Interestingly, transport of lactate via MCT1 is stereoselective. In oocytes, the $K_m$ for D-lactate (> 60 mM) is ~ 17-fold higher than that for L-lactate (3.5 mM). Meanwhile, the transport of L-lactate is 2.7 times faster than that of D-lactate at 10 mM. MCT1 shows a higher affinity for pyruvate ($K_m$ = 1 mM), but the transport rate of pyruvate is only about half of that of L-lactate at 10 mM [85].

Several MCT inhibitors have been identified, such as 2-Cyano-4-hydroxycinnamate (CHC) and its analogs, phloretin, flavonois, stilbene disulphonates and AR-C155858. In particular, CHC has been widely used for the discovery and characterization of MCTs [85–90]. The $K_i$ value of CHC is 166 μM for MCT1 in mouse breast tumor cells, which is higher than that of phloretin (5.1 μM) [91]. AR-C155858 developed by AstraZeneca recently exhibits high specificity and affinity for MCT1 but no
activity for MCT4 [92]. It also can inhibit MCT2 only when it is associated with basigin rather than embigin. The inhibition is time dependent and also exists when injected intracellularly, suggesting that AR-C155858 probably binds to an intracellular site of MCT1.

Besides MCT1-4, there are ten other transmembrane proteins encoded by the SLC16 family of genes in mammals, named MCT5-14. Only MCT11 has been shown to be a proton-coupled MCT and most recently has been associated with type 2 diabetes [93]. MCT6 is likely to be a proton-coupled transporter, as it has been reported to facilitate the proton-linked transport of bumetanide but its natural substrate remains unknown [94]. The other identified MCTs are not proton-coupled and are either a thyroid hormone transporter (MCT8) [95] or a carnitine transporter (MCT9) [96] or an aromatic amino acid transporter (MCT10) [97]. MCT7 has been implicated in the hepatocyte secretion of ketone bodies during fasting [98]. Thus far, the substrates and functions of MCT12-14 are still unknown.

MCTs also exist in other organisms, such as Drosophila melanogaster, which is a useful genetic model organism for studying autophagy. There are 14 genes in Drosophila with variable homology to the members of the mammalian SLC16 family. However, only two have been functionally identified to transport monocarboxylates. Silnoon/dMCT1 mediates transport of butyrate and lactate and is crucial for triggering LKB1 dependent apoptosis [99]. Chaski that is enriched in glial cells has been identified more recently as a lactate/pyruvate transporter [100]. Baehrecke’s group in UMass Medical School also discovered a gene, CG11665/hrm that shows significant sequence similarity to SLC16
genes and is required for both autophagy during steroid-triggered salivary gland cell death and TNF-induced non-apoptotic eye cell death (unpublished). However, it is still unknown whether it functions as MCTs and what its substrates are.

Unlike ClC-5, transport of proton-coupled MCTs is non-electrogenic, making it challenging to measure MCTs activities. Radiolabeled substrates have been commonly used for activity measurements due to their high specificity and sensitivity [83,86,101–104]. The transport rate is measured by determination of intracellular labeled substrates after rapid termination. However, the diffusion and metabolism of substrates during the procedures may generate substantial errors. Genetically-encoded pyruvate and lactate fluorescent sensors have been developed for intracellular substrate measurements [93,105,106]. They enable a real-time measurement of transport, but their $K_D$ values limit the detectable concentration range of substrates. Because of the associated proton transport, the activity of MCTs can also be measured in real time by monitoring $pH_o$ or $pH_i$ using $pH_o$ or $pH_i$ electrodes [79,85,107,108] or $pH_i$ sensitive dyes, such as BCECF-AM [70,89,91,109–111]. Although electrodes and dyes enable determination of $pH_i$ and $pH_o$, the spatiotemporal visualization of MCTs activities at the plasma membrane has not been tenable with existing tools.

**Fluorescent pH Sensors**

Proton fluxes through ion channels and transporters are not only essential for regulation of intracellular pH, but also are involved in many cellular activities, such as signaling and motility [4,112–114]. Patch clamp is a common technique in
electrophysiology for studying ionic currents. It can detect proton currents from e.g. proton channels (Hv1) but is unable to directly reveal the identity of the transported ion(s) or the subcellular location(s) of activity. In contrast, proton-sensitive microelectrodes provide a direct readout of the extracellular pH, but only at a single macroscopic location abutting the cell. Despite their high sensitivity and precision, both techniques are time-consuming and challenging, and provide negligible spatial information. Given these constraints from electrical recordings, various fluorescent pH sensors have been developed and employed to visualize changes in intra- and extra-cellular pH.

**Small-molecule Fluorescent pH Sensors**

At present, many pH-sensitive fluorescent dyes have been developed and can be classified into two groups based on their sensing mechanisms. Fluorescent pH sensors, such as fluorescein, BCECF, SNARF and HPTS belong to the first group having fluorescence controlled by the ionization of phenolic hydroxyl groups (Figure I-4A). In particular, 2’, 7’-Bis-(2-carboxyethyl)-5-(and-6-)carboxyfluorescein (BCECF) developed by Roger Tsien in 1982 is the most widely used pH sensor [115]. Because it is a fluorescein-based dye, it forms a non-fluorescent lactone upon protonation. As pH increases, it becomes a fluorescent phenolate anion, and the wavelengths of the maximum absorption (\(\lambda_{\text{max} \text{ abs}}\)) and emission (\(\lambda_{\text{max} \text{ em}}\)) are 503 and 525 nm, respectively. The pK\(_a\) of BCECF is 7.0 which makes it suitable for sensing intracellular pH (6.8-7.4). Ratiometric pH measurements can be done with BCECF by measuring the ratio of emission intensity excited at \(\sim 490\) nm versus the emission intensity excited at its isosbestic point (\(\sim 440\))
nm). The commercially available acetoxyethyl ester of BCECF enters cells more easily and is retained inside cells after deacetylation by esterase. This ester version (BCECF-AM) has been used for monitoring pH$_i$ in real time when proton fluxes through membrane proteins, such as proton pumps [116], Hv1 channels [19], MCTs [73] and sodium-proton exchangers (NHEs) [117].

In the second group, the fluorescent dyes have an amine group that serves as a proton-sensing moiety and responds to pH changes under a Photoinduced Electron Transfer (PET) signaling mechanism (Figure I-4B). Because protonation of the amine group blocks PET-quenching, these pH sensors become more fluorescent as pH decreases. For example, pHrodo red and pHrodo green are rhodamine-based pH sensors introduced by Life Technologies [118]. With a pKa of ~ 6.5, both sensors have dim fluorescence above pH 8 and become more fluorescent in an acidic environment. Moreover, they do not photobleach significantly after 12 min of imaging and have been used for determining the engulfment of apoptotic cells by macrophages [119]. Recently, Aigner et al. have developed another rhodamine-based pH sensor that can be synthesized in only three steps [120]. It has a pH$_{1/2}$ of 6.9 and absorption/fluorescence maximum at 561 nm and 591 nm, respectively. Because it has two amine groups, protonation of both amines is required to block PET quenching completely (Figure I-4B). An extra carboxyl group in this sensor enables conjugation with other biomolecules of interest.
Figure I-4. Two pH-sensing mechanisms for small-molecule dyes. (A) Fluorescein-based sensors form non-fluorescent lactones upon protonation and become fluorescent with pH increasing. (B) Amine groups highlighted with red circles function as proton-sensing moieties and have PET-quenching effect in the unprotonated form. Protonation of both amine groups is required in this rhodamine-based sensor to block PET-quenching [120].
Genetically Encoded Fluorescent pH Sensors

The green fluorescent protein (GFP) and its variants have become an essential toolbox in biological research over the past two decades. Because GFPs are genetically encodable, they have extremely high specificity and have been widely used as reporters for gene expression, localization markers and biochemical monitors. All GFPs are pH sensitive, allowing them to serve in pH sensing applications. The first two GFP-based pH sensors, named ‘ratiometric’ and ‘ecliptic’ pHluorins are developed by Rothman group using structure-directed combinatorial mutagenesis [121]. Like native GFP, both pHluorins have dual excitation peaks at 395nm and 475nm. Upon acidification, the absorbance of ecliptic pHluorin decreases at both excitation peaks; ratiometric pHluorin, by contrast, exhibits a decrease in the excitation at 395 nm and an increase at 475 nm. Targeting ecliptic pHluorin to a vesicle membrane protein enables monitoring of vesicle exocytosis and recycling in real time. Although various GFP-based pH sensors have been introduced since then, the small range of fluorescence wavelengths (from 509 to 598 nm) limits their applications.

Monitoring Extracellular pH

Intracellular pH measurements are used for proton flux quantification. However, these measurements are not able to detect the kinetics of proton fluxes accurately, because the cell volume and intracellular buffering slow down the pH change. Furthermore, monitoring global pH changes does not directly reveal the subcellular location(s) of proton transport activities. Sensing extracellular pH, therefore, is a more
appropriate approach for detecting proton fluxes. Based on either genetically encoded fluorescent sensors or small-molecule fluorescent sensors, several strategies can be applied for attaching sensors to the plasma membrane.

**Expression of Genetically Encoded Fluorescent Sensors at Extracellular Surface**

One strategy is to fuse a GFP-based fluorescent sensor to an extracellular domain of a membrane protein (Figure I-5a). For example, Du et al. fused SE-pHluorin to syndecan 2, a postsynaptic membrane protein, and found a transient acidification followed by a slower alkalization at the extracellular space of spines and the neighboring dendrites after stimulating cortical inputs [112]. Because this approach is accomplished by gene editing, it has high specificity and enables long-term imaging *in vitro* and *in vivo*. Limitations of this approach include possible poor trafficking and low signal to noise due to low expression after fusion.

**Conjugation of Small-Molecules Fluorescent Sensors to The Extracellular Surface**

Small-molecule fluorescent sensors can be modified through synthesis and can be conjugated to either membrane proteins, lipids, or carbohydrates at the extracellular surface by using semisynthetic approaches. For example, to image exocytosis and endocytosis, Martineau et al. attached pH-sensitive dyes to either expressed self-labeling tags such as SNAP-tag on the membrane proteins of synapse vesicles, or to antibodies that recognize native vesicular proteins [122]. Similar strategies can also be applied at the plasma membrane (Figure I-5c and d).
Small-molecule dyes can be anchored to lipids directly (Figure I-5b). Stock et al. labeled extracellular lipid head groups with DHPE-fluorescein for studying pH nanoenvironment at the extracellular surface of single melanoma cells [123]. To make a comparison, they used a wheat germ agglutinin (WGA)-fluorescein conjugate for glycocalyx labeling and found that pH\textsubscript{o} in melanoma cells is higher right at the plasma membrane than in the glycocalyx (Figure I-5f). Ke et al. developed a lipid-DNA scaffold consisting of a hydrophobic diacyllipid tail and a hydrophilic DNA strand [124]. For extracellular pH sensing, this scaffold is further modified with two fluorescent dyes: one is pH sensitive and the other is pH insensitive as internal reference.

Besides WGA conjugates, a glycocalyx engineering methodology has been applied for covalently binding small-molecule dyes to the glycocalyx (Figure I-5e). In this method, cells are incubated with synthetic monosaccharides having similar structures to natural precursors but bearing unnatural functional groups, such as azido-, alkyne-, ketone-, and thiol- [125]. These unnatural sugars enter the cell and are utilized as the natural precursors for the biosynthesis of cell surface glycans. The resulting glycans carry the functional groups that can covalently binding with small-molecule dyes. Bertozzi’s group, for instance, has developed tetraacetylated N-Azidoacetyl-D-Mannosamine (Ac\textsubscript{4}ManNAz) to incorporate azido- groups into the cell’s glycocalyx which can be subsequently labeled with fluorescent dyes via bioorthogonal reactions, such as copper-free ‘click’ chemistry for imaging glycans [126].
Figure I-5. Strategies for cell-surface labeling with fluorescent pH sensors.
Genetically-encoded pH sensors can be fused to a membrane protein (a). Small-molecule pH sensors can be conjugated to artificial lipids (colored pink) (b) or bind to a membrane protein with SNAP-, CLIP- or Halo-tag (c) or be conjugated to an antibody for a membrane protein (d) or covalently bind to an unnatural functional group (colored blue) after glycocalyx engineering (e) or be conjugated to WGA that binds to terminal sugar residues.
Sensing the extracellular pH changes via fluorescent methods can provide spatiotemporal information of proton-transport activities to understand their roles under physiological and pathophysiological roles. Although several fluorescent methods have been developed for monitoring pH changes at various location in and around a cell, the real-time and spatiotemporal visualization of proton fluxes through proton channels or proton-coupled transporters has not yet been reported.

**Outline of Thesis**

In my thesis work, I focus on developing optical approaches for visualizing cellular proton fluxes through ion channels and transporters.

Chapter II illustrates the development of an optical approach by means of glycocalyx engineering and copper-free ‘click’ chemistry. The dense coating of extracellular facing pH sensors provides real-time detection of proton accumulation and depletion from proton channel Hv1, H⁺/Cl⁻ antiporter ClC-5 and omega mutants *Shaker-IR* R362H and R371H. Moreover, the proton wavefront emanating from one cell is clearly visible when it envelopes neighboring cells.

Chapter III describes the utilization of the approach developed in Chapter II for monitoring non-electrogenic transport of proton-coupled MCTs. Compared to intracellular pH measurement, our approach shows proton depletion followed by a recovery at the cell surface upon extracellular perfusion of L-lactate or pyruvate in HEK293T cells expressing MCT1. Furthermore, by using this approach, we functionally identified a putative MCT, Hrm that is involved in autophagy in *Drosophila*. 
Because the Chapter II approach is not applicable in all cell types, Chapter IV describes the development of another approach using a pH sensitive, fluorescent WGA conjugate, WGA-pHRho to measure the extracellular pH. Compared to the Chapter II approach, WGA-pHRho has a similar fluorescent response to proton fluxes resulting from proton channel Hv1 and omega mutant Shaker-IR R362H. By using WGA-pHRho, we monitored proton-coupled MCT1 activity in MCT1-expressing INS-1 cells and isolated rat cardiomyocytes that cannot use unnatural sialic acid into their glycocalyx.

The development of these two optical approaches provides new tools for further understanding functions of cellular proton fluxes and functional identification of proton-coupled transporters.
CHAPTER II. A BIOORTHOGONAL CHEMISTRY APPROACH FOR VISUALIZATION OF PROTON FLUXES AT THE PLASMA MEMBRANE

Summary

Cells use plasma membrane proton fluxes to maintain cytoplasmic and extracellular pH and to mediate the co-transport of metabolites and ions. Because proton-coupled transport often involves movement of multiple substrates, traditional electrical measurements provide limited information about proton transport at the cell surface. To visualize proton fluxes specifically over the entire landscape of a cell, in Chapter II we synthesized a small-molecule fluorescent pH sensor and attached it to the cell surface by means of glycocalyx engineering and copper free ‘click’ chemistry. The extracellularly facing sensors enable real-time detection of proton accumulation and depletion at the plasma membrane, providing an indirect readout of channel and transporter activity that correlated with whole-cell proton current. Moreover, the proton wavefront emanating from one cell was readily visible as it crossed over nearby cells. Given that any small-molecule fluorescent sensor can be covalently attached to a cell’s glycocalyx, our approach is readily adaptable to visualize most electrogenic and non-electrogenic transport events at the plasma membrane.
Introduction

Proton fluxes mediated by voltage-gated proton channels and proton-coupled transporters are critical for maintaining intracellular pH [30], driving co-substrate transport [127], regulating gastric [128] and airway mucosal acidity [129] and providing electrogenic shunts for phagocytic and endocytic processes [130]. In addition, Gain-of-function mutations that create unregulated proton pores in voltage-gated cation channels have been implicated in myotonias, periodic paralysis, and some forms of long QT syndrome [46]. Given their varied physiological and pathological roles, proton transport proteins are viable therapeutic targets for treating human diseases - the most successful target has been the gastric proton pump, where irreversible inhibition of this proton flux mitigates gastrointestinal reflux disease [131].

Electrical recordings have unequivocally established that ion accumulation and depletion occur at the plasma membrane during normal and pathophysiological activity. However, these electrical measurements do not directly reveal the identity of the transported ion(s) or the subcellular location(s) of activity, nor do they report on non-electrogenic transport events. In contrast, proton-sensitive microelectrodes provide a direct readout of the extracellular pH [132,133], but only at a single macroscopic location abutting the cell. Because both techniques are time consuming, challenging and provide negligible spatial information, various small-molecule and genetically encoded fluorescent pH sensors have been developed for visualizing pH changes in the cytoplasm and intracellular compartments [121,134–137]. Moreover, extracellular pH has been measured by using WGA-fluorescein conjugates [123], lipid-anchored pH sensors
[123,124,138] and genetically encoded pH sensors fused with membrane proteins [112]. Although these reagents have enabled pH determinations at various locations in and around a cell, the real time visualization of voltage-dependent proton fluxes at the plasma membrane has not yet been reported.

Here we used glycocalyx engineering and copper-free ‘click’ chemistry to covalently label the cell surface with small-molecule fluorescent pH sensors to visualize proton fluxes at the plasma membrane. Using voltage-clamp fluorometry to simultaneously control and visualize proton transport, we observed robust fluorescent signals that corresponded to cellular proton fluxes in both directions: outward and inward. The real-time kinetics of proton accumulation and depletion at the cell surface directly correlated to current density and buffer capacity, permitting the fluorescent signal to serve as a surrogate of ion channel and membrane transporter activity. The proton selectivity and sensitivity of the fluorescent signal enabled the determination of the proton current through H+/Cl− antiporter ClC-5 and the visualization of omega proton fluxes in the presence of a fully functioning voltage-gated Shaker potassium channel, where 6% of the total current was carried by protons. Strikingly, the resultant proton wavefront emanating from one cell was readily detected as it enveloped adjacent cells that were covalently modified with pH sensors.
Results

Chemistry, Cell Surface Labeling and Characterization

Compared to targeting or overexpressing a specific membrane protein, we hypothesized that a bioorthogonal chemical reaction with the cell’s glycocalyx would result in a uniform and dense coating of fluorescent pH sensors at the cell surface to enable visualization of extracellular proton fluxes. To covalently modify the cell surface with small molecule fluorescent pH sensors, we used metabolic engineering with a membrane permeant peracetylated unnatural sugar to install azido groups into the cell’s glycocalyx [139,140], which were subsequently modified with pH-DIBO — an azido-reactive, rhodamine-based pH sensor (Figure II-1A). The photoinduced electron transfer (PET) rhodamine pH sensor (pH-DIBO) was synthesized using a modified literature procedure [120] where the piperazine groups were Boc-protected to simplify purification (Figure II-1B). A pendant amine group was attached to 2 by first reaction with 2-ethylcarboxymercaptan followed by peptide coupling with 1,2-bis(2-aminoethoxy)ethane. Reaction of 2 with the 4-nitrobenyl ester of DIBO, and subsequent removal of the Boc groups with TFA yielded pH-DIBO in six steps from 3-(1-piperazinyl)phenol.
Figure II-1. Glycocalyx engineering strategy to visualize plasma membrane proton fluxes. (A) Cells expressing ion channels or membrane transporters are incubated with an azidosugar (azido group in blue) and the cell surface is subsequently labeled with a fluorescent pH sensor (pH-DIBO) to detect proton fluxes. The azide-reactive and fluorescent pH-sensitive groups in the chemical structure and label are colored blue and red, respectively. (B) Scheme for the synthesis of pH-DIBO.
To fluorescently visualize proton fluxes using pH-DIBO, Chinese hamster ovary (CHO) cells were transiently transfected with a GFP-tagged, human voltage-gated proton channel (Hv1) and incubated with tetraacetylated N-azidoacetyl-D-mannosamine (azidosugar) [139,140]. After 2 days, the cells were labeled with pH-DIBO for 30 min and the currents and fluorescence were measured using patch-clamp fluorometry in a bath solution with a low buffer capacity (0.1 mM). Figure II-2B (left panel) shows families of currents and fluorescent signals from voltage-clamped CHO cells expressing Hv1; HEK293 cells are shown in the right panel of Figure II-2B. Voltage-activation of Hv1 resulted in channel opening with voltage-dependent kinetics that reached a steady state with test voltages greater than 20 mV. Simultaneous fluorescent imaging revealed that the fluorescent signals mirrored both the current magnitude and kinetics of voltage-activation at the various depolarizations. In contrast, Hv1 channel closing at –80 mV was much faster than the decaying fluorescent signal, consistent with the accumulated protons diffusing into bulk solution. Both the voltage-dependent currents and fluorescence signals were specific for Hv1 channels because they were inhibited by Zn$^{2+}$ (Figure II-3A) and required transfection with Hv1 DNA (Figure II-3B). In addition, incubation with azidosugar was required to observe a significant change in fluorescence upon Hv1 channel opening (Figure II-3C and D). Cells incubated with azidosugar showed diffuse fluorescence over the entire cell with some higher intensity clusters (Figure II-2A, 3C and 3D). In contrast, the fluorescent signals from vehicle-treated cells were challenging to detect except for a few bright puncta (Figure II-3C and 3D). Because DIBO has been shown to react with thiols [141], we chemically-inactivated pH-DIBO and repeated the
labeling experiments. Comparing TIRF images of cells labeled with chemically-inactive or active pH-DIBO (Figure II-3C) revealed that the punctate labeling in the absence of azidosugar was due to pH-DIBO non-specifically modifying the cell surface. Although some background labeling occurs with the DIBO group, the cells must be incubated with azidosugar to coat the glycocalyx with enough pH sensors to fluorescently detect cellular proton fluxes (Figure II-3D).
Figure II-2. Visualization of proton efflux from CHO and HEK cells expressing Hv1
(A) GFP and pH-DIBO fluorescence of CHO cells expressing Hv1. (B) Voltage-clamp fluorometry current and fluorescence traces of CHO (left) and HEK293 (right) cells expressing Hv1. Cells were held at –80 mV, and currents and fluorescence were elicited from 4-s command voltages from 0 to 100 mV in 20 mV increments. (C) ∆F/∆F₀ snapshots of pH-DIBO fluorescence at 100 mV at time points indicated in (B). pH₀/pHᵢ = 7.5/6.0; 0.1 mM HEPES in bath solution. Voltage-clamp fluorometry scale bars represent 50 pA, 2% ∆F/∆F₀, and 1 s; fluorescent image scale bars represent 5 µm.
Although the observed change in fluorescence was consistent with an outward proton flux, we wanted to confirm that the voltage-dependent changes in fluorescence were due to proton accumulation on the extracellular side of the membrane. To test this, we increased the buffer capacity in the external bath solution to compete with extracellularly-facing pH-DIBO sensors (Figure II-3E). To normalize the cellular proton flux under each buffer condition, the cells were depolarized such that the same steady-state current (~ 250 pA) was reached after 4 s. As expected for extracellular proton accumulation, increasing the buffer capacity from 0.1 to 10 mM monotonically reduced the change in fluorescence until no change was detectable even though Hv1 channels were open and conducting during the depolarizing test pulse (currents not shown). The competition between buffer and the glycocalyx-attached pH-DIBO sensors was also visualized by rapidly jumping to different depolarizing voltages and monitoring the rates to reach steady-state fluorescence (Figure II-4). These experiments also ruled out saturation of the glycocalyx-attached sensors as the cause of the steady-state fluorescence.

To convert the steady-state fluorescence into pH at the cell surface, we varied the pH of the bath solution and measured the change in fluorescence (Figure II-3F). The fluorescence response of glycocalyx-attached pH-DIBO and pH-DIBO sensors in solution was linear over the same pH range, indicating that cell surface attachment did not appreciably change the pKₐ of the sensor. Thus, the 20% change in fluorescence in Figure II-2B corresponded to a pH of ~ 6.5 at the extracellular side of the membrane.
Figure II-3. Fluorescent signals require Hv1 transfection and azidosugar. Voltage-clamp fluorometry current and fluorescence traces of Hv1-expressing CHO cells in the presence (left panel) or absence (middle panel) of 1 μM Zn^{2+} (A) and pcDNA-transfected CHO cells (B). Cells were held at –80 mV, and currents and fluorescence were elicited from a 4-s command voltage (100 mV). pH_{o}/pH_{i} = 7.5/6.0; 0.1 mM HEPES in bath solution. Scale bars represent 50 pA, 2% ΔF/F_{0}, and 1 s. (C) TIRF images of cells incubated with or without azidosugar for 48 h and treated with either pH-DIBO or inactivated pH-DIBO (50 μM, 30 min). pH-DIBO was inactivated by 3-azido-1-propanol (100 eq, 24 h). (D) Average normalized fluorescence at –80 mV and after a 4-s depolarization (100 mV) in the presence (+) or absence (–) of azidosugar. Data were averaged from five cells; error bars are ±SEM (*p < 0.05). (E) Fluorescence traces of cells expressing similar total current (150-300 pA) in bath solutions with various buffer concentrations (HEPES), n = 5; error bars denote ±SEM. (F) Change in pH-DIBO fluorescence attached to the cell surface (filled circles) and in a solution (open squares) versus pH. Buffer concentration 10 mM; ΔF/F_{0} at pH 7.5 was defined as 0. Data were averaged from 5-10 experiments; error bars are ±SEM.
Figure II-4. The competition between buffer and the glycocalyx-attached pH-DIBO sensors. Voltage-clamp fluorometry current and fluorescence traces of Hv1-expressing CHO cells for a series of voltage step-ups (left) and step-downs (right). Scale bars represent 100 pA, 2% ΔF/F₀ and 2 s; pHₒ/pHᵢ = 7.5/6.0, 0.1 mM HEPES in bath solution.
One component of the fluorescent signal that could not be easily explained was
the biexponential decay kinetics that were observed when the channels were closed by
repolarization (Figure II-2B, 3A, 3E, 4 and 5). The slower time constant was consistent
with proton diffusion because it was dependent on buffer concentration (Figure II-3E);
however, the initial drop in fluorescence was independent of buffer concentration. We
initially dismissed inward proton currents through Hv1 channels because the outward
proton gradient used would require hyperpolarization less than –90 mV. However, it has
been recently shown that Hv1 channels can deplete the local proton concentration on the
intracellular side of the channel [39], raising the possibility that the local proton driving
force could be vastly different than the bulk proton gradient. Indeed, holding Hv1
channels open for 4 s at 100 mV in the absence of a pH gradient created a substantial
inward proton gradient, which was detected as an inward tail current when the channels
were closed at 0 mV (Figure II-5, blue trace). Under these experimental conditions, the
fast component of the fluorescent decay was directly proportional to the channel closing
rate, demonstrating that the initial drop in fluorescence was in fact due to protons re-
entering the cell through Hv1 channels before they closed (Figure II-5).
Figure II-5. Tail currents and pH-DIBO fluorescence decay at different closing voltages. Cells were held at –80 mV, depolarized to 100 mV, and tail currents were elicited at 0 mV (blue), –40 mV (red), and –80 mV (black). Current and fluorescence traces of the entire voltage protocol are shown on the left. Scale bars represent 50 pA, 2% ΔF/F₀ and 2 s. The tail regions are enlarged on the right. Scale bars represent 25 pA, 2% ΔF/F₀ and 0.5 s. No pH gradient was used: pHₒ/pHᵢ = 7.0/7.0, 0.1 mM HEPES in bath solution.
We next examined whether the pH-DIBO signals correlated with Hv1 channel localization by comparing the whole-cell fluorescent images. The pH-DIBO fluorescent image shows diffuse staining over the entire cell surface, though some large clusters of sensors were always observed as shown in the exemplar in Figure II-2A. Surprisingly, Hv1 channel localization was similar to pH-DIBO labeling, including the overlapping smiley emoji observed in both panels (Figure II-2A). Because Hv1 channels cannot be N- or O-glycosylated (they do not contain any extracellular serines or threonines), we hypothesized that the unusual convergence was due to pinocytosis of pH-DIBO-labeled glycoproteins into acidic compartments that overlapped with intracellular Hv1 channels. Although the images collected contained both in- and out-of-focus light, we used the voltage-dependent change in the pH-DIBO signal to isolate the population of sensors that were facing the extracellular milieu. Figure II-2C shows three F–F₀ snapshots before, during, and after a 100-mV test pulse (Movie II-1). In contrast to the clusters observed in Figure II-2A, the pH-DIBO signal in these processed images was scattered over the entire cell, indicating that functioning Hv1 channels were not clustered, but were randomly distributed over the cell surface.

**Disentangling Proton Efflux from Ionic Currents**

To characterize the glycocalyx-attached pH sensors, we used a perfectly proton selective channel, Hv1 [30], However, many membrane transport proteins permit the simultaneous passage of a proton with a different ion or ions, which will contaminate or completely obscure the proton current. Omega currents from voltage-gated cation
channels are a class of proton currents that have been postulated to cause several human diseases [46–48,50], but have not been observed without destroying or blocking the ion conducting pore domain. Therefore, we used our approach to determine whether these gain-of-function voltage sensor domain mutations create omega proton currents in a channel with a functioning central pore. For the omega proton current, we used a Shaker-IR K⁺ mutant (R371H) [45], which is expected to form four voltage-dependent proton pores that circumscribe the central potassium pore (Figure II-6A). Figure II-6B shows current traces and fluorescent signals from cells expressing Shaker-IR R371H and WT channels. Depolarization-elicited Shaker-IR R371H currents activated rapidly and slowly inactivated over the 4-s depolarization. The rapid activation kinetics, large currents, and hallmark voltage-dependent C-type inactivation indicated that the majority of the current was flowing through the central pore. In contrast to the current traces, the changes in pH-DIBO fluorescence were indicative of a small, but relatively constant voltage-dependent proton current that modestly lowered the extracellular pH. Using the calibration in Figure II-3F, the ~ 3% change in fluorescence at the end of the 120-mV pulse equated to a ~ 0.05 pH change on the extracellular side of the membrane. No omega proton fluxes were fluorescently detected with WT Shaker-IR, which expressed 10-fold more total current than the R371H mutant and had unusual inactivation kinetics with a 120 mV 4-s depolarization.
Figure II-6. Visualization of proton efflux from co-transport proteins expressed in CHO cells. (A) Cartoons of Hv1, ClC-5 and Shaker omega mutant (R371H). (B) Voltage-clamp fluorometry current and fluorescence traces of cells expressing Shaker-IR R371H (scale bars: 100 pA, 1% ΔF/F₀, 1 s) from 0 to 120 mV in 40-mV increments and WT Shaker-IR at 120 mV (scale bars: 1 nA, 1% ΔF/F₀, 1 s); holding potential – 80 mV, pHₒ/pHᵢ = 7.5/6.0, 0.1 mM HEPES in bath solution. (C) Voltage-clamp fluorometry current and fluorescence traces of cells expressing ClC-5 from 60 to 140 mV in 20-mV increments and ClC-5 E211A at 140 mV. Holding potential – 80 mV; pHₒ/pHᵢ = 7.5/7.5, 0.1 mM HEPES in bath solution. Scale bars represent 50 pA, 2% ΔF/F₀, and 1 s. (D) Plot of ΔF/F₀ as a function of current for Hv1 and ClC-5.
Because we could specifically visualize proton efflux in the presence of a functioning potassium pore, it was possible to calculate the omega proton current’s contribution to the total current. To convert the pH-DIBO signal into current, we needed to determine whether the fluorescent signal could serve as a surrogate for the whole-cell proton current. Therefore, we compared the current traces and fluorescent signals from cells expressing the CIC-5 antiporter that transports two chloride ions for every exported proton (Figure II-6A) [67]. Cells expressing CIC-5 gave rise to near instantaneous voltage-dependent currents that do not inactivate (Figure II-6C). Both the magnitude and rate of \( \Delta F/F_0 \) proportionally increased with the total current, indicating that a constant proton source was activated with depolarization. These changes in fluorescence were proton-specific because they were not observed with a CIC-5 mutant (E211A) [63] that only conducts chloride (Figure II-6C, lower panels). To calibrate the fluorescent signals, we plotted the linear fits of \( \Delta F/F_0 \) vs. current for Hv1 and CIC-5 expressing cells (Figure II-6D) and found that the slope of the CIC-5 data was approximately 1/3rd of Hv1.

Because the fluorescent pH-DIBO signal was directly proportional to the whole-cell proton current, the maximum change in fluorescence for Shaker-IR R371H corresponded to ~ 40 pA of proton current (Figure II-6B). Thus, the omega proton current is only 6% of the total Shaker-IR R371H current.

**Visualizing Extracellular Proton Depletion and Proton Wavefronts**

In addition to the selective visualization and quantification of proton efflux in the presence of other ion currents, glycocalyx-attached pH-DIBO sensors should faithfully
report on changes in proton concentration at the cell surface regardless of the proton source or direction. Therefore, we labeled cells expressing \textit{Shaker-IR R362H} [32] with pH-DIBO to visualize inward proton currents at hyperpolarizing potentials (Figure II-7A). To directly compare the inward fluorescent signals to the outward Hv1 signals, we intentionally blocked the potassium channel pore (W434F) [142]; thus, the currents shown in Figure II-7A are only omega currents. The change in fluorescence with \textit{Shaker-IR R362H} was the mirror opposite to Hv1 (Movie II-3): the cell surface fluorescence became dimmer upon hyperpolarization, reached steady-state, and then rapidly recovered with biexponential kinetics when the cell was returned to the 30-mV holding potential (Figure II-7A, bottom panel). In addition, the absolute value of the current-\(\Delta F/F_0\) slope (0.82 ± 0.09) was similar to Hv1, indicating that \textit{Shaker-IR R362H} is a proton selective omega current that readily depletes protons on the extracellular side of the membrane.

While performing the proton flux experiments, we noticed the pH-DIBO-labeled cells adjacent to the patch-clamped cell fluoresced with a delayed synchrony, as if the neighboring cells were reporting on the proton efflux of the depolarized cell (Movie II-5). To test this supposition, we measured the change in fluorescence on each half of a neighboring cell (near and far) and plotted it against the voltage-clamped cell expressing Hv1 channels (Figure II-7B). The clamped, near, and far fluorescent signals were consistent with a proton wavefront originating from the clamped cell that first reached the near side of the adjacent cell and then gradually enveloped the far side of the cell. After a 4-s depolarization, the extracellular proton concentration was the greatest around the clamped cell, but a substantial proton gradient was also detected over the surface of the
neighboring cell that was four microns away. Upon turning off the proton source (clamped cell), the extracellular proton gradient across the neighboring cell decayed with a single time constant (Figure II-7B, right panel), reaffirming that the rapid drop in fluorescence observed with the patch-clamped cells was due to protons rapidly entering the cytoplasm before Hv1 channel closure. As expected for an extracellular proton wavefront, the pH surrounding a neighboring cell was titratable with buffer concentration (Figure II-7C). In total, these data demonstrated that the glycocalyx-attached pH-DIBO sensors exquisitely report on the extracellular pH at the plasma membrane, enabling the visualization of both proton fluxes and wavefronts.
Figure II-7. Visualization of extracellular proton depletion and wavefronts in CHO cells. (A) Voltage-clamp fluorometry traces of cells expressing Shaker-IR R362H/W434F. Cells were held at 30 mV, and currents and fluorescence were elicited from 4-s command voltages from –40 to –120 mV in 20-mV increments. pH_o/pH_i = 6.0/7.5, 0.1 mM MES in bath solution. Scale bars represent 50 pA, 2% ΔF/F_0, and 1 s. (B) Left: cartoon of proton diffusion to a neighboring cell. Right: fluorescent signals of pH-DIBO-labeled cells. A voltage-clamped Hv1-expressing cell (~80 mV) was depolarized to 100 mV and the fluorescent signals from the clamped (d = 23 μm) and a neighboring cell (near and far side, d = 18 μm) were plotted versus time. The distance of the two cells is ~4 μm. pH_o/pH_i = 7.5/6.0, 0.1 mM HEPES in bath solution. Scale bars represent 2% ΔF/F_0 and 1 s. (C) Buffer concentration dependence of the fluorescent signals of a neighboring cell ~8 μm away from an Hv1-expressing cell. The clamped cells were held at ~80 mV, depolarized to 100 mV, and the fluorescence of the clamped and neighboring cells were plotted versus time. pH_o/pH_i = 7.5/6.0, 0.1 or 1 mM HEPES in bath solution. Scale bars represent 2% ΔF/F_0 and 1 s. (D) Cartoon and proton diffusion equation of the concentric volume model with an unstirred layer (USL). (E) Fits of the fluorescent data in (A). Bulk = 1 mL; USL = 1.5 fL; shell = 0.08 fL; K_{USL} = K_B = 1; k_f = k_f = 1.4 s^{-1}; k_{in} (black) = 0.090 s^{-1}; k_{in} (red) = 0.070 s^{-1}; k_{in} (blue) = 0.050 s^{-1}; k_{in} (magenta) = 0.030 s^{-1}; k_{in} (green) = 0.013 s^{-1}.
Discussion

By covalently attaching small molecule pH-sensitive fluorophores (pH-DIBO) directly to the cell’s glycocalyx, we observed both inward and outward proton fluxes, extracellular pH gradients, and proton wavefronts enveloping neighboring cells. Although a subpopulation of sensors is internalized, these luminally-facing fluorescent sensors are unresponsive to plasma membrane activity. As designed, the glycocalyx-attached pH-DIBO sensors did not report on ion channel gating or transporter activation/deactivation, but rather the kinetics of proton accumulation or depletion on the extracellular side of the membrane. The juxtaposition of the covalently-attached pH-DIBO sensors to the extracellular vestibules of membrane transport proteins provided remarkable insight into the extracellular pH environment abutting a mammalian cell. Previous determinations of extracellular pH at the cell surface required either extremely large cells [67] or mathematical modeling [41] to estimate the radiating pH gradient. Our results suggest that Hv1 proton channels create a much more acidic extracellular environment than was previously calculated. For example, the pH surrounding the clamped cell in Figure II-7B is ~ 6.3 whereas the calculation [41] for a similarly-sized spherical cell is ~ 7.2. Part of the difference may be due to limited proton diffusion in the restricted space between the cell and the glass coverslip. However, the pH gradient (6.8 – 7.2) observed across neighboring cells indicates that the majority of the protons emanating from a patch clamped cell are freely diffusing in the bath solution. These proton accumulation and diffusion results also highlight that open Hv1 channels not only
deplete the local intracellular proton concentration [39], but also raise the extracellular proton concentration tens of microns away from the cell perimeter.

Because the glycocalyx-attached pH sensors are well within the experimentally and computationally determined unstirred layer (USL) for protons [143], our approach provides an opportunity to monitor proton diffusion over the entire cell surface during proton transport. The slow opening and closing of Hv1 channels confounds modeling these data; however, the fluorescent data from the rapidly closing Shaker-IR R362H omega pores (Figure II-7A) are amenable to fitting. Proton depletion from the glycocalyx shell upon channel opening could be well fitted with a simple equation:

$$[\text{cell}] \xleftarrow{k_{\text{in}}} [\text{shell}] \xrightarrow{K_B} [\text{bulk}]$$

where shell corresponded to the approximate volume of the cell’s glycocalyx, $K_B$ was the proton equilibrium constant between bulk and shell, and $k_{\text{in}}$ correlated with the steady state current at each depolarizing voltage. However, upon channel closing, the model failed to recapitulate the slow phase of the biphasic kinetics of proton replenishing shown in Figure II-7A (bottom panel); therefore, an additional layer (USL) and equilibrium constant ($K_{\text{USL}}$) between the glycocalyx shell and an unstirred layer was added to the model (Figure II-7D). For the modeling, we varied $k_{\text{in}}$, the volumes of the USL and shell, and the rate of proton entry and exit into a layer. In addition, we assumed the equilibrium constant between any layer was equal to one and the forward and backward proton rates into a layer (shell, USL, and bulk) was the same. At each voltage (Figure II-7E), $k_{\text{in}}$ was varied to achieve the steady state fluorescence after 4 s, which correlated well with the total number of protons that entered the cell (average current)
during the hyperpolarizing pulse (Figure II-7A, top panel). In contrast to the simple shell model, this concentric volume model fit the biphasic return of protons to the glycocalyx shell (Figure II-7E). Because $k_{in}$ was held constant at each voltage, the model poorly fit the kinetics of proton influx at hyperpolarizing voltages where channel gating, and thus current, was not constant during the test pulse (Figure II-7A, top panel). In addition, the local proton concentration in the shell and equilibrium between the layers is likely more complicated due to the cell surface proteins, carbohydrates, and covalently-bound pH sensors in the glycocalyx.

Although we could fit the fluorescent signal kinetics of proton replenishing at the cell surface, the model does not speak to how the unstirred layer fills (or empties) during proton transport. Several studies [144–147] suggest that during proton efflux the protons diffuse rapidly and parallel to the plasma membrane before entering the unstirred layer and bulk solution. The neighboring cell experiments in Figure II-7B and C demonstrate that it is experimentally feasible to utilize glycocalyx-attached pH sensors to spatiotemporally detect pH differences at the plasma membrane. However, in our experiments, the proton channels and transport proteins were randomly distributed over the entire cell surface; thus, we did not detect rapid proton diffusion parallel to the plasma membrane upon activating proton efflux with voltage. Visualization of proton efflux from a proton transport protein that localizes to a specific region of the plasma membrane would provide insight on whether protons rapidly circumscribe a living cell before diffusing into the unstirred layer and bulk solution.
An unexpected advantage of directly attaching the sensors to the glycocalyx was that the change in pH-DIBO fluorescence was an accurate approximation of the whole cell proton current in voltage-clamp experiments. By comparing the CIC-5 current-ΔF/F₀ slope to Hv1, we showed that only 1/3rd of the CIC-5 current is carried by protons, fully consistent with the known 2:1 stoichiometry of its transport cycle [67]. In contrast to CIC-5, Shaker-IR omega currents were as proton-selective as Hv1 channels. Using the current-ΔF/F₀ relationship, we estimated that the proton current accounted for ~ 6% of the total current for a Shaker-IR VSD omega mutant. If all four VSDs pass the same amount of proton current, then our experimental results indicate that a single omega pore contributes 1 - 2% of the total current in a voltage-gated K⁺ channel. Because VSD mutations affect the potassium current through the central pore by shifting the voltage-activation, deactivation, and inactivation of the channel, we expect the proton current’s contribution to the total current will vary for the different omega pore mutants that cause human disease. No proton fluxes were ever observed with wild-type voltage-gated K⁺ channels, confirming that the histidine residues in the VSDs are required for the omega current in full-length Shaker-IR channels. In addition to being a surrogate for proton current, voltage-clamp fluorometry with pH-DIBO will also be useful for identifying residues required for proton permeation and exploring the voltage-dependency of non-electrogenic proton transport proteins.

Because proton transport is involved in a wide range of cellular processes, chemical tools that specifically detect plasma membrane proton fluxes have broad utility. The diverse color and pKₐ palette of small molecule proton sensors [134] provide
additional flexibility to coat cells with fluorophores with desirable pH-sensing and photophysical properties. Combining our chemical approach with state-of-the-art imaging techniques will allow for high-resolution spatiotemporal imaging over the entire landscape of a cell and possibly visualization of single-channel fluxes. Given that the glycocalyces of living organisms can be engineered with unnatural sugars [126,148,149], visualization of rapid efflux from cells, tissues, and model organisms is tenable by modifying metabolically-labeled glycocalyces with small molecule ion- or metabolite-sensitive fluorophores.
Material and Methods

Molecular Biology

Plasmids containing human Hv1, ClC5, and Shaker-IR omega mutants were gifts from David Clapham (Harvard Medical School), Michael Pusch (Istituto di Biofisica), and Baron Chanda (University of Wisconsin–Madison), respectively. Mutations were introduced by QuikChange site-directed mutagenesis (Agilent) and confirmed by DNA sequencing the entire gene.

Cell Culture, Transfections and pH-DIBO Cell Surface Labeling

Chinese Hamster Ovary-K1 (CHO-K1) cells were cultured in F-12K nutrient mixture (Invitrogen); HEK293 cells were cultured in high glucose DMEM medium. All media were supplemented with 10% fetal bovine serum (Hyclone) and 100 units/mL penicillin/streptomycin (Invitrogen). The cells were plated at 60 ~ 75% confluency in 35 mm dishes. Glass bottom culture dishes (MatTek) were used for HEK293 cells. 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 6μL PLUSTM reagent (Invitrogen) for HEK293 cells in Opti-MEM (Invitrogen). To visualize transfected cells, 0.25 μg of pEGFP-C3 was added to transfections that did not contain GFP-tagged channels/transporters. After terminating the transfections, the cells were incubated in media containing 50 μM azido-sugar for 2 days, which was replenished after 24 hrs.
Whole Cell Patch Clamp Fluorometry

Transfected CHO cells were trypsinized and seeded on a glass bottom culture dish for 2 hrs and then labeled with pH-DIBO (50 μM) in Opti-MEM at r.t. for 30 min; HEK293 cells were directly labeled with pH-DIBO in Opti-MEM. Transfected cells were identified using an inverted light microscope (Axiovert 40 CFL; Carl Zeiss, Inc.) and the currents were recorded in the whole cell patch configuration at room temperature (24 ± 2°C) using a glass electrode (pipette resistance: 2.5 – 3.5 MΩ) filled with (in mM): 126 KCl, 2 MgSO₄, 0.5 CaCl₂, 5 EGTA 4 K₂-ATP, 0.4 GTP and 25 buffer (HEPES for pH = 7.5 and 7.0, MES for pH = 6.0) with KOH; bath solution contained (in mM): 145 NaCl, 5.4 KCl, 5 CaCl₂, 0.1 buffer (TAPS for pH = 8.0, HEPES for pH = 7.5 and 7.0, Bis-Tris for pH = 6.5, MES for pH = 6.0) with NaOH. Cells were imaged at 10 Hz using a CoolLED pE-4000 light source, 63 x 1.4 N.A. oil immersion objective, and a Zyla sCMOS camera (ANDOR). 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). F₀ is the average fluorescence intensity of first five data points.

TIRF Imaging

Images were acquired with an Olympus IX71 microscope with a 60 X 1.49 Olympus objective and a 1.6 X optivar with TIRF illumination. Exposure was set to 100 ms and the excitation light was provided by a Colbolt Jive 561 laser set to 20 mW. Emitted light was first passed through a dual dichroic (525/50 nm, 645/140 nm) and then
a 525/50 nm band-pass. An Andor iXon EM+ 885i CCD (1004 X 1002 with 8 mM 2 pixels) was used to collect the light.

**Synthesis Procedures**

Tetraacetylated N-Azidoacetyl-D-Mannosamine (azidosugar), and 5,6-dihydro-11,12-didehydro-dibenzo[a,e]cycloocten-5-yl ester, 4-nitrophenyl ester (DIBO-4-nitrophenyl ester), and Compound 1 were synthesized and purified as previously described.

Unless otherwise stated, all other reactions were run under an inert environment of argon (Ar) from which water and oxygen were rigorously excluded. Pentafluorobenzaldehyde and methanesulfonic acid were purchased from Acros. 3-(1-Piperazinyl) phenol was from Alfa Aesar. All other reagents and solvents were obtained from Sigma-Aldrich. 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 x 10 mm (RS-2510-W181) on a Hewlett Packard Agilent 1100 HPLC instrument equipped with G1315A DAD absorbance detector. NMR spectra were recorded in CDCl₃, CD₃OD on a Varian 400 MHz spectrometer; ¹H NMR and ¹³C NMR signals are reported in chemical shift relative to the NMR solvent peak; ¹⁹F NMR are reported in chemical shift relative to an internal trifluoroacetic acid (TFA) standard.
Coupling constants are reported as J values in Hz. NMR splitting patterns are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Coupling constants (J) are reported in Hz. 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).

Compound 2 (Boc-pH)

Crude 1 was synthesized following procedure described in the literature without further purification. Di-tert-butyl dicarbonate (4g, 18 mmol) was added to a stirred solution of crude 1 (4 g) and NaHCO₃ (2.7 g, 32 mmol) in H₂O (100 mL). After being stirred at r.t. overnight, the reaction mixture was extracted with CH₂Cl₂ (3 x 100 mL). The combined organic layers were washed with H₂O (3 x 100 mL), dried (Na₂SO₄), filtered and the filtrate was concentrated under reduced pressure. The crude product was purified by chromatography over silica gel (50:1, v/v, CH₂Cl₂/Methanol) to give compound 2 (1.54 g, two steps yield 28%). ¹H-NMR (400 MHz, CD₃OD) δ = 7.53 (d, J = 9.6 Hz, 2H), 7.36 (dd, J = 9.6, 2.4 Hz, 2H), 7.25 (d, J = 2.4 Hz, 2H), 3.93 – 3.84 (m, 8H),
3.68 (s, 8H), 1.50 (s, 18H). $^{19}$F-NMR (376 MHz, CD3OD) $\delta = -138.80$ (d, J = 17.6 Hz, 2F), -150.66 (t, J = 20.3 Hz, 1F), -160.47 (dt, J = 20.3, 5.6 Hz, 2F). $^{13}$C-NMR (101 MHz, CD3OD) $\delta = 159.58, 158.91, 156.17, 131.93, 117.14, 115.59, 98.82, 81.91, 48.06, 28.60$.  

HRMS (ESI): m/z calculated for C$_{37}$H$_{40}$F$_{5}$N$_{4}$O$_{5}$ (M$^+$): 715.2913; found: 715.2890.

Compound 3 (Boc-pH-COOH)

![Chemical Structure](image)

Compound 2 (580 mg, 0.8 mmol), N,N-dimethylacetamide (25 mL) and trimethylamine (720 µL) were heated to 50 °C and 3-mercaptopropionic acid (100 µL) was added dropwise. After being stirred at 50 for 3h, the solvents were evaporated under reduced pressure. The residue was dissolved in CH$_2$Cl$_2$ (100 mL) and the resulting solution was washed with H$_2$O (3 x 50 mL), which was back extracted with CH$_2$Cl$_2$ (3 x 30 mL). The combined organic layers were dried with Na$_2$SO$_4$, filtered and concentrated under reduced pressure. The crude was purified by chromatography over silica gel (50:1 -> 10:1, v/v, CH$_2$Cl$_2$/Methanol) to give compound 3 (0.3 g, 46%). $^1$H NMR (400 MHz, CD$_3$OD) $\delta = 7.54$ (d, J = 9.5 Hz, 2H), 7.36 (dd, J = 9.5, 2.4 Hz, 2H), 7.26 (d, J = 2.4 Hz, 2H), 3.95 – 3.81 (m, 8H), 3.68 (s, 8H), 3.39 – 3.33 (m, 2H), 2.73 – 2.64 (m, 2H), 1.50 (s, 18H). $^{19}$F-NMR (376 MHz, CD$_3$OD) $\delta = -131.87$ (2F, dd, J = 25.8, 12.4 Hz, 2F), -138.96
(dd, J = 26.8, 14.5 Hz, 2F). $^{13}$C NMR (101 MHz, CD$_3$OD) δ = 159.52, 158.85, 156.16, 132.04, 117.14, 115.38, 98.84, 81.86, 48.06, 31.25, 28.60. HRMS (ESI): m/z calculated for C$_{40}$H$_{45}$F$_{4}$N$_{4}$O$_{7}$S (M$^+$): 801.2940; found: 801.2933.

Compound 4 (Boc-pH-NH$_2$)

N,N-Diisopropylethylamine (90 mg, 0.7 mmol) and 1-
[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid
hexafluorophosphate) (171 mg, 0.45 mmol) was added slowly to a stirred solution of
compound 3 (300 mg, 0.37 mmol), 2, 2’-(Ethylenedioxy) bis(ethylamine) (555 mg, 3.7
mmol), DMF (5mL) and CH$_2$Cl$_2$ (5 mL) at 0 ℃. After being stirred at r.t. for 2 h, the
solvents were evaporated under reduced pressure. The residue was dissolved in CH$_2$Cl$_2$
(50 mL) and the resulting solution was washed with brine (3 x 50 mL) and H$_2$O (3 x 50
mL) which were back extracted with CH$_2$Cl$_2$ (3 x 30 mL). The combined organic layers
were dried (Na$_2$SO$_4$), filtered and concentrated under reduced pressure. The crude was
purified by chromatography over silica gel (50:1 -> 10:1, v/v, CH$_2$Cl$_2$/Methanol) to give
compound 4 (0.2 g, 57%). $^1$H NMR (400 MHz, CD$_3$OD) δ = 7.58 (d, J = 9.6 Hz, 2H),
7.38 (dd, J = 9.6, 2.4 Hz, 2H), 7.26 (d, J = 2.4 Hz, 2H), 3.93 – 3.82 (m, 8H), 3.72 – 3.64
(m, 12H), 3.58 (t, J = 5.7 Hz, 4H), 3.37 (dd, J = 9.7, 4.1 Hz, 4H), 3.11 – 3.05 (m, 2H),
2.67 (t, J = 6.8 Hz, 2H), 1.50 (s, 18H). $^{19}$F NMR (376 MHz, CD3OD) δ = -131.63 (dd, J = 25.1, 12.6 Hz, 2F), -138.89 (dd, J = 24.5, 11.9 Hz, 2F). $^{13}$C NMR (101 MHz, CD3OD) δ = 173.21, 159.57, 158.90, 156.18, 117.11, 115.41, 98.82, 81.92, 71.35, 70.55, 67.93, 48.06, 40.67, 40.31, 37.46, 31.52, 28.60. HRMS (ESI): m/z calculated for C$_{46}$H$_{59}$F$_4$N$_6$O$_8$S (M$^+$): 931.4046; found: 931.4034.

Compound 5 (Boc-pH-DIBO)

DIBO-4-nitrophenyl ester (196 mg, 0.51 mmol) was added to a stirred solution of compound 4 (240 mg, 0.26 mmol) and triethylamine (36 µL, 0.26 mmol) in CH$_2$Cl$_2$ (30 mL). After being stirred at r.t. for 16 h, the solvent was evaporated under reduced pressure. The residue was dissolved in CH$_2$Cl$_2$ (50 mL) and the resulting solution was washed with H$_2$O (3 x 50 mL), which was back extracted with CH$_2$Cl$_2$ (3 x 30 mL). The combined organic layers were dried with Na$_2$SO$_4$, filtered and concentrated under reduced pressure. The crude was purified by chromatography over silica gel (50:1 -> 20: 1, v/v, CH$_2$Cl$_2$/Methanol) to give compound 5 (110 mg, 36%). $^1$H NMR (400 MHz, CD$_3$OD) δ = 7.48 (dd, J = 12.3, 8.7 Hz, 4H), 7.35 – 7.21 (m, 8H), 7.14 (d, J = 2.1 Hz, 2H),
5.29 (s, 1H), 3.89 – 3.77 (m, 8H), 3.63 (d, J = 6.2 Hz, 12H), 3.54 (t, J = 5.1 Hz, 4H), 3.37 – 3.33 (m, 4H), 3.26 (t, J = 5.2 Hz, 2H), 3.12 (dd, J = 15.1, 2.1 Hz, 1H), 2.68 (dd, J = 15.1, 3.9 Hz, 1H), 2.60 (t, J = 6.7 Hz, 2H), 1.51 (s, 18H).  

$^{19}$F NMR (376 MHz, CD$_3$OD) $\delta = -131.27$ (dd, J = 27.3, 14.7 Hz, 2F), -138.41 (dd, J = 26.8, 14.2 Hz, 2F).  

$^{13}$C NMR (101 MHz, CDCl$_3$) $\delta =$ 171.40, 158.10, 157.33, 155.94, 154.44, 152.42, 151.37, 132.65, 130.24, 128.17, 128.14, 126.97, 126.94, 126.02, 125.84, 124.36, 123.78, 121.09, 116.32, 114.74, 112.90, 110.12, 97.45, 81.07, 70.46, 70.34, 70.22, 70.05, 47.19, 46.24, 40.99, 39.22, 36.61, 30.43, 28.48. HRMS (ESI): m/z calculated for C$_{63}$H$_{69}$F$_4$N$_6$O$_{10}$S (M+): 1177.4727; found: 1177.4729.

Compound 6 (pH-DIBO)

Trifluoroacetic acid (5mL) was added dropwise to a stirred solution of compound 5 (100 mg, 0.085 mmol) in CH$_2$Cl$_2$ (5 mL), H$_2$O (1 mL). After being stirred at r.t. for 1.5 h, the solvents were evaporated under reduced pressure. The residue was dissolved in H$_2$O (25 mL) and the resulting solution was washed with CH$_2$Cl$_2$ (3 x 20 mL). The aqueous layers were concentrated under reduced pressure and purified by HPLC. 25 mg crude yielded 15 mg pure compound 6. If up-scaled, 60 mg (0.051 mmol, 72%).  

$^1$H NMR (400 MHz, CD$_3$OD) $\delta =$ 7.59 (d, J = 9.5 Hz, 2H), 7.51 (d, J = 7.9 Hz, 1H), 7.38 (d, J = 9.6
Hz, 3H), 7.33 (s, 4H), 7.30 – 7.24 (m, 4H), 5.31 (s, 1H), 4.07 (d, J = 4.6 Hz, 8H), 3.62 (s, 4H), 3.54 (d, J = 2.6 Hz, 4H), 3.44 (d, J = 4.7 Hz, 8H), 3.35 (m, 4H), 3.27 (t, J = 5.5 Hz, 2H), 3.15 (dd, J = 15.1 Hz, 1H), 2.75 – 2.67 (m, 1H), 2.61 (t, J = 6.7 Hz, 2H). 19F NMR (376 MHz, CD3OD) δ = -132.77 (dd, J = 27.2, 14.4 Hz, 2F), -139.95 (dd, J = 27.1, 14.4 Hz, 2F). 13C NMR (126 MHz, CD3OD) δ = 173.04, 159.86, 158.97, 157.95, 153.54, 152.46, 145.26, 132.40, 131.09, 129.32, 129.26, 128.29, 128.26, 127.19, 126.91, 124.99, 124.88, 122.26, 117.57, 116.14, 113.78, 110.97, 99.66, 77.87, 71.37, 71.30, 70.94, 70.51, 47.19, 45.34, 44.04, 41.76, 40.43, 37.62, 31.41. HRMS (ESI): m/z calculated for C53H53F4N6O6S (M+): 977.3678; found: 977.3669.
CHAPTER III. VISUALIZATION OF PROTON FLUXES FROM MCT1 AND IDENTIFICATION OF HRM, A PROTON-COUPLED MONOCARBOXYLATE TRANSPORTER IN DROSOPHILA

Summary

The transport of monocarboxylates, such as lactate and pyruvate, is essential for cellular metabolism and is mainly mediated by proton-coupled monocarboxylate transporters (MCT1-4). There are ten other MCTs in mammals: MCT6 and MCT11 are proton-coupled transporters; the others are either non-proton-coupled (MCT8-10) or orphan transporters (MCT12-14). Although pH electrodes and intracellular pH sensors have been widely used for measuring the transport of proton-coupled MCTs, they provide limited information about the subcellular activities and underestimate the transport rate. Here we used the approach developed in Chapter II to monitor proton fluxes from MCT1 at the cell surface. Upon extracellular perfusion of L-lactate or pyruvate, we observed proton depletion followed by a rapid recovery over the entire landscape of cells expressing MCT1. Furthermore, we demonstrated that the membrane protein in Drosophila, Hrm, which has been implicated in autophagy, is a proton-coupled MCT that preferentially transports pyruvate.
Introduction

Monocarboxylates play a major role in cellular metabolism. For example, pyruvate, a product of glycolysis, is used as an energy source for cells. In anaerobic conditions, pyruvate is converted into lactate which can then negatively regulate glycolysis when intracellular concentrations increase [150]. Furthermore, lactate and ketone bodies can be oxidized as respiratory fuels in the heart, brain and red skeletal muscle [73]. Monocarboxylate transport in and out of cells is mainly mediated by proton-coupled monocarboxylate transporters (MCT1-4) encoded by the SLC16 family of genes in mammals.

Besides MCT1-4, there are ten other MCTs: MCT11 has been shown to be a proton-coupled pyruvate transporter implicated in type 2 diabetes [93]; MCT6 facilitates the proton-coupled transport of bumetanide, but its natural substrate remains unknown [94]; MCT7 is involved in the hepatocyte secretion of ketone bodies during fasting but has not been functionally characterized [98]; MCT8-10 transport thyroid hormones, carnitines and aromatic amino acids, respectively and are not proton-coupled [95–97]. The substrates and functions of MCT12-14 are currently unknown.

In *Drosophila melanogaster*, there are 14 genes belonging to the SLC16 family. However, only two of them have been functionally characterized. Sln/dMCT1 mediates transport of butyrate and lactate and is crucial for triggering LKB1 dependent apoptosis [99]. The second gene, Chaski, which is enriched in glial cells has been identified as a lactate/pyruvate transporter [100]. The Baehrecke laboratory at UMMS has identified CG11665/hrm, a gene that shows significant sequence similarity to SLC16 genes. They
found that Hrm is required for both autophagy during steroid-triggered salivary gland cell death and TNF-induced non-apoptotic eye cell death (unpublished). However, it is still unknown whether Hrm functions as a proton-coupled MCT or what its substrates are.

As the transport of proton-coupled MCTs is non-electrogenic, it is difficult to use electrical recordings to monitor their activities. Radiolabeled substrates are commonly used for the activity measurement due to their high specificity and sensitivity [73]. The transport rate is measured by determination of intracellular labeled substrates after rapid termination. However, the diffusion and metabolism of substrates during the procedures may generate substantial errors. In recent years, genetically encoded fluorescent sensors have been introduced for the real time measurement of intracellular substrates, such as pyruvate and lactate [105,106], but their $K_D$ values limit the detectable concentration range of substrates. Because the activities of MCT1-4 are associated with proton transport, they also have been measured by monitoring $pH_i$ using pH-sensitive dyes, such as BCECF-AM [73]. Although these tools enable the real-time measurement of transport, they provide limited spatial-information and underestimate the transport rate due to the cell’s volume and intracellular buffering. Many of these constraints can be overcome by visualizing changes in extracellular pH.

Here, we used the approach developed in Chapter II to detect proton fluxes from MCT1 expressed in HEK293T cells. Upon addition and removal of extracellular L-lactate or pyruvate, we observed a rapid decrease in fluorescence followed by a fast recovery corresponding to proton fluxes and diffusion. The kinetics of proton depletion and accumulation at the cell surface revealed MCT1 activity in real time. Moreover, Hrm,
which was implicated in mTOR-regulated autophagy in Drosophila, was identified as a proton-coupled MCT.
Results

Visualizing Proton-coupled Transport of MCT1

Since transport of substrates by proton-coupled MCTs is not voltage-dependent and non-electrogenic, proton fluxes through MCTs may behave differently from that through Hv1 and ClC-5. To verify the utility of the Chapter II approach on MCTs transport, we transiently transfected HEK293T cells with MCT1, the most well-studied MCT, and incubated with azidosugar, which cells metabolize and incorporate an unnatural azido-group in the terminal sugar residues of the glycocalyx. After 2 days, the cells were labeled with an azido-reactive, fluorescent pH sensor, pH-DIBO for 30 min and the fluorescence change (ΔF/F₀) was monitored over time upon addition and removal of substrates of interest by using a gravity-perfusion system (Figure III-1A). To determine the perfusion rate, we decreased the external pH from 7.5 to 7.0 by using the perfusion system. The fluorescent signal decreased and reached to a steady state in ~ 5s, indicating that the external solution was completely replaced (Figure III-1B). Figure III-1C shows the fluorescent signals in MCT1-expressing and mock transfected (control) cells upon addition and removal of 10 mM L-lactate in a bath solution with a constant pH (7.0) and a low buffer capacity (0.1 mM). In control cells, the fluorescent signals decreased rapidly due to endogenous MCTs and then slowly recovered as net transport activity slowed and protons in the media reprotonated the cell-attached pH-sensitive fluorophore. In contrast, the fluorescent signals in MCT1-expressing cells recovered faster and reversed direction, implying that the exogenous expression of MCT1
significantly increased proton-coupled transport of L-lactate into the cell such that proton efflux became thermodynamically favored (Figure III-1D, a and b). Washout of L-lactate resulted in a rapid efflux of protons from the cell followed by proton diffusion to the bulk solution (Figure III-1D, c). The transport rate for MCT1-expressing cells was 2-fold faster than control cells upon removal of L-lactate (Figure III-1C, inset).
Figure III-1. Visualization of proton fluxes in control and MCT1-expressing HEK293T cells. (A) Schematic representation of the gravity-perfusion system. (B) Fluorescence traces were recorded during perfusion of external solutions with different pH indicated by black and grey bars, n = 4; error bars displaying in grey area denote ± SEM. (C) Fluorescence traces of MCT1-expressing (black) and control (grey) cells upon perfusion of 10 mM L-lactate (dash bar), inset: transport rates upon removal of L-lactate, n = 8; error bars denote ± SEM. (D) Schematic cartoon of proton-coupled L-lactate transport through MCT1 and the plasma membrane at time points indicated in (C).
We hypothesized that the fluorescent recovery is partially due to either the reprotonation of sensors from the medium (L-lactate addition) or proton diffusion to the bulk (L-lactate removal). To confirm this hypothesis, we performed the perfusion experiment under different external buffer capacities. By increasing the buffer capacity from 0.1 to 3 mM, the fluorescence change in both directions reduced monotonically and reached the peak and recovered faster (Figure III-2A), implying the buffer-dependent change in fluorescence is due to the competition between buffer and the glycocalyx-attached pH sensors.
Figure III-2. H⁺/L-lactate co-transport at different external buffer capacity and pH in MCT1-expressing HEK293T cells. (A) Fluorescence traces of MCT1-expressing cells when perfusing 10 mM L-lactate in bath solutions with different external buffer capacity ($B_o$), n = 8; or (B) with different external pH ($pH_o$), n = 6 - 8; error bars denote ± SEM.
Partial Characterization of MCT1 by Monitoring Extracellular pH Changes

MCT1 has a broad range of substrates. To demonstrate the utility of our approach on proton-coupled transport of other monocarboxylates, we replaced L-lactate with pyruvate, D-lactate and butyrate and measured the fluorescence changes in MCT1-expressing and control cells (Figure III-3A, B and C). To determine relative transport rates, we measured the rate of proton efflux upon removal of the extracellular monocarboxylate, subtracted the background rate (control cells), and normalized the transport rates to the transport of L-lactate (Figure III-3D). The result showed that at a concentration of 10 mM, the MCT1 transport of L-lactate was approximately twice as fast as that of pyruvate, and 2.5-fold faster than that of D-lactate which is consistent with previous studies [85]. Because the transport of butyrate is mainly mediated by passive diffusion due to its high pKₐ [73], the transport rate of butyrate in MCT1-expressing cells is almost same as the background rate.
Figure III-3. Substrate specificity of proton-coupled transport in control and MCT1-expressing HEK293T cells. Fluorescence traces of MCT1-expressing (black) and control (grey) cells during perfusion of 10 mM (A) pyruvate, (B) D-lactate or (C) butyrate, n = 6 - 10; (D) Relative transport rates for different substrates. Error bars denote ±SEM.
As the driving force of MCT transport is dependent on the concentration of substrates and pH, next we examined the effect of these both factors on the fluorescent signal. By increasing the concentration of substrates from 1 to 30 mM, the inward peak of fluorescence increased monotonically for L-lactate (Figure III-4, top panel) and reached a maximum at 3 mM for pyruvate (Figure III-4, middle panel). Because the transport of D-lactate is slower than that of L-lactate and pyruvate, the fluorescent signal decreased slowly and did not exhibit a recovery until 10 mM (Figure III-4, bottom panel). Changing external pH influenced the fluorescent signal as well. In 0.1 mM HEPES buffer, decreasing the pH from 7.0 to 6.5 increased the inward ΔF/F₀ but decreased and slowed the outward ΔF/F₀ (Figure III-2B). In contrast, increasing the pH from 7.0 to 7.5 accelerated the recovery in both inward and outward ΔF/F₀ (Figure III-2B).
Figure III-4. Concentration-dependent transport in MCT1-expressing HEK293T cells. Fluorescence traces were recorded during perfusion of L-lactate (top panel), pyruvate (middle panel) or D-lactate (bottom panel) with different concentration, n = 6; error bars denote ±SEM.
Although MCT1 expression resulted in a faster recovery in fluorescence, we wanted to confirm that the fluorescence change was caused by the proton-coupled transport activity of MCT1. To test this, we inhibited MCT1 by adding the known MCT inhibitor, CHC. Compared to the control experiment (Figure III-5A), the fluorescent signal reduced significantly in the presence of 0.1 or 0.3 mM CHC and recovered after washing out CHC (Figure III-5C). However, we noticed that perfusion of CHC alone quenched the fluorescence (Figure III-5B). To correct for this quenching effect, we performed the inhibition experiment using butyrate, which does not rely on MCT1 for transport (Figure III-3C). After correction, the MCT1 activity was reduced by 43 ± 4% and 13% ± 2% at 0.3 and 0.1 mM CHC (Figure III-6C), respectively, which is in agreement with previous studies (Figure III-6C, dash line) [89].
Figure III-5. Inhibition of MCT1 transport by CHC. (A) Representative fluorescence traces of a MCT1-expressing HEK293T cell when repeat perfusion of 10 mM L-lactate three times. (B) Representative fluorescence traces during perfusion of 0.3 mM CHC (black) or 10 µM phloretin (grey) only. (C) Representative fluorescence traces of a MCT1-expressing HEK293T cell during perfusion of 10 mM pyruvate in the absence of (left, black), in the presence of (middle, red) and after washing out (right, blue) 0.1 mM (top panel) or 0.3 mM CHC (bottom panel).
Identification of Hrm, a Putative MCT in Drosophila

The gene CG11665/hrm is predicted to encode an MCT, Hrm in Drosophila. Hrm is required for both autophagy during steroid-triggered salivary gland cell death and TNF-induced non-apoptotic eye cell death (Baehrecke laboratory, unpublished data). However, it is still unknown whether Hrm is a proton-coupled MCT or what its substrates are. To determine whether Hrm mediated proton-coupled transport of monocarboxylates, we transiently transfected HEK293T cells with Hrm and extracellularly labeled cells with fluorescent pH sensors. Compared to MCT1-expressing cells, we observed similar fluorescent signals in cells transfected with Hrm upon extracellular perfusion of 10 mM pyruvate (Figure III-6A, bottom panel). The transport rate was ~ 2-fold faster than control cells, the same as MCT1. However, Hrm expression did not change the transport rate of L-lactate significantly compared to control cells (Figure III-6A, top panel). The determination of relative transport rates showed that only proton-coupled pyruvate transport was detectable for Hrm (Figure III-6B).

In Hrm-expressing cells, proton-coupled transport was also inhibited by CHC. At 0.3 mM CHC, Hrm activity was reduced by 60 ± 3% whereas both overexpressed and endogenous MCT-1 were inhibited to a lesser extent: 43 ± 3% and 39 ± 3%, respectively (± SEM; n = 6 - 8 cells), indicating that the IC₅₀ of CHC is lower for Hrm (Figure III-6C). Overall, these data demonstrate that Hrm is a bona fide proton-coupled monocarboxylate transporter that transports pyruvate faster than lactate.
Figure III-6. Proton-coupled transport in Hrm-expressing HEK293T cells. (A) Fluorescence traces were recorded during perfusion of 10 mM L-lactate (top) or pyruvate (bottom) in control (grey), MCT1- (black) and Hrm-expressing (red) cells, n = 8 - 10. (B) Relative transport rates of L-lactate and pyruvate in MCT1- and Hrm-expressing cells, n = 8 - 10. (C) Transport inhibition by CHC in control (grey), MCT1- (black) and Hrm-expressing (red) cells, n = 6; the dash line indicates the reported MCT1 inhibition by CHC [14]. (D) Sequence alignment of human MCT1-4, 11 and Hrm; conserved key residues were highlighted in yellow; sequences were aligned using ClustalW multiple alignment in BioEdit software with default parameters. Error bars denote ± SEM.
Figure III-7. Proton-coupled transport of L-lactate and pyruvate measured by BCECF-AM. Cells were pre-loaded with BCECF-AM. Normalized fluorescence traces of control (grey), MCT1- (black) and Hrm-expressing (red) HEK293T cells during perfusion of 10 mM L-lactate (left) or pyruvate (right), n = 6; error bars denote ±SEM.
**Discussion**

Compared to Hv1 and ClC-5, proton-coupled transport by MCTs is non-electrogenic. Previously, it has been demonstrated that extracellular addition of L-lactate or pyruvate to cells expressing MCT1 resulted in pH decreasing rapidly at the beginning, then reaching a steady state [85,89]. Here, by covalently attaching a pH-sensitive dye to the cell’s glycocalyx, we observed different kinetics of pH changes at the outer surface of the plasma membrane: a rapid proton depletion followed by a recovery which is consistent with intracellular acidification, slowed MCT1 activity and proton diffusion from the media.

Although there was no significant pH gradient observed within cells, a previous report using pH electrodes demonstrated that pH changes at the inner face of the cell membrane occurred faster and more significantly compared to those in the cytoplasm in response to L-lactate [85]. Because our pH-DIBO was located at the cell’s glycocalyx, nanometers away from the transporter, it had more sensitivity than intracellular pH-sensitive dyes. Indeed, when we measured changes in pH using BCECF-AM in cells expressing Hrm, we did not detect a change as significant as that using pH-DIBO (Figure III-7).

Because a low buffer capacity in the bath solution was required to detect the fluorescent signal in response to MCT activity, the extracellular pH measurement was particularly sensitive to the buffer capacity changes. It is challenging to determine $K_m$ values for substrates by using the extracellular facing pH sensor, as monocarboxylates themselves contain carboxylic acid and serve as weak buffers. Furthermore, because
buffer capacity is dependent on the ratio of salts to acid/base, changing external pH influences the buffer capacity as well. Therefore, it will overestimate the roles of monocarboxylates and external pH on MCT activity without consideration of their effect on buffer capacity.

As reported previously, exposure of cells to the known MCT inhibitor, CHC did not cause intracellular acidification, indicating that there is no proton transport induced by CHC [85]. In our experiments, however, we observed that the fluorescence decreased upon addition of CHC due to intermolecular PET quenching. Phloretin, another known MCT inhibitor with a lower Kᵢ (~ 5 μM) [91] quenched the fluorescence more significantly at 10 μM (Figure III-5B), possibly because it has three more phenolic hydroxyl groups as electron donors than CHC. When studying MCT inhibitors, a quenching correction needs to be taken into consideration for the extracellular pH measurement if inhibitors are extracellular binding, and vice versa.

In proton-coupled MCTs, there are three conserved charged residues that appear to be crucial for the proton-coupled transport (K38, D302/R306 in human MCT1). Interestingly, sequence alignment of Hrm with human proton-couple MCTs revealed that Hrm possesses these three residues (K74, D503 and R507) as well (Figure III-6D). MCT11, the closest human homolog of Hrm, performs proton-coupled transport of pyruvate and has been associated with type 2 diabetes [93]. However, it remains unknown weather MCT11 transports other substrates, such as L-lactate. Here we observed that Hrm transported pyruvate preferentially. No significant L-lactate transport was observed in Hrm-expressing cells compared to the control. One possibility is that L-
lactate transport through Hrm is slower than that through MCT1 and was disguised by the endogenous MCT1. The Baehrecke group has showed that basigin, an ancillary protein for MCTs was required for translocation of Hrm to the plasma membrane in Drosophila. However, co-transfection of Hrm with either human or Drosophila basgin in HEK293T did not significantly increase the transport rate (data not shown).

MCTs are expressed at specific locations of cell membranes [151,152]. For instance, the highest density of MCT1 in cardiomyocytes occurs in the intercalated disk and T-tubules regions [84]. Combining our approach with state-of-the-art imaging techniques will allow for detection of MCT activity over the entire landscape of a cell with high spatiotemporal resolution. Moreover, as extracellular pH measurement is more sensitive than intracellular pH measurement, this approach will support the functional identification of orphan MCTs and other proton-coupled transporters in mammals and other organisms. Given that the glycocalyces of living organisms can be engineered with unnatural sugars [148,149,153], visualization of proton-coupled transport events from cells, tissues, and model organisms is tenable by modifying metabolically labeled glycocalyces with pH-sensitive dyes.
Materials and Methods

Chemicals

Azidosugar (tetraacetylated N-Azidoacetyl-D-Mannosamine) and pH-DIBO were synthesized as previously reported [154,155]. BCECF-AM was obtained from life technologies. Sodium pyruvate and sodium butyrate were obtained from Alfa Aesar. L-lactic acid, sodium salt, 60 wt% solution in water was obtained from Acros. Sodium D-lactate, α-Cyano-4-hydroxycinnamic acid (CHC) and phloretin were obtained from Sigma.

Plasmids

pGHJMCT1 containing rat MCT1 (rMCT1) was a gift from Sebastián Brauchi (Universidad Austral de Chile). CG11665/Hrm was a gift from Eric Baehrecke (Umass Medical School). rMCT1 and CG11665 were cloned into pcDNA3.1 for mammalian cells expression.

Cell Culture, Transfections and pH-DIBO Cell Surface Labeling

HEK293T cells were cultured in high glucose DMEM medium (Gibco) with 10% fetal bovine serum (Hyclone) and 100 units/mL penicillin/streptomycin (Invitrogen) at 37°C in 5% CO₂. The cells were seeded at 60-75% confluency in 35 mm dishes. After 24 hrs, cells were transiently transfected with 1 μg of transporter DNA and 5 μL of Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen) for 6 hours. To visualize transfected cells, 0.25 μg of pEGFP-C3 or mCherry was added to transfections that did
not contain FP-tagged transporters. After removing the transfection mixture, the cells were transferred to glass bottom culture dishes (MatTek) coated by fibronectin and incubated in media containing 50 μM Ac4ManNAz for 2 days, which was replenished after 24 hrs.

*Extracellular pH Measurement*

After 2 days, transfected HEK293T cells were directly labeled with pH-DIBO (50 μM) in Opti-MEM at 37°C in 5% CO₂ for 30 min, then they were washed 3 times with a bath solution (145 mM NaCl, 5.4 mM KCl, 5 mM CaCl₂ and 0.1 mM HEPES, pH 7.0). A 35-mm chamber insert (RC-33DL, Warner) was put into the glass bottom dish to decrease volume. The dish was then mounted onto a Quick Exchange Platform QE-1 (Warner) for imaging. Monocarboxylates and inhibitors were perfused in/out by using a gravity perfusion system at about 10 mL/min. Cells were imaged at 1 Hz using a CoolLED pE-4000 light source (550 nm excitation), 63 x 1.4 N.A. oil immersion objective, DAPI/FITC/TRITC/Cy5 filter set (CHROMA) and a Zyla sCMOS camera (ANDOR). Fluorescent images were collected and processed using open source software (micro-manager and ImageJ). F₀ is the average fluorescence intensity of first five data points.

*Intracellular pH Measurement*

After 2 days without azidosugar treatment, the transfected cells were directly labeled with BCECF-AM (1 nM) in Opti-MEM at 37°C in 5% CO₂/95% air for 30 min, then were washed 3 times with bath solution. Cells were imaged at 1 Hz using a
CoolLED pE-4000 light source (490 nm excitation), 63 x 1.4 N.A. oil immersion objective, DAPI/FITC/TRITC/Cy5 filter set (CHROMA) and a Zyla sCMOS camera (ANDOR).

**Calculations**

Transport rate: From MCT1, Hrm and mock transfected cells, the increment parts of fluorescence upon removal of monocarboxylates were fitted to the exponential growth equation. The rate constant $k$ was treated as a transport rate. To determine a relative transport rate, the rate from MCT1 or Hrm was first subtracted by the background rate (control cells), then normalized the transport rates to the rate of MCT1 transport L-lactate.

Remaining activity: amplitudes of inward peaks before ($A_0$) and after ($A_{CHC}$) adding CHC were measured. The ratio of $A_0$ and $A_{CHC}$ was obtained as the remaining activity of transporters. The CHC quenching effect was corrected by $A_0/A_{CHC}$ values of butyrate.
CHAPTER IV. VISUALIZATION OF MCT1 ACTIVITY IN CARDIOMYOCYTES USING WGA-CONJUGATED FLUORESCENT PH SENSORS

Summary

Cardiac metabolism requires transport of lactate and ketone bodies that are mainly mediated by MCT1. Previous studies reveal that MCT1 is densely distributed in intercalated disc and T-tubule regions in isolated rat cardiomyocytes. However, monitoring the subcellular activity of MCT1 has not been tenable with existing tools. In Chapter II and III we described a bioorthogonal chemistry approach to covalently attach a fluorescent pH sensor at the cell surface and applied it in monitoring proton fluxes through MCT1 in HEK293T cells. However, this approach is not applicable in cardiomyocytes, which cannot incorporate the unnatural sialic acid precursor into their glycocalyx. To address this, here we made a pH-sensitive fluorescent WGA conjugate, WGA-pHRho to visualize extracellular proton accumulation and depletion. Compared to the glycocalyx-attached pH-DIBO, WGA-pHRho has similar fluorescent signals in response to the changes in the extracellular proton concentration caused by proton channel Hv1 and omega mutant Shaker-IR R362H. Furthermore, with WGA-pHRho, we visualized the proton-coupled transport of MCT1 in INS-1 cells and cardiomyocytes. Given that WGA binds to endogenous glycoproteins at the plasma membrane, this straightforward approach will enable us to fluorescently visualize proton fluxes from most cell types.
Introduction

In all animals, cardiomyocytes, which constitute the heart tissue, require a precisely tuned metabolism for proper function. Under normal conditions, cardiomyocytes take up lactate as a respiratory substrate, but under hypoxic conditions, they instead produce and release lactate [109]. Proton-coupled monocarboxylates (MCTs) play a major role in catalyzing transport of lactate into and out of cardiomyocytes. In particular, MCT1 is abundantly present in mammalian hearts [152]. MCT2 has been detected in the hamster heart [156], but not in the rat and mouse heart [157]. In rats, MCT4 is detected in the neonatal heart but expression decreases or is absent in the adult heart [158].

To provide flexibility in lactate transport, cells often express MCTs at specific domains of the plasma membrane. In cardiomyocytes, MCT1 is found on the regions of the plasma membrane that are near capillaries and other myocytes and has the highest density in the intercalated disks suggesting H⁺/lactate fluxes between cells [159]. In addition, high MCT1 expression is also observed in the T-tubules which are highly branched invaginations of the plasma membrane in close proximity to mitochondria, implying that the lactate transported by MCT1 may be used for oxidative metabolism [159]. As MCT1 is not expressed uniformly along the plasma membrane of cardiomyocytes, the biochemical characteristics and functions may vary between different subdomains of the plasma membrane. Thus, it is of great interest to monitor MCT1 activity spatiotemporally over the entire plasma membrane in cardiomyocytes.
Currently, because lactate fluxes through MCTs are associated with proton transport, intracellular fluorescent pH sensors such as BCECF-AM have been widely used to monitor the transport activity of proton-coupled MCTs [73]. Although this approach enables the transport detection in real-time, it is unable to distinguish MCT activity at different plasma membrane domains. In contrast, extracellularly attached fluorescent pH sensors will allow for monitoring MCTs activities at different regions of the plasma membrane. In Chapter II and III, we described a bioorthogonal chemistry approach to target a pH-sensitive dye to the cell’s glycocalyx and applied it in real-time visualization of extracellular proton accumulation and deletion in HEK293T cells expressing MCT1. However, we found that this approach is not tenable in some cells, such as INS-1 cells and cardiomyocytes that do not incorporate the unnatural sialic acid precursor into their glycocalyx. Therefore, we sought to develop an approach that was more universally applicable.

Wheat germ agglutinin (WGA) binds to N-acetyl-glucosamine and sialic acid at the cell’s glycocalyx [160] and has been routinely used to demarcate the plasma membrane of live and fixed cells [161]. A WGA-fluorescein conjugate has been used to approximate pH changes in the extracellular region of single melanoma cells [123], raising the possibility that pH-sensitive WGA-conjugates may enable the visualization of proton fluxes from ion channels, transporters and omega mutants in the extracellular nanodomains.

Herein we described the synthesis of WGA-pHRho (Figure IV-1A), a pH sensitive, red fluorescent WGA conjugate that binds to cell’s glycocalyx (Figure IV-1B).
Despite visual signs of endocytosis, proton fluxes from proton channel Hv1 and omega mutant Shaker-IR R362H/W434F can be detected within 3 hrs after labeling with the localized WGA-pHRho. Since many ion channels are glycosylated, and WGA is able to bind to these modifications, we confirmed that WGA labeling did not appreciably alter the activities of glycosylated or non-glycosylated ion channels as measured by the current density and voltage-activation. Compared to commercially-available WGA-fluorescein, WGA-pHRho is photostable, increases fluorescence upon protonation, and has a pKa of ~7 which is well matched for extracellular pH measurements. Furthermore, with WGA-pHRho, we monitored the transport activity of MCT1 on the entire plasma membrane of INS-cells and cardiomyocytes. Given that any small-molecule fluorescent sensor can be conjugated to WGA and all cells have terminal sugars for WGA binding, this straightforward approach will enable physiologists to visualize any transport events at the plasma membrane from most cell types.
Figure IV-1. The synthesis of WGA conjugates to visualize plasma membrane proton fluxes. (A) Scheme for the synthesis of WGA-pHRho. (B) Cells expressing ion channels or transporters are labeled with WGA-pHRho to detect proton fluxes by using patch clamp fluorometry. (C) pH standard curves of WGA-pHRho in solution (top, n = 6) or attached to the plasma membrane of CHO cells (bottom, n = 22). Buffer concentrations are 10 mM; ΔF/F₀ at pH 7.5 was defined as 0; error bars are ±SEM.
Results

*Synthesis and characterization of a pH sensitive WGA conjugate, WGA-pHRho*

Compared to small-molecule dyes with molecular weight <1000, WGA exists mostly as a heterodimer of 38,000 daltons in solution and is cationic at physiological pH [161]. In order to confirm that WGA labeling would not alter the activity of membrane transport proteins, we recorded families of currents from cells transiently transfected with voltage-gated proton channels Hv1 or *Shaker*-IR potassium channels before and after treatment with WGA (Figure IV-2A). Hv1 cannot be N- or O-glycosylated as it doesn’t contain any extracellular serine or threonine residues [162]. In contrast, *Shaker*-IR contains two N-linked glycosylation sites in the S1-S2 loop of the voltage-sensor [163]. After WGA treatment, the current density of Hv1 was reduced by ~30% at 60 mV depolarization, but that of *Shaker*-IR did not change significantly. In contrast, tail current analysis showed that WGA treatment did not significantly affect the voltage-activation of Hv1; a small, but statistically insignificant shift was measured for *Shaker*-IR (Figure IV-2B). All these results indicate that unglycosylated and glycosylated ion channels were negligibly affected by WGA binding.
Figure IV-2. Effects of WGA-binding on the activities of unglycosylated and glycosylated ion channels. (A) Voltage-clamp current traces of CHO cells expressing Hv1 (top panel) and Shaker-IR (bottom panel) before (black) and after WGA treatment (red). Cells were held at –80 mV. Currents were elicited from –50 mV to 100 mV for Hv1 (scale bars: 1 nA, 100 ms), and from –70 to 60 mV for Shaker-IR (scale bars: 2 nA, 50 ms) in 20-mV increments. Middle panel shows a comparison of currents before and after WGA treatment at 60 mV depolarization. (B) G-V curves of Hv1 (triangles) and Shaker-IR (circles) before (solid) and after WGA treatment (open). $V_{0.5} = 68.32 \pm 6.38$ mV (n = 8, solid triangles), 67.40 ± 7.82 mV (n = 9, open triangles), –18.01 ± 1.15 mV (n = 5, solid circles) and –10.39 ± 1.37 (n = 6, open circles); error bars are ±SEM.
Next, we determined whether pH-sensitive WGA conjugates would effectively report on proton accumulation and depletion on the extracellular side of the plasma membrane. We made a red fluorescent, pH sensitive WGA-conjugate (WGA-pHRho) by activating the carboxylic acid of pHRho with dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) (Figure IV-1A), incubating it with WGA for 2 hrs, and separating out the unreacted dye with a gravity-fed size exclusion column. Although attaching WGA-pHRho to the cell surface decreased the pH sensitivity, the ΔF/F₀ and pH exhibited a linear relationship in the range between 6.0 and 8.5, indicating conjugation of the small molecule pH-sensitive fluorophore to WGA did not change the apparent pKₐ of 6.9 [120] (Figure IV-1C). To visualize proton efflux with WGA-pHRho, Chinese hamster ovary (CHO) cells were treated with 50 μg/mL WGA-pHRho for 10 min and the current and fluorescence were measured using patch-clamp fluorometry in a bath solution with a low buffer capacity (0.1 mM) (Figure IV-1B). Figure IV-3A (left panel) shows families of currents and fluorescent signals from a CHO cell expressing a C-terminally GFP-tagged, human voltage-gated proton channel (hHv1). Under low buffer conditions, voltage-activation of Hv1 channels resulted in small currents (~ 200 pA at 100 mV) that reached steady state faster with stronger depolarizations. Simultaneous imaging of the cell’s fluorescence at 10 Hz showed that the change in fluorescence was similar to voltage-dependence currents of Hv1; however, the rate to reach steady state was slower as it corresponds to proton accumulation at the cell surface rather than Hv1 channel gating. This distinction between channel gating and proton accumulation (or depletion) was clearly evident upon Hv1 deactivation at – 80 mV: no current remained after 300 ms
whereas it required seconds for the fluorescent signal to fully decay. As observed in Chapter II, the fluorescent decay has two time-constants: the fast component corresponding to protons rushing into the cell before Hv1 channel closing and a slow component corresponding to proton diffusion into the bulk solution. \( \Delta F \) snapshots before, during and after a 100-mV test pulse highlight the kinetic differences in proton accumulation and depletion at the cell surface (Figure IV-3B, left panel).
Figure IV-3. Visualization of proton accumulation and depletion at the cell surface with WGA-pHRho. (A) Voltage-clamp fluorometry traces from CHO cells transfected with Hv1 (left panel), Shaker-IR R362H/W434F (middle panel) or empty vector (control, right panel). Hv1-expressing and control cells were held at –80 mV. Currents and fluorescence were elicited from 4-s command voltages from 0 to 100 mV in 20-mV increments for Hv1, and at 100 mV depolarization for control cells. Shaker-IR R362H/W434F-expressing cells were held at 30 mV and currents and fluorescence were elicited from 4-s command voltages from –120 to –20 mV in 20-mV increments. (B) ∆F snapshots of WGA-pHRho fluorescence at time points indicated in (A). pH_o/pH_i = 7.5/6.0 for Hv1 and control, and 6.0/7.5 for Shaker-IR R362H/W434F; 0.1 mM HEPES in the bath solution. Voltage-clamp fluorometry scale bars represent 100 pA, 2% ∆F/F_0 and 2s; the scale bar in images represent 10 µm.
To visualize proton depletion, we set up an inward proton gradient and used a potassium pore-blocked (W434F) Shaker-IR R362H mutant that creates an omega proton pore upon hyperpolarization [32]. The fluorescent signal mirrored the current: it became dimmer and reached steady state upon hyperpolarization, and then recovered with biexponential kinetics when the cell was returned to the 30-mV holding potential (Figure IV-3A, middle panel). ΔF snapshots before, during and after a – 120 mV test pulse showed an inverse fluorescent change at the cell surface compared to Hv1 (Figure IV-3B, right panel). In empty-vector control cells, no fluorescence change was observed, indicating that all fluorescence changes were due to proton fluxes from Hv1 or Shaker-IR R362H/W434F mutant (Figure IV-3A, right panel).

All above images were collected within 1 hr of labeling when the majority of the WGA-pHRho appeared to be on the cell surface as evidenced by the circular labeling pattern (Figure IV-4A). However, we observed that bright puncta became evident 1 hr after labeling, and visually overwhelming at 3 hrs, implying that WGA-pHRho was internalized and localized in low pH internal compartments (endosomes and lysosomes). To determine whether this internalization would affect the fluorescent signals, we recorded the current and fluorescence until 4 hrs after labeling. The fluorescent signal still had a response to the current (Figure IV-4B), but the ratio of ΔF/F₀ over the current was decreased ~ 20% after 1 h, and ~ 50% after 3 hrs post-labeling, implying that some of the extracellular pH sensors were internalized (Figure IV-4C). Although the signal intensity decreases overtime, this approach is viable for longer timepoints. In addition, the data in the latter time points likely favored cells with less internalized WGA-pHRho,
because it became noticeably harder over time to make and maintain a gigaohm seal in the whole cell configuration. For experimental ease, it was experimentally prudent to image and record immediately after labeling.
**Figure IV-4. Internalization of WGA-pHRho in CHO cells.** (A) Fluorescent images of CHO cells were collected at 0, 1, 2 and 3 hours after WGA-pHRho labeling, the scale bar represents 10 µm. (B) An exemplar of current-fluorescence traces from a Hv1-expressing cell at 3 hrs after labeling, scale bars represent 100 pA, 1% ∆F/F₀ and 2 s. The cell was held at –80 mV, and currents and fluorescence were elicited from 4-s command voltages from 0 to 100 mV in 20-mV increments. (C) A bar graph shows the ratio of ∆F/F₀ and current (I) in 1, 2, 3, 4 hours after labeling, n = 5; error bars represent ±SEM.
WGA-fluorescein is commercially available and has been used to approximate pH changes at the surface of single melanoma cells [123]. To make a fair comparison with WGA-pHRho, we made WGA-fluorescein by reacting FITC with WGA and removing the unreacted fluorophore with a gravity-fed size exclusion column. Compared to WGA-pHRho (Figure IV-3A), homemade WGA-fluorescein yielded the mirror opposite response to extracellular proton accumulation (Hv1) and depletion (Shaker-IR R362H/W434F) with approximately the same current-ΔF/F₀ relationship (Figure IV-5A and C). As expected, photobleaching was problematic with WGA-fluorescein (Figure IV-5B), especially for extracellular proton accumulation where the fluorescent signal was also quenched by protons. There was no noticeable difference between commercially-available and homemade WGA-fluorescein. Thus, for extracellular proton depletion near fluorescein’s pKₐ of 6.4, commercially-available WGA-fluorescein was a satisfactory reagent when photobleaching could be reliably corrected.
**Figure IV-5. Visualization of proton fluxes with WGA-fluorescein.** (A) Voltage-clamp fluorometry traces from CHO cells transfected with Hv1 (left panel), Shaker-IR R362H/W434F (right panel). The Hv1-expressing cell was held at – 80 mV, and currents and fluorescence were elicited from 4-s command voltages from 0 to 100 mV in 20-mV increments. The Shaker-IR R362H/W434F-expressing cell was held at 30 mV, and currents and fluorescence were elicited from 4-s command voltages from – 120 to – 20 mV in 20-mV increments. (B) Fluorescence signals without photobleaching correction of WGA-pHRho (red) and WGA-fluorescein (green) during voltage-clamp experiment in Hv1-expressing CHO cells; cells were held at – 80 mV, and currents and fluorescence were elicited from 4-s command voltages from 0 to 100 mV in 20-mV increments. (C) ΔF/F₀-current curves show the current sensitivity of WGA-pHRho (solid circle, red) and WGA-fluorescein (open square, green). pH₀/pHᵢ = 7.5/6.0 for Hv1, and 6.0/7.5 for Shaker-IR R362H/W434F; 0.1 mM HEPES in the bath solution. Scale bars represent 200 pA, 2% ΔF/F₀ and 2 s.
Visualization of proton-coupled transport of MCT1

INS-1 cells, a pancreatic β cell line, have very low endogenous MCT1 levels and have no MCT2 or MCT4 [90,164]. We attempted to label INS-1 cells with azidosugar and pH-DIBO to monitor proton fluxes from MCT1 but they also cannot use the unnatural sialic acid precursor. To determine whether WGA-pHRho can bind to the plasma membrane of INS-cells and detect proton fluxes from MCTs, we transiently transfected MCT1 into INS-1 cells. After 48 hrs, cells were treated with 50 μg/mL WGA-pHRho for 10 min and the fluorescence was recorded during extracellular perfusion of 10 mM L-lactate or pyruvate in a bath solution with a constant pH (7.0) and a low buffer capacity (0.1 mM) (Figure IV-6A and B). Upon perfusing L-lactate, the fluorescence of control cells had a small increase initially and then decreased slowly. In contrast, the fluorescence of MCT1-expressing cells decreased rapidly, reaching peak in 5 sec and then recovered and reversed the direction indicating the exogenous MCT1 significantly increased proton-coupled transport of L-lactate into the cell. Washout of L-lactate resulted in a rapid efflux of protons from the cell followed by proton diffusion to the bulk. A similar result was obtained during perfusion of 10 mM pyruvate, another physiologically important substrate for MCT1 (Figure IV-6B).
Figure IV-6. Visualization of proton-coupled transport of L-lactate and pyruvate in MCT1-expressing INS-1 cells. Fluorescence traces of MCT1-expressing (black) and control (grey) cells upon perfusion of 10 mM (A) L-lactate or (B) pyruvate, n = 6; error bars denote ± SEM.
MCT1 is critical for the oxidative capacities of cardiomyocytes and is more expressed in intercalated disc and T-tubule regions. Visualizing the transport activity of MCT1 in cardiomyocytes spatiotemporally is of high interest. Similar with INS-1 cells, we found that cardiomyocytes cannot use the unnatural sialic acid precursor either. To determine whether we can label cardiomyocytes with WGA-pHRho and monitor the transport activity of endogenous MCT1, we isolated cardiomyocytes from rat hearts and treated them with WGA-pHRho for 30 min after 2 hrs culture. Fluorescent images show that WGA-pHRho was distributed at the sarcolemma and T-tubule regions in cardiomyocytes after labeling (Figure IV-7A). During L-lactate perfusion, we observed a rapid decrease followed by a recovery in fluorescence (Figure IV-7B). However, compared to the fluorescent signals in HEK293T (Chapter III) and INS-1 cells expressing MCT1, the recovery did not reverse direction in cardiomyocytes. Perfusion of D-lactate on the same cell resulted in a smaller fluorescent change and a slower recovery in fluorescence, demonstrating that the transport rate of D-lactate is lower than L-lactate. To confirm the role of MCT1 on the fluorescence change, we pre-treated cardiomyocytes with 50 nM AR-C155858, a specific MCT1 inhibitor binding to MCT1 intracellularly [92] for 30 min. After AR-C155858 treatment, perfusion of L-lactate on the same cell caused a much slower and smaller decrease in fluorescence, and no recovery was observed. As adult rat cardiomyocytes only express MCT1 and have no other proton-coupled MCTs, this fluorescence change is mainly due to passive diffusion of lactic acid.
Figure IV-7. Visualization of MCT1 transport in cardiomyocytes. (A) The left fluorescent image shows WGA-pHRho distribution in cardiomyocytes after labeling. The schematic cartoon on the right shows MCT1 transport in sarcolemma and T-tubule regions in cardiomyocytes; the scale bar represents 10 µm. (B) Fluorescence traces of a cardiomyocyte upon perfusion of 10 mM L-lactate (black) and pyruvate (red) successively. (C) Fluorescence traces of a cardiomyocyte upon perfusion of 10 mM L-lactate before (black) and after (red) 50 µM AR-C155858 treatment.
Discussion

In Chapter II and III, we described an approach to covalently attach pH-DIBO to the cell’s glycocalyx that was engineered by an azidosugar. However, not all cell types can utilize this unnatural sialic acid precursor. For instance, we did not observe pH-DIBO labeling in INS-1 cells or cardiomyocytes after 48 hrs incubation with azido-sugar. One advantage of WGA-conjugates is that WGA binds to mammalian glycocalyces without the need of an unnatural sialic acid precursor.

By labeling cells with pH-sensitive, fluorescent WGA conjugates, we observed proton accumulation and depletion at the plasma membrane resulting from proton channel Hv1, omega mutant Shaker-IR R362H/W434F and MCT1. Although the molecular weight of a WGA-conjugate is ~ 40-fold higher than that of pH-DIBO used in Chapter II and III, the fluorescent signals corresponding to proton fluxes are both similar. Previous studies showed that the terminal sialic acids in voltage-gated cation channels modulate channel gating and function [165,166]. Even though our approach utilized WGA-sialic acid binding, we did not observe any significant activity changes in either unglycosylated or glycosylated membrane transport proteins.

Compared to the labeling time for pH-DIBO (30 min), WGA effectively labeled cells in only 10 min. Such fast labeling reduces background signal caused by endocytosis; the cells after labeling exhibit a bright circular fluorescence and have very few puncta even after 1 hr of labeling. Although a subpopulation of sensors is internalized over time, it doesn’t affect the fluorescent signal significantly, as the luminally facing fluorescent
sensors are unresponsive to the transport events occurring at the cell surface and there are enough uninternalized sensors to detect extracellular pH changes 3 hrs after labeling.

Perfusion of L-lactate or pyruvate in mock-transfected HEK293T cells caused the fluorescence signal to decrease rapidly, followed by a slow and partial recovery (Chapter III). Surprisingly, when compared to HEK293T cells we observed slower and smaller changes in fluorescence when perfusing L-lactate or pyruvate in mock-transfected INS-1 cells. Additionally, we did not observe a recovery of fluorescence, indicating that INS-1 cells have much lower endogenous MCT activity than HEK293T cells, making them an appropriate model system for functional characterization of MCTs in mammalian cells.

MCT1 is abundantly expressed in cardiomyocytes and is crucial for cardiac physiology. Here we labeled cardiomyocytes with WGA-pHRho and monitored the MCT1 activity in real-time. During perfusion of L-lactate, the fluorescent signal is similar to that in HEK293T and INS-1 cells. Interestingly, in cardiomyocytes we noted that the fluorescence recovery did not reverse the direction, which may be due to the large volume of the cell. According to immunofluorescence staining studies, MCT1 is densely localized in T-tubule regions that are near mitochondria, implying that MCT1 promotes the delivery of lactate to the oxidation site [167]. Given that WGA-pHRho can label sarcolemma and T-tubules in cardiomyocytes, our approach will allow for monitoring MCT1 activity in these specific regions with high spatiotemporal resolution when combined with the state-of-the-art imaging.

Proton fluxes also exist in other proton-coupled transporters and are involved in a wide range of cellular processes. For instance, Na+/H+ exchanger 1, NHE1 is implicated
in regulation of transepithelial transport, cell volume, cell death/survival balance and cell
motility [168]. Therefore, pH sensitive, fluorescent WGA conjugates that work with a
majority of cell types have broad utility. Moreover, when combined with a pH-insensitive
WGA conjugate, a pseudo-ratiometric pH sensor will allow for calibrated pH
determinations. Given a wide variety of ion- and metabolite-sensitive small-molecule
dyes, WGA-conjugates can be used for studying any transport events at the plasma
membrane.
Materials and Methods

Materials

WGA was obtained from Vector Laboratories. FITC and commercial WGA-fluorescein were obtained from Invitrogen. Sodium pyruvate was obtained from Alfa Aesar. L-lactic acid, sodium salt, 60 wt% solution in water was obtained from Acros. Sodium D-lactate was obtained from Sigma. AR-C155858 was obtained from Torcis.

Synthesis Procedures

pHRho (7)

\[
\begin{align*}
\text{3} & \quad \text{4M HCl, r.t.} \quad \text{7}
\end{align*}
\]

Compound 3 (50 mg, 0.06 mmol) synthesized in Chapter II was dissolved in 2 mL 4M HCl. After being stirred at r.t. for 4 h, the solvents were evaporated under reduced pressure. The residue was purified by HPLC.

WGA Conjugates

pHRho-COOH (1.5 mg, 2.1 µmol) was reacted with dicydohexylcarbodiimide (DCC: 4.0 mg, 19.8 µmol) in anhydrous N, N-dimethylformamide (DMF; 0.75 mL) at r.t. for 15 min, and then N-hydorxsuccinimide (NHS; 3 mg, 17.4 µmol) was added and
stirred at r.t. 2 hrs [120]. In 200 μl WGA solution (1 mg WGA at 5mg/ml, in 0.1 M sodium bicarbonate pH 8.3) containing 15 mg N-acetylglucosamine (0.14 μmol), the activated pHρHO-COOH was added at a dye-to-protein molar ratio of 5. Reaction mixture was stirred at r.t. for 2 hrs, and the reaction was terminated by hydroxylamine to a final concentration of 0.1 M. For the synthesis of WGA-fluorescein, FITC stock solution was prepared at 10 mg/mL in DMF immediately before starting the reaction, and 0.26 μmol FITC dye was added to WGA solution with N-acetylglucosamine (pH 9.0). The final molar ratio of dye: protein was 10:1. WGA-conjugates were separated from free unreacted reagent by gel filtration on a Sephadex G-25 Fine (20~50 mm) column equilibrated by PBS and were lyophilized down to a powder (ref. patent 5846737, Kang).

The degree of labeling (D.O.L.) was calculated using the following equation: the absorbance of WGA-conjugates was measured at 280 nm (A280) and the λmax (Amax).

\[ \text{DOL} = \frac{A_{\text{max}} \times MW}{[\text{WGA}] \times \varepsilon_{\text{dye}}} \]

where MW= the molecular weight of WGA, \( \varepsilon_{\text{dye}} \) = the extinction coefficient of dye at its absorbance maximum, and the WGA protein concentration is in mg/mL.

Molecular Biology

Plasmids containing human Hv1, Shaker-IR R362H/W434F mutant and rat MCT1 were gifts from David Clapham (Harvard Medical School), Baron Chanda (University of Wisconsin–Madison) and Sebastián Brauchi (Universidad Austral de Chile), respectively. All DNA was cloned into pcDNA3.1 for mammalian cells expression.
Cell Culture and Transfections

Chinese Hamster Ovary-K1 (CHO) cells were cultured in F-12K nutrient mixture (Gibco); INS-1 cells were cultured in RPMI medium 1640 (Gibco). All media was supplemented with 10% fetal bovine serum (Hyclone) and 100 units/mL penicillin/streptomycin (Gibco). RPMI medium 1640 was additionally supplemented with 1 mM sodium pyruvate and 55 μM 2-Mercaptoethanol (BME). Cells were plated at 60 ~ 75% confluency in 35 mm dishes. After 24 h incubation, cells were transiently transfected with 1 μg of ion channel or transporter DNA and 5 μL of Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen). To visualize transfected cells, 0.25 μg of pEGFP-C3 or mcherry was added to transfections that did not contain GFP- or mcherry-tagged channels/transporters.

Isolation and Culture of Cardiomyocytes

Sprague–Dawley rat heart cells were isolated by enzymatic digestion using a Langendorff perfusion apparatus. The heart was perfused with oxygenated solutions at 37°C: (1) Ca²⁺ free buffer pH7.4 and contained (mM) NaCl 118, KCl 4.8, HEPES 25, K₂HPO₄ 1.25, MgSO₄ 1.25, and Glucose 10 for 5 min. (2) Liberase TM research grade enzyme blend at 34ug/ml in Ca²⁺ free buffer for 15min. (3) Finally, a high K⁺ KB solution was used to wash out the enzyme, and single cells were stored at room temperature in KB until plated onto the glass bottom dishes with 5% serum Medium 199 and incubated at 37°C with 5% CO₂. (4) After 2hrs, cells were ready for WGA labeling.

Cell Surface Labeling
Transfected CHO and INS-1 cells were trypsinized and seeded on 35 mm glass bottom culture dishes (MarTek) for 2 hrs before labeling. All cells were rinsed with HBSS three times and then labeled with WGA-pHRho or WGA-fluorescein (50 µg/mL) in HBSS at r.t. for 10 min (CHO and INS-1 cells) or 30 min (cardiomyocytes). After labeling, cells were rinsed with recording or perfusion solution three times.

**Whole Cell Patch Clamp Fluorometry**

Transfected cells were identified using an inverted light microscope (Axiovert 40 CFL; Carl Zeiss, Inc.) and the currents were recorded in the whole cell patch configuration at room temperature (24 ± 2°C) using a glass electrode (pipette resistance: 2.5 – 3.5 MΩ) filled with (in mM): 126 NaCl, 2 MgSO₄, 0.5 CaCl₂, 5 EGTA 4 K₂-ATP, 0.4 GTP and 25 buffer (HEPES for pH = 7.5 and 7.0, MES for pH = 6.0) with NaOH; bath solution contained (in mM): 145 KCl, 5.4 KCl, 5 CaCl₂, 0.1 buffer (TAPS for pH = 8.0, HEPES for pH = 7.5 and 7.0, Bis-Tris for pH = 6.5, MES for pH = 6.0) with KOH. Cells were imaged at 10 Hz using a CoolLED pE-4000 light source, 63 x 1.4 N.A. oil immersion objective, and a Zyla sCMOS camera (ANDOR). 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). F₀ is the average fluorescence intensity of first five data points.

**Extracellular pH Measurement with Gravity Perfusion**
A 35-mm chamber insert (RC-33DL, Warner) was put into the glass bottom dish containing HEK293T cells or cardiomyocytes in order to decrease the bath volume. The dish was then mounted onto a Quick Exchange Platform QE-1 (Warner) for imaging. Monocarboxylates and inhibitors were perfused in/out by using a gravity perfusion system at ~10 mL/min. Cells were imaged at 1 Hz using a CoolLED pE-4000 light source (550 nm excitation), 63 x 1.4 N.A. oil immersion objective, DAPI/FITC/TRITC/Cy5 filter set (CHROMA) and a Zyla sCMOS camera (ANDOR). Fluorescent images were collected and processed using open source software (micro-manager and ImageJ). F0 is the average fluorescence intensity of first five data points.
Proton fluxes at the plasma membrane are created not only by proton channels and omega mutants, but also by various proton-coupled transporters. They play a crucial role in regulating intra- and extra-cellular pH and facilitating co-transport of other ions and metabolites. Therefore, chemical tools which visualize proton fluxes specifically at the plasma membrane have broad utility. Although intracellular pH measurements have been performed using both genetically-encoded and small-molecule fluorescent pH sensors, these approaches underestimate the density and kinetics of plasma membrane proton fluxes and provide little spatial information. Targeting fluorescent pH sensors to the extracellular surface has become of great interest, because it will allow for monitoring the local pH changes that more closely mirror proton fluxes.

Genetically-encoded and small-molecule fluorescent pH sensors have both been targeted to membrane proteins for studying endocytosis and exocytosis [112,122]. However, proton flux detection using this approach is dependent on the expression level of the membrane proteins and its proximity to the transporter of interest. Moreover, it is difficult to target pH sensors directly to the extracellular side of membrane transporting proteins which do not have extracellular termini such as Hv1, ClC-5, Shaker-IR and MCT1.

Besides membrane proteins, lipids and carbohydrates at the plasma membrane also can be used as targets for small-molecule fluorescent pH sensor labeling. Compared to pH sensors conjugated to lipids which have high flexibility [169], in my thesis we hypothesized that conjugation of sensors to the cell’s glycocalyx would be more stable
and would result in a uniform and dense coating of fluorescent pH sensors at the cell surface to enable visualization of extracellular proton fluxes.

In Chapter II, we described a bioorthogonal chemistry approach to covalently attach a small molecule, fluorescent pH sensor to the cell’s glycocalyx. Using this approach, we were able to monitor the changes in proton concentration at the plasma membrane resulting from the voltage-gated proton channel Hv1, the voltage-gated H^+/Cl^- antiporter ClC-5 and mutants of the Shaker channel R362H and R371H that produce omega current. Our results provide direct evidence of extracellular proton accumulation and deletion caused by proton efflux and influx, respectively. These results, combined with a recent study that showed proton depletion on the intracellular side of Hv1 channels [39], suggest that the local proton driving force could be vastly different from the bulk proton gradient.

It is notable that proton dynamics at the extracellular surface are different from those in bulk solution because of microenvironment complexity. For example, experimental and theoretical studies demonstrated that lipid head groups can act as a proton-collecting antenna accelerating proton uptake from the bulk solution to membrane-anchored proton acceptors [144,170]. Here we observed proton wavefronts emanating from one cell altering the local pH environment of nearby cells. Combining our approach with high-resolution spatiotemporal imaging will allow for monitoring proton diffusion along the cell surface and into the bulk solution. This will answer the question as to whether protons rapidly circumscribe a living cell before diffusing into the bulk solution.
Extracellular protons can not only modulate the activity of some membrane proteins, such as voltage-gated calcium channels [171,172], N-methyl-D-aspartate (NMDA) [173,174] and g-amino butyric acid (GABA) receptors [175,176], but also act as an agonist for acid-sensing ion channels (ASICs) which are voltage-insensitive sodium channels and involved in pathological states such as retinal damage, seizures, and ischemic brain injury [177,178]. Moreover, protons are important neurotransmitters. They can mediate muscle contraction in Caenorhabditis elegans [179], regulate synaptic plasticity in the lateral amygdala [112], as well as convey excitatory stimuli from inner ear type I vestibular hair cells to postsynaptic calyx nerve terminals [113]. By using our approach, monitoring the proton wavefronts will shed light on how the emanated protons modulate or activate nearby membrane proteins or downstream signaling. One limitation of our approach is that factors such as excitation source fluctuations and sensor concentration would affect the fluorescent intensity of pH-DIBO, which is a single-emission sensor, and make it difficult to measure extracellular pH accurately. To address this, it is required to develop an azido-reactive, ratiometric pH sensor.

In Chapter III, we applied this approach to detect the activity of MCT1 which mediates non-electrogenic, proton-coupled transport of monocarboxylates such as lactate and pyruvate. Compared to intracellular pH measurement (BCECF-AM) that shows intracellular acidification upon addition of L-lactate or pyruvate in MCT1-expressing cells, we observed proton depletion followed by a recovery at the cell surface due to proton influx and sensor re-protonation. Furthermore, by using this approach we identified a putative MCT, Hrm in Drosophila as a proton coupled MCT that
preferentially transports pyruvate. Our results clearly demonstrate that pH measurement at the cell surface is more sensitive than monitoring intracellular pH changes.

MCTs belong to solute carrier proteins (SLCs) that are essential for nutrient uptake and waste removal in cells. A quarter of the more than 400 SLC genes are associated with human diseases and are important drug targets [180]. However, compared to other membrane proteins, SLCs are the most neglected group of genes and many of them have not been functionally identified yet. The transport events in some SLCs often induce changes in the extracellular pH, such as H⁺/oligopeptide cotransporter, H⁺/Na⁺ exchanger, Na⁺/HCO₃⁻ cotransporter and Cl⁻/HCO₃⁻ exchanger. Glycocalyx-attached pH sensors not only provide a tool for functional identification of proton-involved SLCs, but also can be used for real-time and spatiotemporal measurements of SLC transport to help us better understand their physiological and pathophysiological roles.

Because glycan engineering has been applied in various mammalian cell lines [139,140,181], tissues [182] and living organisms [126,149,183,184], we expected that the Chapter II approach can be broadly applicable in vitro and in vivo. However, not all cell types can incorporate the unnatural sugar into their glycocalyx. For example, INS-1 cells, which are one of pancreatic beta cell lines, and cardiac myocytes did not be labeled successfully by using azidosugar and pH-DIBO (data not shown). It is possibly due to the low permissivity of the sialic acid biosynthetic pathway or the slow metabolism of the sialic acid in these cell types. In addition, in vivo delivery of the unnatural sugar and the small-molecule dye to the target of interest is difficulty in some cases. Although pH-DIBO has a certain solubility in water, its two large hydrophobic regions slow the
diffusion in aqueous phase. For example, we attempted to inject pH-DIBO into the pseudocoelomic space of C-elegans, but the fluorescent signal was only observed in the injection site and not in coelomocytes indicating pH-DIBO did not diffuse to the pseudocoelomic fluid (data not shown). Another challenge is the in vivo delivery of azidosugar, especially in the brain because of its inability to cross the blood-brain barrier (BBB) and a further modification, e.g. liposome encapsulation, was required in azidosugar for facilitating the delivery [148].

Since not all cell types are amenable to surface glycan engineering, we sought to develop an approach that would work with a majority of cell types. In Chapter IV, we conjugated pH sensors to WGA that binds to endogenous terminal sugar residues at the cell surface. Despite visual signs of endocytosis, the fluorescent signals of the pH-sensitive, WGA-pHRho conjugate are similar to that of pH-DIBO in response to proton efflux and influx from Hv1 and Shaker-IR R362H/W434F. We successfully used WGA-pHRho to label the plasma membrane of INS-1 cells and rat cardiomyocytes which cannot incorporate the unnatural sialic acid precursor into their glyocalyx and monitored proton fluxes from MCT1 in these cells. This straightforward approach and the detailed procedures of WGA conjugation and cell surface labeling will enable physiologists to employ these reagents to visualize plasma membrane proton fluxes in most cell types.

Cells often express ion channels and transporters at specific locations of the plasma membrane to regulate ion and metabolite fluxes. For example, T-tubules in cardiomyocytes are important regions at the cell membrane for rapid communication between extracellular and cytoplasmic environments because they are highly invaginated
into the center of cells and contain abundant ion channels, transporters and pumps. In the meantime, this special morphology also separates T-tubule regions from the bulk space and slows the solution change between T-tubules and the bulk space [185]. Monitoring this solution change event can be accomplished by using fluorescent sensors attached to the plasma membrane of T-tubules and sarcolemma regions and will help us better understand how T-tubules communicate with the outside environment.

Overall, this thesis describes the development of chemical tools that allow for visualization of proton fluxes specifically at the cell surface. Combined with state-of-the-art optogenetic tools and imaging modalities, our approaches will allow for high-throughput screening for ion channels and transporters that represent the second biggest group of membrane proteins as drug targets [186]. In theory, the pH sensor can be replaced with any ion- or metabolite- sensitive fluorescent sensor. Therefore, our approaches are readily adaptable to visualize most electrogenic and non-electrogenic transport events at the plasma membrane. Given that glycocalyces of living organisms can be engineered with unnatural sugars or labeled with WGA, further application and optimization of these approaches will allow for real-time and spatiotemporal measurement of ions or metabolites transport \textit{in vitro} and \textit{in vivo}. 
APPENDICES
Compound 2 (Boc-pH) $^1$H-NMR in MeOD (Chapter II)
Compound 2 (Boc-pH) $^{19}$F-NMR in MeOD (Chapter II)
Compound 2 (Boc-pH) $^{13}$C-NMR in MeOD (Chapter II)
Compound 3 (Boc-pH-COOH) $^1$H-NMR in MeOD (Chapter II)
Compound 3 (Boc-pH-COOH) $^9$F-NMR in MeOD (Chapter II)
Compound 3 (Boc-pH-COOH) $^{13}$C-NMR in MeOD (Chapter II)
Compound 4 (Boc-pH-NH2) $^1$H-NMR in MeOD (Chapter II)
Compound 4 (Boc-pH-NH2) $^9$F-NMR in MeOD (Chapter II)
Compound 4 (Boc-pH-NH2) $^{13}$C-NMR in MeOD (Chapter II)
Compound 5 (Boc-pH-DIBO) $^1$H-NMR in MeOD (Chapter II)
Compound 5 (Boc-pH-DIBO) $^9$F-NMR in MeOD (Chapter II)
Compound 5 (Boc-pH-DIBO) $^{13}$C-NMR in MeOD (Chapter II)
Compound 6 (pH-DIBO) $^1$H-NMR in MeOD (Chapter II)
Compound 6 (pH-DIBO) $^9$F-NMR in MeOD (Chapter II)
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