

TECHNIQUES FOR IMPROVING ACTIVITY BASED BIOSENSORS

A KUHL PLATFORM FOR ENGINEERING

by
Merrilee Anne Thomas

A dissertation submitted in partial fulfillment
of the requirements for the degree

of
Doctor of Philosophy

In
Neuroscience

MONTANA STATE UNIVERSITY
Bozeman, Montana

DECEMBER 2020

©COPYRIGHT

by

Merrilee Anne Thomas

2020

All Rights Reserved

DEDICATION

To my father, David Michael Thomas, a fountain of optimistic knowledge.
To Jack Quatman, I miss you everyday friend.
To Shanika Thomas, and all the people of Whitefish who have suffered from or lost someone because of mental illness, may we one day find a cure.

ACKNOWLEDGEMENTS

Science doesn't exist in a vacuum. I would like to thank the following people: Paul Tewson for HEK-293 cells. Elsa Roush for HEK-293 plates. Scott Martinka for great high titer virus. Kevin Harlett for having a deep love of analyzing data. Anne Marie because science needs more star power. Kaspar Podgorski, Steven Hoffman, Dr. Mark Owkes, Jamie Mazer, and Nadav Oakes for MATLAB wizardry. Huge thanks to Joan Lemire at Tufts University for HCN2 plasmid. This body of work was funded by a NIH/NINDS BRAIN initiative grant (grant number 1U01NS099691). To Jamie Mazer, Anne Marie, and MBI for additional funding and support.

To my greatest teachers, Ray Boksich, Katie Phelan, and Thom Hughes for believing in me and pushing me to be the best I could be. Susy Kohout for being a really tough teacher and a great friend. I would also like to thank the graduate committee for their time, commitment, and tireless source of knowledge and willingness to help.

To CC Haworth, thank you for serving this community, never forget. To all of my friends, but especially, Rosana Molina, Faith Doty, Kayleen Offringa, Conlin Ritter, Sam Starr (Ski MO 4life), Peter Sandstorm, Rachel Glass, Clint Hoon & Co., Neal & Missy, KT. G, Grace .A, Cody.B, Dale. G. To the INDO crew, Jersey Boyz crew, Buffalo Crew, friends of Bill. W, and the Cooley Crew! I would not be where I am today without every single one of you.

To the Kuhlest parents I know. To my sisters, Sarah, Alison, Kjerstin, & Melissa, and to all of my brothers for being an endless supply of knowledge, guidance, and friendship. For laughter and tough love.

TABLE OF CONTENTS

1. THE POTENTIAL OF SYNTHETIC BIOLOGY	1
2. BIRTH OF THE EXCITABLE CELL	4
The Investigator's Nascency	4
Birth of the Excitable Cell	5
Cell Theory	8
The Neuron Doctrine	9
Establishing the Membrane Potential	10
Axoplasm and birefringence	10
A Brief History of the Patch Clamp	12
The Giant Squid Axon	12
The Ionic Nature of the Action Potential	13
The Patch	16
Ion Channels	18
The Action Potential	18
Voltage-Gated Sodium Channels	20
Voltage-Gated Potassium Channels	21
ATP Pump (Transport Proteins)	22
Sodium-Potassium Pump	22
Serca Pump	22
Gap Junctions	22
Neuronal Transduction	23
Synapt-pHluorins	24
Activation Systems for Excitable Cells	25
Photodynamics	25
Bacterial Rhodopsin	26
Photo-regulation and Caged Compounds	27
Optogenetics is Coined	30
bPAC	30
Recording Activity with Fluorescence	31
Activity-Based Biosensors	31
Why are Ca^{2+} Sensors Important	31
Genetically Encoded Voltage Sensors	33
De Novo Models of Excitable Cells	33
Catechism of Synthetic Biology	33
The Kuhl Model	36
References Cited	40

TABLE OF CONTENTS CONTINUED

3. OPTICALLY ACTIVATED, CUSTOMIZABLE, EXCITABLE CELLS	55
Abstract	56
Introduction	57
Why are Biosensors Important?	57
Results	59
Establishing an Optical Actuator	59
Dose Dependence.....	62
Coupling the Actuator with an Ion Channel	63
Whole-Cell Ca^{2+} Transients with the addition of Kir2.1 Coupled with bPAC.....	66
Optimizing the Activity	68
Voltage-gated Sodium Channel NavD creates Faster Ca^{2+} Transients.....	70
Further Optimization with Connexin-43 and Kir2.1 and NavD	72
Optimizing Activity with Connexin-43	74
Optimizing Activity with CNG.....	76
Optimizing Activity with HCN2.....	77
A Kuhl-KCNaCx	79
Comparing Green and Red Sensors in the Same Cell.....	83
Green Voltage Sensors Can be Screened in Kuhl-CKNaCx Cells	84
Discussion	85
Methods	87
Growth Conditions for HEK293	87
Plasmids	87
Baculovirus	87
Transduction	88
An ASI Modular Microscope.....	88
Optosplitter	89
Analysis.....	89
Acknowledgements	91
References Cited.....	92
Supplemental Material	96
 3. FUTURE RESEARCH DIRECTIONS	 114
Further Kuhl Optimization	114
Screen Activity-Based Biosensors	115
Using the Kuhl System to Screen Drugs	116
A Need for a Voltage-clamp Protocol Using Electrophysiology	116

TABLE OF CONTENTS CONTINUED

Subcellular Flashes in the Kuhl Cells.....	118
REFERENCES CITED.....	119

LIST OF TABLES

Table	Page
1. Dunnett Multiple Comparison Test	111
2. Experimental Setup for Each Figure.....	112

LIST OF MOVIES

Movie	Page
1. Bright subcellular flashes of Ca^{2+}	
2. The temporal/spatial movement of CNG in Kuhl-CKNaCx cells	
3. The temporal/spatial movement of HCN2 in Kuhl-HKNaCx cells	
4. A well from the Kuhl-CKNaCx 10 well Experiment	
5. G-GEQO1 and R-GEQO1 can be imaged simultaneously	
6. Whole well image of ArcLight activity	
7. Combined images of ArcLight activity	
8. Combined images of ArcLight activity	

LIST OF FIGURES

Figure	Page
1. 20ms of blue light repeatedly activates bPAC	61
2. Dose/response relationship between the intensity of blue excitation of bPAC and fluorescence response of R-caDDis	63
3. Blue light activation of bPAC slowly increases whole-cell Ca^{2+} when CNG or HCN2 is expressed	65
4. Addition of Kir2.1 leads to fluctuating whole-cell Ca^{2+} transients in the Kuhl-C/HK system	67
5. Features of prolonged Ca^{2+} transients in the Kuhl cells shown in Figure 4 differ depending on the presence of CNG or HCN2	69
6. The Kir2.1 and NavD concentration were optimized	72
7. Optimizing Kir2.1 and NavD with connexin-43 increases the average number of Ca^{2+} transients	73
8. The gap junctions between cells using connexin-43 was optimized by varying the levels of viral titer	75
9. The amount of CNG channel was optimized	77
10. HCN2 can be exchanged for the modified CNG channel	78
11. A Kuhl-CKNaCx – Consistent activity with stable viral concentration	81
12. Both red and green Ca^{2+} sensors can be recorded from the same Cell and imaged simultaneously using an OptoSplit II	83
13. The Kuhl-CKNa-Cx system undergoes voltage changes that are Visible with ArcLight	85
S1. Fluorescence trace of cells with Kir2.1, CNG, R-GECO1 (Kuhl-CK	96
S2. Representative Traces	97

LIST OF FIGURES CONTINUED

Figure	Page
S3. Introduction of NavD to Kuhl and Cx-43 increases speed of the Ca^{2+} transients	98
S4. Features of the [Kir2.1]:[NavD] optimized Kuhl-CKNa cells and their Ca^{2+} transients.....	99
S5. Features of the Features of the [Kir2.1]:[NavD] optimized Kuhl-CKNaCx cells and their Ca^{2+} transients.	100
S6. Features of the Cx-43 optimized Kuhl-CKNaCx cells and their Ca^{2+} transients, corresponding figure 8	102
S7. Features of the CNG optimized Kuhl-CKNaCx cells and their Ca^{2+} transients, corresponding figure 9	104
S8. Features of the HCN2 optimized Kuhl-HKNaCx cells and their Ca^{2+} transients, corresponding figure 10	106
S9. Features of the optimized Kuhl-CKNaCx cells and their Ca^{2+} transients, corresponding figure 11a.....	108
S10. Features of the optimized Kuhl-CKNaCx cells and their Ca^{2+} transients, corresponding figure 11b	110

ABSTRACT

According to Kuhn, “there are three classes of problems - determination of significant facts, matching of facts with theory, and articulation of that theory([Kuhn 2012](#)).” The current paradigm in molecular neuroscience is that there is a need for revolutionary tool development in neuroscience. Interestingly, the need for better tools in neuroscience is to answer neuroscience theories and provide the determination and articulation of those theories.

Currently, the neuroscientist’s toolbox is growing and the ways in which those tools are used is rapidly changing. Neuroscience underwent a revolution when we were able to take single-cell recording in vivo and then assign field properties to individual neurons based upon those responses([O’Keefe and Bouma 1969](#); [O’Keefe and Dostrovsky 1971](#); [Moser et al. 1995](#)). Scientists became adept at imaging increasingly smaller regions of the functioning human brain ([Price 2012](#)). We have since been able to genetically encode and manipulate proteins and pathways while recording from them using fluorescence([Southern and Berg 1982](#); [Chalfie 2009](#)). In vitro and in vivo we have harnessed the use of light to stimulate or inhibit specific neurons or ligands([Boyden 2011](#); [Adamantidis et al. 2007](#)). These tools are just the beginning and by no means is this an exhaustive list.

We introduce the Kuhl synthetic cell system that provides a customizable de-novo excitable cell. The Kuhl system is activated using a blue light photo activated cyclase bPAC. It can be used to create better tools to image the brain and can be used to screen multi-color fluorescent sensors. Interestingly, sensors that are within bPACs activation spectrum can be used in these synthetic cells. We show that both red and green Ca^{2+} sensors can be imaged simultaneously, and both Ca^{2+} and Voltage sensors can be screened in the Kuhl system. The Kuhl system has the potential to be used to screen for drug compounds and in theory, they could be used in studying pathways that are less understood, such as the mTOR pathway.

CHAPTER ONE

THE POTENTIAL OF SYNTHETIC BIOLOGY

Our understanding of the electrical signaling in the brain and nervous system has advanced with technology. Luigi Galvani in the 18th century showed the effects of electrical stimulation on muscles. Since then we have built many tools to further our understanding of the brain that range from electrophysiology to genetically encoded biosensors. Biosensors are used consistently in neuroscience today to help untangle the intricate signaling pathways of the brain. Biosensors are fluorescent proteins attached to a protein that provides an optical fluorescent readout in response to an analyte of interest. They enable scientists to build maps of activity between individual neurons as well as entire subsets of neurons within the brain. To image the entire brain scientists need multi-color biosensors without spectral overlap. This requires that there are a wide array of fluorescent proteins with different spectral properties. In addition, these fluorescent proteins need to maintain their spectral properties when attached to an analyte-sensitive protein and have the ability to undergo fluorescence changes when the analyte of interest increases or decreases in some way. Importantly, these sensors can be genetically encoded providing highly specific placement within the brain or body.

In the second chapter, I will go through the history of neuroscience and some of the tools that are currently in use. I will also outline the Kuhl system for building novel tools to image the brain. I propose that we can improve the speed at which genetically encoded biosensors are built and in doing so scientists might also uncover the underlying mechanisms that cause changes in a biosensors fluorescence, specifically genetically encoded indicators. My work builds on the work

of pioneers such as Lester, Bursac, and Cohen among others ([Hsu et al. 1993](#); [Park et al. 2013](#); [Kirkton and Bursac 2011](#)). They have carved the idea that simpler tools can be used as a facsimile of a neuron. Genetically encoded voltage sensors are behind compared to Ca^{2+} sensors in their use in the neuroscientific field. However, if they were built with the necessary kinetics, large change in fluorescence, and proper membrane trafficking they would provide the ideal readout needed to understand the intricate electrical network system of the brain.

In chapter three I will present my research regarding the Kuhl cell system. To improve the throughput of activity-based biosensors I have built an electrically active cell line that can be stimulated with blue light. This system can be manipulated by changing the proteins placed into the system, and optimized by changing the expression levels of any particular channel. This screen is an improvement on the previous work because it can screen both red and green sensors simultaneously. This is significant because it provides ease of use when comparing the modified sensor to the gold standard. Somewhat surprisingly, it can be used to improve both voltage and Ca^{2+} biosensors. Finally, I will end with future research directives for the improvement of genetically encoded biosensors and other optogenetic tools.

REFERENCES CITED

1. Kuhn TS. *The Structure of Scientific Revolutions: 50th Anniversary Edition*. University of Chicago Press; 2012.
2. O'Keefe J, Bouma H. Complex sensory properties of certain amygdala units in the freely moving cat. *Exp Neurol*. 1969;23: 384–398.
3. O'Keefe J, Dostrovsky J. The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. *Brain Res*. 1971;34: 171–175.
4. Moser MB, Moser EI, Forrest E, Andersen P, Morris RG. Spatial learning with a minislab in the dorsal hippocampus. *Proc Natl Acad Sci U S A*. 1995;92: 9697–9701.
5. Price CJ. A review and synthesis of the first 20 years of PET and fMRI studies of heard speech, spoken language and reading. *Neuroimage*. 2012;62: 816–847.
6. Southern PJ, Berg P. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J Mol Appl Genet*. 1982;1: 327–341.
7. Chalfie M. GFP: Lighting up life. *Proc Natl Acad Sci U S A*. 2009;106: 10073–10080.
8. Boyden ES. A history of optogenetics: the development of tools for controlling brain circuits with light. *F1000 Biol Rep*. 2011;3: 11.
9. Adamantidis AR, Zhang F, Aravanis AM, Deisseroth K, de Lecea L. Neural substrates of awakening probed with optogenetic control of hypocretin neurons. *Nature*. 2007;450: 420–424.
10. Piccolino M. Animal electricity and the birth of electrophysiology: the legacy of Luigi Galvani. *Brain Res Bull*. 1998;46: 381–407.
11. Hsu H, Huang E, Yang XC, Karschin A, Labarca C, Figl A, et al. Slow and incomplete inactivations of voltage-gated channels dominate encoding in synthetic neurons. *Biophys J*. 1993;65: 1196–1206.
12. Park J, Werley CA, Venkatachalam V, Kralj JM, Dib-Hajj SD, Waxman SG, et al. Screening fluorescent voltage indicators with spontaneously spiking HEK cells. *PLoS One*. 2013;8: e85221.
13. Kirkton RD, Bursac N. Engineering biosynthetic excitable tissues from unexcitable cells for electrophysiological and cell therapy studies. *Nat Commun*. 2011;2: 300.

CHAPTER TWO

BIRTH OF THE EXCITABLE CELL

The Investigator's Nascency

The textbook is the technique that academia has taken to effective science pedagogy. Prior to the birth of modern textbooks, many scholars would have books such as Aristotle's *Physica*, Ptolemy's *Almagest*, Newton's *Principia*, and *Optiks*, Franklin's *Electricity*, Lavoisier's *Chemistry*, and Lyell's *Geology* lining their bookshelf (Kuhn 2012). These early textbooks served as a starting point for proceeding generations as they combined the current compounding knowledge and unanswered questions to be defined and solved by the current investigator. The literature review is the combination of known and defined unknown phenomena giving rise to the current paradigm in any given doctrine of science (Kuhn 2012).

The confidence in the current paradigm makes textbooks possible. Textbooks are re-written continuously leading the student to believe that science is largely cumulative. They relay to the reader only the most recent paradigm, and in doing so, eliminate the history that built the current understanding. As Whitehead said, “A science that hesitates to forget its founders is lost” (Kuhn 2012).

Science doesn't forget its heroes; science revises its heroes' works to fit into the current paradigm. The scientist who gains the recognition, however, is not always the one who answered the question first. Science is not always fair in this way. All too often, the recognition doesn't merit the investigator who answered the question first, but instead the investigator who did it best

or loudest. The corollary to this is that there will always be scientist's scientists, people that practicing scientists know as the folks who should be recognized yet they simply aren't as flashy.

Birth of the Excitable Cell

The birth of the excitable cell began when a cell first acquired a lipid membrane. The lipid membrane acts as a barrier separating the cells' organelles, cytoplasm, proteins, nucleus, and many other parts from the surrounding environment. Once a membrane was established the cell evolved to have membrane transport mechanisms to move nutrients and ions across the membrane. This flow of ions creates an electrical potential difference across the membrane that is highly specific to each cell type (Nicholls and Ward 2000).

The resting membrane potential is crucial for the function of excitable tissue, muscle, and nerve. The resting membrane potential is specifically controlled by the ion channels and ATP pumps expressed in each cell type. It is a conduit for the propagation of action potentials in excitable cells (Abdul Kadir et al. 2018). An action potential is the sending of an electrical signal along a cell. The electrical signal is then passed to neighboring cells through gap junctions and neurotransmitters. Excitable cells define who we are. Can you imagine your heart laying limp in your chest unable to pump blood through your entire vasculature? Or if your sensory neurons were slow to react to the hot flame? How would your body regulate insulin levels without the bursting activity found in pancreatic β -cells? In the absence of excitable cells, we would cease to exist.

Every living mammal has an average of about 1 billion heartbeats within its lifetime. This was true until modern medicine started helping humans outlive this average reaching close to 2.5 billion heartbeats in a lifetime (Bryson 2019; Dunn 2015). How does the heart, a muscle,

contract continuously throughout your life? The heart contracts by propagating an action potential across the myocardial tissue and is similar to the electrical potential that crosses the neuronal membrane.

The transmembrane ionic currents in myocardial cells are initiated by the Sinoatrial Node SAN and choreograph the cardiac electrical impulse. The SAN action potential has a slow depolarization that is regulated by hyperpolarization cyclic nucleotide-gated HCN channels. Then T-type Ca^{2+} channels open and further depolarize the cell. When the transmembrane potential reaches -40mV the L-type Ca^{2+} channels open and depolarize the cell to $+40\text{mV}$. Following this, the delayed rectifier K^+ channels hyperpolarize the cell as the Ca^{2+} channels close. The pacemaker cells of the heart are electrically coupled to the cardiomyocytes through gap junctions. This allows all neighboring cells to depolarize and coordinate the heart as one unit.

The myocardial action potential can be thought of in four parts. First, there is the rising edge also called the depolarization that is initiated by the neighboring SAN action potential. The initial phase of the myocardial cell depolarization is controlled by fast activating Na^+ voltage-gated channels and slow L-type Ca^{2+} channels. Activating these ion channels brings Na^+ and Ca^{2+} ions into the cell. Then, as the cell reaches maximum depolarization or early repolarization, the Na^+ channels close, and the K^+ channels open. There are two K^+ currents, the IK_S and the IK_R . The IK_S channel is made from the KCNQ channel family and slowly repolarize the cell. The IK_R current is encoded by the Human Erythro-Gogo HERG channel rapidly repolarizes the cell. The second is the plateau phase when the IK_S current conductance drives the action potential slowly back to baseline but the Ca^{2+} channels stay open. The third phase is repolarization when

the Ca^{2+} channels close, and the IK_R current drives the cells back to a hyperpolarized state. During the repolarization period, the leaky K^+ channels open and ATP ion pumps restore the Na^+ , Ca^{2+} , and K^+ ions to resting transmembrane ionic concentration gradients (Milstein et al. 2012).

When we eat glucose enters our bloodstream and pancreatic β -cells respond by releasing insulin. Insulin is a hormone that signals cells to absorb the sugar in the bloodstream so it can be used for energy or stored. Insulin acts to regulate blood sugar levels. Humans diagnosed with type 1 and 2 diabetes have significantly reduced function in their beta cells, leading to insufficient insulin secretion and hyperglycemia. The β -cells have a complex response to the metabolism of glucose that affects ion channels, transporters, and pumps that create spatiotemporally coordinated electrical activity resulting in the secretion of insulin. Glucose initiates the release of insulin which induces depolarization and the firing of an action potential (Jacobson and Philipson 2007). The falling phase of the β -cells action potential is due to the voltage-gated K^+ channels (Jacobson and Philipson 2007).

A notable example of an organelle with a membrane potential is the mitochondria. Mitochondria are essential to the life and death of a cell because they generate most of the chemical energy, adenosine triphosphate (ATP), needed to power the biochemical reactions within the cell (Mammucari et al. 2016). The biochemical reaction is driven by mitochondria's ability to regulate the flow of ions across its membrane by using proton pumps. These proton pumps work to create an H^+ proton gradient and a pH gradient that is essential for the process of energy storage during oxidative phosphorylation thus driving the synthesis of ATP (Nicholls and Ward 2000).

Interestingly, mitochondria are also a sink for Ca^{2+} within the cell, however, the endoplasmic reticulum is the greatest intracellular Ca^{2+} storage organelle. Mitochondria control cellular Ca^{2+} levels by regulating the sequestration and release of Ca^{2+} . The uptake of Ca^{2+} by the mitochondria is driven by the membrane potential difference which is generated by the electron transport chain. This affects cellular signaling which is important in muscle function, cell migration and growth, neuronal excitability, and muscle contraction (Giorgi et al. 2018).

Cell Theory

Cell theory can be captured in three parts. First, all living organisms are composed of one or more cells. Second, the cell is the most basic structure of organization within an organism. Finally, the cell must come from pre-existing cells. The requirements for cells to function include the ability to self-organize, self-regulate, contain self-support structure and movement, and self-replicate. Although all cells have the same general moving parts, such as mitochondria, nucleus, and membranes, not all cells are equal (Deiters and Guillery 2013).

Robert Hooke was the first to coin the term “cell” while examining the dead cells of cork. He had done significant work using microscopy and published a book called *Micrographia* that described his observations (Mazzarello 1999). However, it was Theodor Schwann who originally described that all tissues of living organisms were made up of cells. But this theory didn’t hold up when it came to the nervous system. Karl Dieters images showed a cell that included an axon and dendrites. It wasn’t clear what part of the image was the cell. How did the cells of the nervous system connect to other cells? How did they communicate? Was it one single connected

mass of cells that shared cytoplasm or discrete cells that shared borders (Probst and Langui 1994)?

The neuron doctrine theory states that the nervous system is made up of many discrete cell types that are anatomically and genetically independent. The neuron doctrine is credited to Ramon y Cajal, however, the theory was borne from the work of many notable scientists such as Camilo Golgi, Franz Nissl, August Forel, Jan Purkinje, among others (Probst and Langui 1994).

The Neuron Doctrine

It is important to remember that science was borne out of religion and the hope to find evidence therein. The Latin word for soul, ‘anima’, means to “have breath or soul”. This later became the derivative of the English word, animal. “Animal Spirits” were the elusive entities that acted as the messenger of the soul, guiding a person to discover sensation according to classical science (Piccolino 2006). A common philosophy at the time was that electricity was responsible for the “animal spirits” and that there was a fluid that propagated along nerves.

In 1791, Luigi Galvani, an Italian physicist and biologist, was the first known man to observe the electrical pulse of the nervous system in frogs. He proposed that the muscles of dead frogs legs twitched when subjected to an electrical pulse because of (Piccolino 2006) “the flow of an extremely tenuous nervous fluid...similar to the electric circuit which develops in a Leyden jar” (Galvani 1791). Alessandro Volta, a physicist that became interested in Galvani’s work, decided that the frog nerve was bereft of electricity itself, but instead acted as a Leyden jar due to the metals that Galvani used to connect the muscle and nerve (Piccolino 2006). Volta argued that an external electric stimulus was necessary to allow for the muscle contraction, while Galvani believed there was a disequilibrium between the animal tissues, driving the electrical pulse.

Interestingly, both men were correct (Piccolino 2006). Alessandro Volta himself would go on to create the electric battery, a monumental moment in science and human life. However, Galvani's experiments led the way in understanding electrical impulses and how they travel. But what did these cells look like upon which electrical pulses traveled?

Following this, German neuroscientist Otto Freidrich Karl Deiters (1869) proposed that the neuron had two distinct parts to its cell body: an axon and a dendrite. Unfortunately for Otto, his famous and detailed studies on the neuron cell, showing that each cell had a single axon and multiple dendrites, were published postmortem (Deiters and Guillery 2013). A revolutionary technique developed by an Italian neuroscientist in 1873 used silver to stain and visualize nerve cells including the axon and dendritic branches in detail. This silver staining technique was termed the “Golgi stain” after its founder Camillo Golgi (The Nobel Prize in Physiology or Medi...). Golgi believed that the nervous system was a single continuous network, also known as reticular theory. However, the Golgi stain technique was then used by Santiago Ramon y Cajal who drew what he saw through his microscope. Cajal wanted to be an artist, but instead gifted the world of neuroscience his draughtsmanship by publishing drawings of neurons with incredible detail. He went on to develop the concept of the “Neuron Doctrine”, a theory that the nervous system is made up of discrete individual cells. Interestingly, both Golgi and Ramon y Cajal were awarded the Nobel prize in 1906 even though their views on the neuronal system differed (The Nobel Prize in Physiology or Medi...).

Establishing the Membrane potential

Axoplasm and birefringence

By the 1900s scientists had discovered that signaling in neurons was electrical while signaling between neurons was chemical. They discovered that there were bursts of activity which was coined the Action Potential AP, by Julius Bernstein. In 1868 Bernstein produced the first accurate description of the action potential and published “*Membrane Theory of Electrical Potentials*” in *Cells and Tissue* in 1902. His fundamental idea drove later experiments on the conduction of the nervous impulse and still holds today. He concluded that the action potential is a self-propagated mechanism driven by the differences between the external and internal surfaces of the neuronal membrane (Seyfarth 2006). It was clear that there was a general flow of charged particles across the membrane but the full details behind the action potential remained unclear.

A landmark paper was published in 1922, “A Study of the Action Currents of Nerves with a Cathode Ray Oscillograph”, by Nobel Prize winners Herbert Gasser and Joseph Erlanger. They developed the time course of the action potential including the refractory period, local graded responses to stimulation, and the ionic nature of the AP. To do this they used a cathode ray oscillograph that was designed by J.B. Johnson (Gasser and Erlanger 1922; Index of Review, 1921 1921), a tool that was sensitive enough to measure small changes in voltage. They attached two recording electrodes to a frog sciatic nerve that sent the electrical signal through a three-stage amplifier onto two horizontal plates within a glass tube encasing an anode and a cathode. The amplified signal from the nerve impinged a vertical movement onto the cathode ray which was read as a change in voltage. The ray is projected onto vertical plates and is processed in a spreading device where standing waves can then be recorded by hand or photographed. The use of this instrument and their subsequent findings that there were multiple nerve fiber types and that there was a correlation between the diameter of the nerve fiber and the speed of the action

potential awarded them the Nobel Prize in physiology in 1944 (Gasser and Erlanger 1922; Nobel Prize for Physiology and Medici...).

Following this, Hill and Keynes described a change in the opacity of a shore crabs nerve when stimulated. They suggested that there was a change in viscosity within the axoplasm of the nerve which was translated as changes in scattering light. However, they were unsure if the optical scattering could otherwise result from optical property changes in the membrane of the axon. They used a photocell that changed its resistance depending on the amount of light that was scattered after passing light through a nerve that was stimulated. A second photocell acted to balance the light component. They measured the resulting differential photocurrent as a voltage across a series resistance that was then recorded by a cathode ray oscilloscope. This would lead to the idea that the axoplasm contained ions that were changing with electrical conductance (Hill and Keynes 1949).

A Brief History of the Patch Clamp

The Giant Squid Axon

Much of what we know about neuroscience has come from sea species such as the *Aquerea Victoria*, *Decapodiforms*, *Aplasia Californica*, *Beggiatoa*, *Discosoma sea anemone*-- i.e. jellyfish, squid, slugs, corals, and sulfur loving bacteria. Reductionist science helps us use these organisms to answer questions about the brain's electrical conductance and guide us to the next logical experiment. By the early 1930s, the axon of the giant squid was consistently being used for exploration of the action potential. Many foundational principles in neuroscience were born at the Marine Biological Laboratory in Woods Hole, MA, where a plethora of squid was

harvested for their giant axons. Two men, George Marmont and Kenneth Cole shared a lab there in the early 1940s (Cole 1982).

The current clamp was invented by Marmont in 1947 allowing researchers to measure transmembrane voltage while simultaneously controlling the transmembrane potential. The method involves injecting positive or negative current constantly while measuring the voltage change across a cellular membrane. In his original design, Marmont had one wire injecting current while the other wire was inserted into the axon for recording the voltage. Outside of the axon was a reference electrode which allowed him to measure the changes in membrane voltage (V_m)(Cole 1982; Marmont 1940).

Meanwhile, Sir Kenneth Cole was deeply invested in getting successful measurements using a similar technique but instead of measuring current, he measured voltage. They worked using the same equipment at different hours of the day, and the circuit configurations were switched easily by a connection block with two plug-ins. By using a voltage clamp Cole could control voltage across the membrane through an electronic feedback circuit. To measure the capacitance of the cell he used voltage stepping, a method that involves holding the voltage at a preset number and increasing or decreasing the voltage over time. Then at each voltage step he measured the amount of current supplied or absorbed by the circuit needed to hold the voltage constant. Therefore, the voltage clamp supplies the experimenter with the ionic current flowing across the membrane in response to the voltage step (Cole 1982; Cole and Baker 1941; Cole and Curtis 1941).

The Ionic Nature of the Action Potential

An initial problem that Hodgkin and Huxley had to fix was that Cole had been investigating the action potential by controlling for voltage using a single axial wire. A single wire created an electrode polarization that is due to the metal exposure to an electrolyte solution. This interface leads to a decreased measurement of an outward current when trying to depolarize the cell. Hodgkin and Huxley optimized this protocol by using two internal electrodes; one to pass current and one to measure voltage.

The initial upstroke of the action potential was computationally predicted by Hodgkin and Katz and later experiments showed this (Hodgkin and Katz 1949). They examined the effect of the sodium current on the electrical activity of the squid giant axon. The hypothesis was built on the ground that the ionic composition of the axoplasm of a squid nerve is different from that of the seawater in which the axon is immersed. The resting membrane theory, proposed by Bernstein, assumes that the membrane is permeable to potassium and prudently permeable to sodium. However, they proposed that the reversal of the membrane potential is obtained if the membrane increases its selective permeability to sodium by becoming highly permeable to sodium. The take-home message was that they proposed that the active membrane had a special mechanism that allowed the sodium ions to cross the membrane at a much greater rate than either potassium or chloride. Therefore, the permeability to sodium would rise to a value that is much greater than that of potassium or chloride (Hodgkin and Katz 1949).

In March of 1952, Hodgkin and Huxley reported a series of experiments that led to the ionic basis of the action potential in a giant squid axon (Alan Lloyd Hodgkin - Nobel Lecture; Hodgkin and Huxley 1952; Hodgkin and Huxley 1952). Initially, they produced two papers examining the components of the membrane and portrayed it as a capacitor and each ion with

varying conductances. They then detailed their experimental procedure and the measurement of current and voltage in the squid giant axon (Hodgkin and Huxley 1952; Hodgkin et al. 1952). Next, they examined the sodium conductance and confirmed what Katz and Hodgkin had hypothesized. They found that the membrane potential directly influences the flow of sodium ions across the membrane. They also found that a membrane can go through a refractory period during which time the sodium conductance is slow, even if the membrane is further depolarized (Hodgkin and Huxley 1952; Hodgkin and Huxley 1952; Hodgkin and Katz 1949).

Finally, they determined the influences of the ionic currents driving the action potential and which currents are active during the various stages of the action potential. They determined that the inward current in the voltage clamp measurement was probably due to sodium ions because the inward current was of the correct magnitude and occurred over the same range of membrane potentials as they had discovered in their previous paper. To test this they placed the axon in a reduced or sodium-free environment and when they did so the inward current tapered off or disappeared. Next, they examined the outward current which they predicted was due to potassium ions. However, because the current is outward, they could not repeat the same experiments that they used to determine the inward sodium current. They then suggested that the system was made of two components, both the sodium current and the potassium current and they determined the time course for both. Amazingly, they found that the current depended on the concentration difference of the ions on either side of the membrane and the electrical potential difference across the membrane. They determined that each ion has an equilibrium potential, E_{ion} , which means that at a certain voltage, the current of the given ion effectively falls

to zero. Then the driving force on the ion can be measured as the difference between the membrane potential and the equilibrium potential (Hodgkin and Huxley 1952).

They concluded their series of five papers by summing up their experiments and expressing a set of nonlinear differential equations that generally characterized excitable cells such as neurons or cardiomyocytes as though they were an electrical circuit (Hodgkin and Huxley 1952). Hodgkin and Huxley won the Nobel Prize in 1963 for their clear and methodical analysis of ionic currents concerning voltage changes across nerve membranes and the differential equations underlying them (Alan Lloyd Hodgkin - Nobel Lecture). Although the concept of the ion channels existed, there wasn't a method available for directly measuring the unit events that made up the action potential. The current of a unit event was drowned out by the background noise associated with measuring the electric current passing through the cell membrane.

Membrane biophysicists believed that if measurements could be taken at the molecular level with increased resolution, an entire world of signaling molecules could be found (Neher and Sakmann 1992). Bert Sakmann and Erwin Neher studied together at the Max Planck, then Sakmann went on to study biophysics as a British council scholar at UC in London under Bernard Katz from 1970-1973. During this time Bernard Katz and Ricardo Miledi published findings using a statistical analysis that indicated that there were electrical events that constituted the voltage fluctuations at the neuromuscular junction. This provided mounting evidence that there were small channels in the membrane causing these electrical signals to propagate (Katz and Miledi 1972).

The Patch

Immediately following this, Erwin Neher and Bert Sakmann discovered the patch-clamp technique which would later gain them the Nobel Prize in 1991 ([Sakmann 1992](#)). They specialized the voltage clamp by creating a micropipette that allowed for a perfect seal between the pipet and the cell membrane. Initially, this micropipette was a heat polished glass pipette that was placed along a running radio player to polish the point of the open tip ([Neher and Steinbach 1978](#)). Using a slight suction through the pipette with a resistance of 10-100G Ω they create a “giga seal” and can isolate a small patch of the membrane and the ion channels that are present ([Sigworth and Neher 1980](#)). The channel's properties are then available for manipulation electrically or chemically. Since the original Whole patch technique was created many types of patch-clamp techniques have been developed such as the inside-out, outside-out, outside-in ([Hamill et al. 1981](#)).

Their original studies were on the role of ion channels in synaptic transmission at the neuromuscular junction of frog skeletal muscle([Neher and Sakmann 1976](#)). Specifically, they were looking at the ionic channel associated with acetylcholine (ACh) receptors. By carefully recording from the membrane and applying different agonists and antagonists to the muscle fiber that they were recording from they were able to see single ionic currents of ACh. The Ohmic seal allowed researchers to apply voltage to even small cells, which was previously impossible to do, due to the need to insert wires into large axons ([Neher and Sakmann 1976](#)). This novel voltage-clamp technique was termed Whole-cell recording and allowed researchers to access the intracellular membrane of the cells that are only a few microns in diameter without sticking in a microelectrode.

A fundamental piece of the patch-clamp invention was the amplifier that Fred Sigworth built by hand (Hamill et al. 1981; Sigworth and Neher 1980). As the patch-clamp technique was perfected hundreds of labs started detailing ionic channel properties in detail. Indeed, a profound paper by Sigworth and Neher detailed the voltage and time-dependent conductance of sodium channels, and their function in propagating the action potential in cultured rat muscle cells. (Sigworth and Neher 1980). By doing voltage-step-clamp experiments they determined that the sodium channel conductance had rapid transitions between closed and open states. They were able to determine the resting potential of the cell as a value V and it was the first time that a cell had been voltage-clamped without the addition of electrodes.

Ion Channels The *basic study and fascination with ion channels was born*. Ion channels are transmembrane proteins that allow for the passage of inorganic ions to cross a cell's membrane. The ions rapidly move across the membrane depending on their electrochemical gradients. These ions are the driving force behind an action potential and the bread and butter of understanding excitable cells. The opening of an ion-selective channel in a cell membrane is the initial driver of everything we experience, from pain to pleasure, thoughts, and sensation. The excitable cell is an aggregate of ion channels, each cell type having a unique combination of channels that complements the function of the cell.

The Action Potential

The Hodgkin-Huxley model states that there is a disequilibrium of electricity along the plasma membrane, which is made of a lipid bilayer. This bilayer is hydrophobic and is impermeable to the flow of electricity or charged ions. However, ions can flow across the

membrane through specialized ion channels. At rest, the membrane potential is negative, meaning the inside of the nerve is more negatively charged than the outside of the membrane. The initial generation of the action potential is due to a slight increase of positive ions across the membrane from the extracellular space. This ionic flow causes a further positive potential or “depolarization” if there is enough passage of positive ions into the nerve. The sodium gated ion channels that line the nerve open when the cell reaches a sufficient depolarization or positive potential further driving the cell's electrical pulse along the membrane. This leads to the “Hodgkin Cycle”, where the initiated zone on the axon leads to a trigger (opening of sodium channels) nearby, thus driving the electrical pulse to that zone and so forth.

The distinguishing ions of the action potential can generally be conceived as Na^+ , K^+ , Cl^- , and Ca^{2+} (Bear et al. 2007). A combination of Na^+ and Ca^{2+} ions enter the cell during the initial response to stimulus in an electrical cell. The voltage-gated sodium channel (Na_vs) is the driver of the action potential, initiating the potential generation (Miller 1992). Following this, the voltage-gated potassium channels open bringing the cells membrane potential back to a negative (hyperpolarized) state. Finally, both the voltage-gated sodium and potassium channels close, and the sodium-potassium pump brings the cell back to the resting state so that the cell can fire an action potential and do it all over again! Amazingly, this happens on the order of milliseconds (Payandeh and Minor 2015).

Voltage-Gated Sodium Channels The eukaryotic voltage-gated sodium ion channel Nav consists of four domains in a single polypeptide chain. In humans, there are nine Nav isoforms that all have differing pharmacology, and functionality and are generally fast inactivating (Catterall et al. 2005). The sodium channel is what brings the cell membrane into a depolarized

state, and fast activation and inactivation of the Na_v channel confers high conduction velocity to the speed of the action potential. Typically, the Na_v channel has three states, activated, refractory (inactive), and closed. The activated state occurs when the membrane potential reaches a sufficient depolarization, and the channel's voltage-gated mechanism can open the pore. The refractory period is when the channel's voltage gate is open, however, the inactivation gate is closed and current cannot flow. Eventually, the channel moves back to its closed state, where the inactive gate is open, but the voltage activation gate is closed, ready to respond to the next depolarization.

The bacterial voltage-gated sodium channel, NaChBac, was first discovered in 2001 and was critical for the ion channel field due to its high potential for delivering a high-resolution crystal structure due to its small size (Ren et al. 2001). This could then be used to give potential understanding to the eukaryotic Na_v s. Greater than 500 members of the bacterial voltage-gated sodium channels (BacNa_v) have been discovered and examined (Koishi et al. 2004). But it wasn't until 2011 that a full-length BacNa_v crystal structure of a mutant Na_vAb from *Arcobacter butzleri* was developed (Payandeh et al. 2011). In recent years, detailed studies on the characteristics of conformation states of the bacterial voltage-gated sodium channel have been published (Amaral et al. 2012; Bagn  ris et al. 2015; Payandeh and Minor 2015). Since the discovery of NaChBac, many bacterial orthologue crystal structures have been determined, as well as the eukaryotic Na_v (Amaral et al. 2012; Payandeh et al. 2012; Shen et al. 2017; McCusker et al. 2012; Shaya et al. 2014).

The human sodium channel has an enormous coding region that exceeds the carrying capacity of most viruses. The BacNa_v is a particularly interesting channel because of its small

(<1 kb) genome compared to its mammalian ortholog, Na_v channel (>6 kb) (Ren et al. 2001).

This small size allows it to be placed in conjunction with other ion channels into a single virus for exogenous expression. One lab went on to engineer the bacterial sodium channel de novo to have specific characteristics such as enhanced conduction velocity which could be used in building synthetic tissues (Nguyen et al. 2016).

Voltage-Gated Potassium Channel The potassium channel brings the cell membrane potential close to the equilibrium potential of potassium and in excitable cells, it concludes the depolarization of the action potential and brings the cell back to “resting state”. The voltage-gated potassium channel is a large family of ion channels that confer rhythmic activity to the excitable cell.

Perhaps most significantly, the *drosophila* has contributed deeply to our understanding of the K⁺ channel. The *drosophila* is a perfect model organism as it has 70% of our genes and many of the signaling pathways between flies and humans are the same (Littleton and Ganetzky 2000). The first cloned voltage-activated K⁺ channel, *Shaker*, was found in *drosophila* and many more since then (Papazian et al. 1987). The *Shaker* channel demonstrates a rapidly activating and inactivating potassium current (Covarrubias et al. 1991). The study of the *drosophilids* K_v channel has accounted for a large body of knowledge in the understanding of the function of the channel. The axonal membrane of a neuron predominantly expresses the delayed rectifier potassium channel, K_v1 (Rogero et al. 1997). This delayed rectifier confers a brief period of “refractory”, where the neuron cannot fire. This is important in allowing the cell to reset before firing the next action potential.

ATP Pump (Transport Proteins)

Sodium-Potassium Pump The sodium-potassium pump was the first ion pump discovered and is found in many cell types. It is an integral membrane protein that is powered by ATP and can move ions against the concentration gradient. For every ATP that is broken down, the pump moves three sodium ions out of the cell, and two potassium ions in. The pump contributes by regulating the resting membrane potential and helps prevent cell lysis. The pump also acts as an enzyme and functions by hydrolyzing ATP which couples ion movement through protein conformational changes (Faller 2008).

Serca Pump The Sarcoendoplasmic reticulum (SR) calcium transport ATPase (SERCA) pump transports calcium ions from the cytoplasm into the SR. It is ATP driven and is found in many different tissue types. By keeping cytosolic calcium levels low it contributes to cellular processes such as synaptic transmission, muscle contraction, and fertilization (Primeau et al. 2018). The SERCA pump plays a large role in muscle contraction, such as cardiac and skeletal muscle, and can promote relaxation by pumping ions into the lumen of the SR. Interestingly, compared to the Sodium pump which has nine small transmembrane peptides, the SERCA only has two regulatory subunits (Primeau et al. 2018).

Gap Junctions Gap junctions are specialized intercellular connections that connect the cytoplasm of the two cells. They are made up of connexin proteins, which is a multigene family in mammals. Connexin-43 Cx43 is one of the most prevalent connexin proteins and allows for exchanges of ions, molecules, and gasses to pass between cells (Laird 2006). Activation of PKA,

specifically its second messenger cAMP, has shown to lead to increased phosphorylation of connexin-43 (Atkinson et al. 1995).

Neuronal Transduction Signals (Excitatory/Inhibitory) Interestingly, imaging with fluorescence came before the patch-clamp, marking the beginning of a long-term effort to find better organic voltage-sensitive dyes and ways to image voltage (Cohen 2011). The study of the changes of light scattering and birefringence in axons marked a radical moment in biology (Cohen et al. 1968). The theoretical concept that a membrane potential could be measured by using changes in fluorescence, specifically using dye, such as merocyanine was conceived in 1973 (Davila et al. 1973). This later became the platform for creating genetically encoded biosensors to provide molecular insights into the brain.

The initial discovery of the Green Fluorescent Protein GFP was crucial to the continued development of imaging techniques. It was first discovered in the bioluminescent jellyfish *Aequorea Victoria* and the work surrounding it was awarded the Nobel prize in 2008 (Chalfie 2009). Surprisingly, the GFP emits green light without needing additional cofactors. The electrons in the GFP protein are excited before they drop to a lower state due to internal conversion, before finally dropping to ground state. At this point, a photon is emitted at a higher wavelength than the original excitation because of lost energy (Chalfie et al. 1994). The GFP can be expressed and detected with high specificity in any gene within a living organism (Chalfie 2009). Today, genetically encoded fluorescent proteins are the backbone for many fluorescent reporters.

Synapto-pHluorins Scientists now had a deeper grasp on how neurons were excited, and how they propagated signals along their axon, but the lacuna was how they communicated. A famous model of neurotransmitter release is based on experiments that determined neurotransmitter is released at the frog neuromuscular junction (MacRae 1967; KATZ and B 1969). Before an action potential, the vesicles that contain neurotransmitters are near the membrane, and each vesicle is a “quanta” (Rosenmund and Stevens 1996). Katz determined that at the neuromuscular junction, per action potential, a quanta of several hundred are released (Yuste et al. 2000; KATZ and B 1969). The vesicle’s membrane is re-internalized into the cells membrane upon release where it is then recycled and its interior is again filled with neurotransmitters (Heuser and Reese 1973).

Early investigation into the neurotransmitter release at the neuromuscular junction functioned on using horseradish peroxidase HRP. The HRP could be visualized using electron microscopy and viewed ex-vivo post sectioning of the tissue (Ceccarelli et al. 1973; Heuser and Reese 1973; Holtzman et al. 1971). Following this, synthetic amphipathic compounds and fluorescent dyes became popular as they became brightly fluorescent after they were inserted into a lipid bilayer. The dye could be viewed after the residual dye had been washed off and was shown to be successful in invertebrates, vertebrates, and neurons in culture (Cochilla et al. 1999; Betz et al. 1992; Ryan et al. 1993; Ryan et al. 1997). However, the dyes often were un-specific and proved to be insufficient in brain slices where many synapses are crowded into small volumes and staining and washing solution was a limiting factor due to probe access (Yuste et al. 2000).

Investigators needed an experimental design to distinguish synaptic membrane proteins as resting or exocytosed in-vivo to determine when and where there is neurotransmitter release. The answer came in the form of a genetic tool that reports the local pH via a pH-sensitive mutant of GFP, termed “pHluorins,” which afford investigators the ability to see where and when a neurotransmitter is being released from the cell (Yuste et al. 2000). Synapto-pHluorins are genetically encoded, therefore the synapse can be labeled specifically (Miesenböck et al. 1998; Miesenböck and Rothman 1997). The structure of the synapto-pHluorin is a chimera between a GFP that reports pH (pHluorin) and a vesicle membrane protein VAMP-2, which binds the pHluorin to the inner vesicle surface. The pHluorins are located inside the synaptic vesicles in an off state. Upon exocytosis, the interior of the vesicle becomes exposed to the extracellular space, where the pH is ~ 7.4 , and the pHluorins excitation spectrum switches to an “on” state (Miesenböck et al. 1998). However, as with most fluorescent genetically encoded reporters, the signal is often much dimmer than the dyes

Activation systems for Excitable Cells

Photodynamics

Photodynamic action arose as a field in the late 1800s and demonstrated that in cell culture, long-wavelength light increased toxic oxidation and free radical formation in the media (Richardson 1893; Blum 1932; Stoien and Wang 1974; Dixit and Cyr 2003; Stockley et al. 2017). Oddly, in 1971 Richard Fork showed that 488 nm laser radiation can excite the *aplysia californica*, although he did not know by what mechanism. To follow up on this, a 2002 study showed that multiphoton lasers induce depolarizations and the authors hypothesized that there

were chromophores, such as flavins that increased H_2O_2 production and therefore the depolarization was mediated by the generation of reactive oxygen species. The other theory of mechanism was, that through the heating by the laser they were causing microholes in the membrane which increased the rate of repolarization (Hirase et al. 2002).

The cautionary tale from a 2019 paper by Gray and Tyssowski shows that blue light increases neuronal activity-regulated gene expression in the absence of optogenetic proteins (Tyssowski and Gray 2019). They found with exposure to blue light wavelengths at time points between 1-6 hours there was an increased expression of activity-regulated genes such as Fos, Npas4, and Bdnf. This study demonstrates that experimenters trying to measure transcription following optogenetic stimulation should be cautious and run light only controls in the absence of optogenetic proteins (Tyssowski and Gray 2019).

Bacterial Rhodopsin

The idea that an inert cell could be made alive was perhaps first introduced by Mary Shelley in 1818, in her wonderful book, Frankenstein. However, she was probably influenced by Luigi Galvani's work. Nearly two centuries later scientists began trying to excite cells that are both typically non-excitable (HEK293/CHO etc.) and excitable (neurons/heart cells) using molecular techniques. In the 1970s, the species archaeon *Halobacterium salinarum* provided neuroscientists with two tools; bacteriorhodopsin and halorhodopsin (Oesterhelt and Stoeckenius 1973). Bacteriorhodopsin has been shown to pump charged ions across neuronal membranes in species such as yeast, and frog oocytes (Nagel et al. 1995; Hildebrandt et al. 1993; Hildebrandt et al. 1989). Meanwhile, optical uncaging of neurotransmitters with ultraviolet laser pulses was

becoming a powerful method for activation of neurons but it was not neuron type-specific (Adams and Tsien 1993; Wang and Augustine 1995). A review by Francis Crick “suggested” that neuroscientists should tell molecular biologists of their problems in hopes that this will stimulate the production of novel biological tools, specifically that light could be used to individually activate and target neurons, turning them on and off (Crick 1999). Indeed, the idea for activating genetically targeted neurons with light was circulating in labs across the globe.

Photo-regulation and Caged Compounds

Neurons often communicate via two routes for the transduction of excitatory signals. As touched on earlier, the neuron signals to other neurons by excreting synaptic vesicles that are filled with neurotransmitters. Neurotransmitters act as ligands when binding to the receptors on neighboring cell membranes. Apart from direct gap junctions that allow ions to flow between cells, there are two major signaling systems, the metabotropic and the ionotropic system. Ionotropic systems immediately affect the cell because they are ligand gated ion channels that open in response to binding. The metabotropic signaling system acts on receptors at the cell membrane that when bound to their specific ligand, stimulate the cell by acting via second messengers and have endless downstream effects within the cell (Bear et al. 2007).

Specifically, when G Protein-coupled receptors GPCRs bind receptor molecules outside the cell, they then modulate activity within the cell through two main pathways, cAMP and IP3/DAG. cAMP is known to bind to the cAMP response element-binding protein (CREB) that is closely related to cell proliferation and is responsible for binding cAMP response elements (CRE) that increase and decrease the transcription of some genes. cAMP is also known to

regulate c-fos and junB. The cAMP/PKA signaling pathway is well characterized as serving several roles such as cell survival, synaptic plasticity, and gene expression. It is also a mediator in the pathogenesis of disorders related to learning, mood, and anxiety ([Alberts 2017](#)). The IP3 pathway activates phospholipase C, which then hydrolyzes phosphatidylinositol 4,5-bisphosphate PIP2. This results in two second messengers inositol 1,4,5,-triphosphate IP3 and diacylglycerol DAG. IP3 binds with IP3 receptors on the ER and Mitochondria which opens Ca^{2+} channels. DAG activates Protein Kinase C which phosphorylates enzymes resulting in regulating their activity. Interestingly, the activation of PIP3/DAG also regulates cAMP and the corresponding phosphodiesterases ([Capel et al. 2019](#); [Mikoshiha 2007](#)).

A pioneer in photo regulation was the Erlanger group, demonstrating a model for light-controlled metabolic systems. The authors synthesized a soluble photochromic ligand that could activate and inactivate chymotrypsin by harnessing the isomerization of the light-sensitive azobenzene linked to diphenylcarbonyl-chloride ([Kaufman et al. 1968](#)). This opened the door for other pharmacophores to be used to activate and inactive enzymes. To this end, groups built photo-regulated adolases, urease, amylase, acetylcholinesterases, and many more ([Bieth et al. 1969](#); [Wainberg and Erlanger 1971](#); [Montagnoli et al. 1976](#); [Hug 1978](#)).

A lab in New York was one of the first to produce a genetically targeted metabotropic method. They built a genetic tool that combined rhodopsin from the *Drosophila* photoreceptor gene combined with the alpha subunit of the cognate heterotrimeric G protein, dubbed “chARGE”. Using chARGE they were able to stimulate photo cascades in rat hippocampal neurons ([Zemelman et al. 2002](#)).

Another technology that arose from this lab was the use of “caged” compounds or ligands. Often the technology involved cognate receptor and agonist to be ectopically expressed and using the light “caged” ligand could then activate the receptor of choice. Gero Miesenbocks lab was ahead of the curve on this technology in many ways. Not only did he design metabotropic light-activated systems, but he also designed ionotropic systems. His lab designed many tools such as the capsaicin receptor (TRPV1), and the ionotropic purinergic receptor (P2x₂) to be controlled optically, by using photorelease of the active compounds for the said receptors (Zemelman et al. 2003). What was incredibly useful was that they could express their optically gated ion channels in groups of neurons in vivo that would evoke behavioral responses that were specific to the site of ectopic expression. This work provided a novel technology that assisted in determining the connectivity and dynamics of neuronal circuits (Lima and Miesenböck 2005). Following the “chARGE” of Misenbock’s group, Isocoff, Trauner, and Kramer created a synthetic photoisomerizable Azobenzene regulated K⁺ channel (SPARK) that could induce mammalian depolarization (Banghart et al. 2004).

Another mentionable scientist in this field was Har Gobind Khorana. He won the Nobel prize in 1968 while working with Marshall Nirenberg and Robert Holley at the University of Wisconsin in Madison for determining the genetic code of DNA. Shortly before he retired from MIT, Khorana produced extraordinary work on rhodopsins. Specifically pertinent to this body of work was a study done by H.G. Khorana et al., where they created a prototype for structure-function studies on GPCRs. Their first chimera was a rhodopsin containing a B2-Adrenergic Receptor, creating a light-activated system that probed if GPCRs contain common activation mechanisms (Kim et al. 2005). Khorana was known for his dedication to not only science, but

his dedication to mentoring young scientists, and his profound humility (Finn and News Office).

Optogenetics is Coined

A revolution was sparked to control neuronal activity with fantastic spatiotemporal precision driving Roger Tsien, Karl Deisseroth, and Ed Boyden, along with other labs, to work on placing channelrhodopsins into mammalian neurons. A landmark paper in Nature Neuroscience by Karl Deisseroth, Ed Boyden, and Feng Zhang showed the first use of a single component, channelrhodopsin-2(H134R)-eYFP construct from Nagel and Hegemann (Nagel et al. 2003), could indeed elicit excitation in rat hippocampal cultured neurons. Compared to other genetically encoded photo methods such as “chARGe” or “SPARK”, the channelrhodopsin provided voltage control on the millisecond time scale (Boyden et al. 2005; Zemelman et al. 2002; Banghart et al. 2004). The term “Optogenetics” was coined following the paper by Boyden, in a paper published by Schnitezer et al., and is used to describe light-driven channels placed exogenously into species through genetic control. (Deisseroth et al. 2006).

Optogenetics is a growing field in Neuroscience with a driving need for a broad spectrum of molecules that respond to light. These highly specific tools in neuroscience provide the ability to make increasingly difficult experiments possible with greater accuracy and temporal-spatial precision.

bPAC

One such molecule is the blue photoactivated cyclase bPAC. It was discovered in 2011 and comes from a bacteria, *beggiatoa*, that thrives next to sulfur vents in the deep sea. It has a

photoactivated adenylyl cyclase that when stimulated with blue light increases cAMP (Stierl et al. 2011). This second messenger can then bind to cyclic nucleotide-gated CNG or HCN channels.

Recording Activity with Fluorescence

Activity-Based Biosensors

Genetically encoded bio-indicators contain fluorescent proteins that change fluorescence intensity as a function of ion concentration or voltage (Kerruth et al. 2019; Huang et al. 2019). Since their development in the late 1990s, fluorescent activity indicators have seen extensive improvements in sensitivity and kinetics (Miyawaki et al., 1997; Romoser et al., 1997; Gong et al., 2015; Chamberland et al., 2017; Kannan et al., 2018; Piatkevich et al., 2018). Activity-based biosensors often use analyte/voltage-sensitive proteins that undergo conformational changes in response to their environment. These conformational changes modulate the level of fluorescence emitted. These sensors are beginning to complement electrode-based studies to understand the fundamental aspects of neurophysiology. There are a wide plethora of sensors available today including sensors for neurotransmitters, GPCRs, voltage changes, and ion levels (Looger and Griesbeck 2012; Marvin et al. 2019; Marvin 2012; Patriarchi et al. 2018; Abdelfattah et al. 2019).

Why are Ca²⁺ Sensors Important?

The Ca²⁺ ion is an important second messenger in cellular processes ranging from neuronal function, muscle contractility, calcium-binding and memory, to fertilization and

embryogenesis (Bear, Connors, and Paradiso 2007). In the mammalian neuron, Ca^{2+} plays a role in many signaling pathways, ultimately controlling the release of neurotransmitters from neurons. Genetically encoded Ca^{2+} indicators contain fluorescent proteins that change fluorescence intensity as a function of Ca^{2+} concentration (Kerruth et al. 2019; Huang et al. 2019). They are minimally invasive, and their expression can be exquisitely targeted to specific cell types and tissues (Agetsuma, Matsuda, and Nagai 2017; Bruton, Cheng, and Westerblad 2020; Kim et al. 2019).

Sensors built with calcium-binding properties such as calmodulin present specific problems *in vivo* such as buffering the internal Ca^{2+} concentration, and activation of kinases and phosphatases, and modulate ion channels (Mank and Griesbeck 2008). The fluorescence of these biosensors is typically captured with wide-field microscopy and standard CCD cameras. However, there are many new imaging approaches to capture the wealth of information they provide. Two-photon microscopes make it possible to image microdomains of Ca^{2+} signals within neurons in thick living tissue (T.-W. Chen et al. 2013; Peron et al. 2015). Light-sheet microscopy makes it possible to now capture entire larval zebrafish brain activity at single-cell resolution at 2 Hz (Ahrens et al. 2013). Recently, Ca^{2+} sensors are being coupled with functional magnetic resonance imaging (fMRI) (Barandov et al. 2019; Ghosh et al. 2018).

There are now several Ca^{2+} sensors that can sense Ca^{2+} transients in cells with millisecond response times and that are bright enough to image in living tissue (Ouzounov et al. 2017). Most of these sensors have blue excitation and green emission peaks. Currently, the sensors with the fastest kinetic response time to Ca^{2+} flux are the Ultrafast GCaMP6f, jRCaMP, and jRGECO1a (Helassa et al. 2016; Kerruth et al. 2019). The recent jGCaMP7 sensor makes it

possible to capture individual action potentials and can track large neuronal populations when using two-photon or wide-field imaging ([Dana et al. 2019](#)).

Genetically Encoded Voltage Sensors

One of the first described genetically encoded voltage indicators GEVIs was a combination of the Shaker potassium channel and a fluorescent protein ([Siegel and Isacoff 1997](#)). Since this initial design, many GEVIs have been designed and undergone extensive improvement to their sensitivity and kinetics.

One of the greatest engineering problems to overcome with GEVIs is increasing their maximal change in fluorescence. Detecting neural activity largely depends on the signal-to-noise ratio of the sensor. The signal-to-noise ratio SNR can be defined as the peak fluorescence divided by the signal standard deviation before stimulation ([Woehler et al. 2010](#)). Furthermore, a better signal to noise ratio can help increase imaging speeds and increase the field of view. Brighter sensors can be detected with lower expression levels. This could be important since overexpression can lead to epileptiform activity ([Dana et al. 2019](#)).

De Novo Models of Excitable Cells

Catechism of Synthetic Excitable Cells

In addition to ion channels being engineered to be controlled by light, some channels are sensitive to small chemicals, voltage, stretch, and temperature that have been customized to enable experimenters to control a tissue's excitability ([Nguyen and Bursac 2019](#)). The vast majority of understanding of the excitable cell has come from direct manipulation of endogenous excitable cells in vivo and in vitro. However, combining ion channels in various combinations

and expressing them exogenously in non-excitable cells has become an area of interest in not only neuroscience but other fields of biology, including drug screening companies, channelopathies, cardiology, and biosensor development (Zhang and Cohen 2017; Kirkton and Bursac 2012; Kirkton et al. 2013; Zhang et al. 2016; Mohanty et al. 2008). The combination of optical activation and imaging will help us to better understand and modulate the physiological functions of various excitable cells in skeletal, cardiac, and neuronal systems.

Synthetic excitable cells are built to mimic the process of an action potential. A voltage-gated sodium channel and an inwardly rectifying potassium channel KIR are the minimal components needed. The goal has become to optically excite mammalian cells with light, instead of using electrophysiology of drugs, that create spontaneous depolarizations. These spontaneously spiking cells have a few major purposes in the world of biology. Currently, there is a great need in the biosensor development community to create a high throughput screen in mammalian cells for biosensors. The next generation of sensors needs to be engineered for greater maximal change in fluorescence, photostability, and fast kinetics (Abdelfattah et al. 2019). These sensors could largely replace traditional tools used by neuroscientists such as electrodes for both stimulation and recording from neurons. Also, if built, the screen could prove useful in building screens for drugs that modulate neuronal and cardiac function (Zhang and Cohen 2017).

One of the first de novo excitable cells was created by expressing rat brain IIA Na⁺ channel and the *Drosophila* Shaker K⁺ channel into Chinese hamster ovary cells via vaccinia virus (Hsu et al. 1993). Bursac and Kirkton built a biosynthetic excitable cell in the hopes it would be used in electrophysiology and cell therapy studies (Kirkton and Bursac 2011). In their

first experiment, they stably expressed three ion channels, Kir2.1, Nav1.5, and Cx-43 in HEK293 cells. Using electrophysiology they measured the membrane potential and found that with all three channels expressed they could create fast and uniform action potential propagation. However, these cells cannot be used to test genetically encoded voltage indicators or other fluorescent markers because each channel was marked with a fluorescent protein spanning the visible spectrum (Kirkton and Bursac 2011).

Current methods to screen voltage sensors include using excitable HEK-293 cells that combine the Kir2.1 and voltage-gated sodium channel NAV1.3 with field stimulation to produce rhythmic depolarization and hyperpolarization in HEK cells (Park et al. 2013; Zhang, Reichert, and Cohen 2016; Shah et al. 2004). The Cohen lab created a line of electrically spiking E.Coli and then to create a line of excitable cells that stably expressed Nav 1.3 and Kir2.1 in HEK293 cells (Kralj et al. 2011; Park et al. 2013). They characterized the cells using patch-clamp electrophysiology and a voltage-sensitive dye. Here they found that using the dye they could determine an AP of 2ms, however, they also developed a protocol in these cells to express GEVIs such as ArcLight, Arch mutants with fluorescence traces that lasted ~ 600ms. However, because the repolarization rate of the HEK293 action potential was variable between cultures, they did not use the spiking HEK293 cells to measure the off rates and instead focused on the 4ms time resolution on the rising edge of the spiking HEK293 cells. Therefore, they were unable to determine multi-exponential kinetics of the sensors but still provide a stringent screen for genetically encoded voltage sensors.

In 2016, Cohen and Zhang demonstrated that they could photoactive cells when provided with Kir2.1, Nav1.7, CheRiff (a channelrhodopsin) while recording the oscillating membrane

voltage with QuasAr2 (GEVI). They found that this could sufficiently act as a high throughput screen for drugs that block Nav1.7. ([Zhang and Cohen 2017](#)).

Similarly, another paper described a cell line that had pacemaker-like activity. They stably expressed the HCN2 and Kir2.1 channels, and transiently expressed a voltage-gated channel Cav3.1 or Nav1.5 ([K. Chen et al. 2018](#)). HCN2 is a hyperpolarization-activated, cyclic nucleotide-gated ion channel ([K. Chen et al. 2018](#)). Nav1.5 is a voltage-gated sodium channel responsible for action potentials in cardiomyocytes ([K. Chen et al. 2018](#)). Chen et al. found that cells expressing HCN2 and Kir2.1 had action potentials with depolarizations that last over ~450 ms, and a hyperpolarization phase that lasts ~737 ms ([K. Chen et al. 2018](#)). With either Cav3.1 or Nav5.1 expressed in conjunction with the Kir2.1 and HCN2, they found that there was an average of one action potential per second. This suggests that even without field stimulation, cells expressing these channels can create auto-depolarization leading to rhythmic oscillations in membrane potential. They also showed that the currents produced by the voltage-gated channels influenced the speed at which the action potentials occur.

The Kuhl Model

Genetic drift and gene silencing are inherent problems when building stable cell lines. Often after several passages, stable cell lines will begin to regulate the expression of the non-endogenous genes ([Khan 2013](#); [Rodin and Riggs 2003](#)). Also, the level of expression of each of the channels cannot be easily changed. Further, in cell lines excited optically only wavelengths outside of the rhodopsin's excitation spectrum can be used for screening. What if we could screen thousands of variants in mammalian cells for sensitivity, speed, and dynamic range?

The Kuhl system uses similar channels to previous studies to create cells that have action potentials, whole-cell Ca^{2+} transients, and can withstand the bandpass problem (Thomas et al. 2020). The system is automatable, inexpensive, and modular. The platform is robust for screening activity-based biosensors such as Ca^{2+} and Voltage. It is consistent, reliable, and capable of quickly screening thousands of prototypes.

To establish the minimal “Kuhl” cell system they used a blue light photoactivated enzyme. From there they show that various channels and their amounts can affect the cell's response to blue light stimulus. Manipulating the relative expression of these channels is an interesting delve into the world of synthetic biology. By creating cells that can be stimulated with blue light while imaging in both red and green wavelengths they have created a system that is useful for multi channel drug screening and biosensor development.

They introduced cAMP gated ion channels, specifically the modified CNG or the HCN2 ion channel. When these channels bind cAMP they open bringing a positive inward current into the cell. This causes a long and slow inward Ca^{2+} current that can be visualized using Ca^{2+} sensors (Thomas et al. 2020).

Next, they optimized the concentrations of two ion channels, the inwardly rectifying potassium channel Kir2.1 and the bacterial sodium channel NavRosDg217A NavD. Kir2.1 is an inwardly rectifying potassium channel that upon hyperpolarization greater than -20mV increases its conductance, producing an outward current (Hille 2001). The mutant NavD is slow, and they originally wanted to create a system with relatively long, slow depolarizations such that membrane depolarization events could be captured with slower imaging speeds (10Hz). The role of Kir2.1 in Kuhl synthetic cells is two-fold. First, it is required in “priming” the NavD for

activation, as Na_v won't be able to make the necessary transition from the deactivated state to the open state. Secondly, after the sodium channel is inactivated, Kir2.1 slowly repolarizes the cell to -70mV, beginning the cycle again.

Upon investigating the various channels of Kir2.1 and NavD they found that the amount of ion channel directly impacts the cells ability to fire and that a ratio close to 1:1 was optimal. While the system is novel it has a lot of room for improvement. Faster Nav channels could increase the speed of the action potential which would aid in finding faster voltage sensors.

Finally, Hek-293 cells have endogenous connexin protein, connexin-45. However, Kirkton and Bursac [18] found that a confluent layer of HEK293 cells expressing Kir2.1 and Nav1.5 had action potentials that did not propagate uniformly. But with the addition of Connexin-43 Cx-43, they recorded rapid and robust action potentials. Also, several other studies have shown that including the Cx-43 protein increases the gap junction coupling of HEK293 cells [18,32,38]. With the addition of Cx-43 Thomas et al. found that the Ca^{2+} transients became more coordinated following blue light stimulus. Potentially, another channel could increase the rhythmicity of these cells so that their depolarization and hyperpolarization was coordinated like a heart cell.

They created a simple, modular, reproducible system to screen Ca^{2+} sensors and voltage sensors. The system can be optimized by varying the ratio of Kir2.1 to NavD channels, thereby changing the type of Ca^{2+} transients that occur and the frequency at which they occur. Therefore, optimizing the ratio of the channels to the experimenter's preference for cell type and sensor can empower an automated screen for bio sensors with different dynamic properties (Thomas et al. 2020).

REFERENCES CITED

1. Abdelfattah, Ahmed S., Takashi Kawashima, Amrita Singh, Ondrej Novak, Hui Liu, Yichun Shuai, Yi-Chieh Huang, et al. 2019. "Bright and Photostable Chemigenetic Indicators for Extended in Vivo Voltage Imaging." *Science* 365 (6454): 699–704.
2. Abdul Kadir, Lina, Michael Stacey, and Richard Barrett-Jolley. 2018. "Emerging Roles of the Membrane Potential: Action Beyond the Action Potential." *Frontiers in Physiology* 9 (November): 1661.
3. Adams, S. R., and R. Y. Tsien. 1993. "Controlling Cell Chemistry with Caged Compounds." *Annual Review of Physiology* 55: 755–84.
4. Agetsuma, Masakazu, Tomoki Matsuda, and Takeharu Nagai. 2017. "Methods for Monitoring Signaling Molecules in Cellular Compartments." *Cell Calcium* 64 (June): 12–19.
5. Ahrens, Misha B., Michael B. Orger, Drew N. Robson, Jennifer M. Li, and Philipp J. Keller. 2013. "Whole-Brain Functional Imaging at Cellular Resolution Using Light-Sheet Microscopy." *Nature Methods* 10 (5): 413–20.
6. "Alan Lloyd Hodgkin - Nobel Lecture." n.d. <https://www.nobelprize.org/uploads/2018/06/hodgkin-lecture.pdf>.
7. Alberts, Bruce. 2017. *Molecular Biology of the Cell*. Garland Science.
8. Amaral, Cristiano, Vincenzo Carnevale, Michael L. Klein, and Werner Treptow. 2012. "Exploring Conformational States of the Bacterial Voltage-Gated Sodium Channel NavAb via Molecular Dynamics Simulations." *Proceedings of the National Academy of Sciences of the United States of America* 109 (52): 21336–41.
9. Atkinson, M. M., P. D. Lampe, H. H. Lin, R. Kollander, X. R. Li, and D. T. Kiang. 1995. "Cyclic AMP Modifies the Cellular Distribution of connexin43 and Induces a Persistent Increase in the Junctional Permeability of Mouse Mammary Tumor Cells." *Journal of Cell Science* 108 (Pt 9) (September): 3079–90.
10. Bagn  ris, Claire, Claire E. Naylor, Emily C. McCusker, and B. A. Wallace. 2015. "Structural Model of the Open–closed–inactivated Cycle of Prokaryotic Voltage-Gated Sodium Channels." *The Journal of General Physiology* 145 (1): 5–16.
11. Banghart, Matthew, Katharine Borges, Ehud Isacoff, Dirk Trauner, and Richard H. Kramer. 2004. "Light-Activated Ion Channels for Remote Control of Neuronal Firing." *Nature Neuroscience* 7 (12): 1381–86.

12. Barandov, Ali, Benjamin B. Bartelle, Catherine G. Williamson, Emily S. Loucks, Stephen J. Lippard, and Alan Jasanoff. 2019. "Sensing Intracellular Calcium Ions Using a Manganese-Based MRI Contrast Agent." *Nature Communications* 10 (1): 897.
13. Bear, Mark F., Barry W. Connors, and Michael A. Paradiso. 2007. *Neuroscience*. Lippincott Williams & Wilkins.
14. Betz, W. J., F. Mao, and G. S. Bewick. 1992. "Activity-Dependent Fluorescent Staining and Destaining of Living Vertebrate Motor Nerve Terminals." *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 12 (2): 363–75.
15. Bieth, J., S. M. Vratsanos, N. Wassermann, and B. F. Erlanger. 1969. "Photoregulation of Biological Activity by Photocromic Reagents. II. Inhibitors of Acetylcholinesterase." *Proceedings of the National Academy of Sciences of the United States of America* 64 (3): 1103–6.
16. Blum, Harold F. 1932. "Photodynamic Action." *Physiological Reviews* 12 (1): 23–55.
17. Boyden, E. S., F. Zhang, and E. Bamberg. 2005. "Millisecond-Timescale, Genetically Targeted Optical Control of Neural Activity, Nat. Neurosci. 8 (9)." *Nature Neuroscience* 8 (9): 1318–27.
18. Bruton, Joseph, Arthur J. Cheng, and Håkan Westerblad. 2020. "Measuring Ca²⁺ in Living Cells." *Advances in Experimental Medicine and Biology* 1131: 7–26.
19. Bryson, Bill. 2019. *The Body: A Guide for Occupants*. Random House.
20. Capel, Rebecca A., Thomas P. Collins, Samuel J. Bose, Skanda Rajasundaram, Thamali Ayagama, Manuela Zaccolo, Rebecca A. B. Burton, and Derek A. Terrar. 2019. "cAMP Signalling Is Required for the Actions of IP₃ on Ca²⁺-Transients in Cardiac Atria and Beating Rate in Sino-Atrial Node." *bioRxiv*. <https://doi.org/10.1101/694349>.
21. Catterall, William A., Alan L. Goldin, and Stephen G. Waxman. 2005. "International Union of Pharmacology. XLVII. Nomenclature and Structure-Function Relationships of Voltage-Gated Sodium Channels." *Pharmacological Reviews* 57 (4): 397–409.
22. Ceccarelli, B., W. P. Hurlbut, and A. Mauro. 1973. "Turnover of Transmitter and Synaptic Vesicles at the Frog Neuromuscular Junction." *The Journal of Cell Biology* 57 (2): 499–524.
23. Chalfie, Martin. 2009. "GFP: Lighting up Life." *Proceedings of the National Academy of Sciences of the United States of America* 106 (25): 10073–80.

24. Chalfie, M., Y. Tu, G. Euskirchen, W. W. Ward, and D. C. Prasher. 1994. "Green Fluorescent Protein as a Marker for Gene Expression." *Science* 263 (5148): 802–5.
25. Chen, Kuihao, Dongchuan Zuo, Sho-Ya Wang, and Haijun Chen. 2018. "Kir2 Inward Rectification-Controlled Precise and Dynamic Balances between Kir2 and HCN Currents Initiate Pacemaking Activity." *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology* 32 (6): 3047–57.
26. Chen, Tsai-Wen, Trevor J. Wardill, Yi Sun, Stefan R. Pulver, Sabine L. Renninger, Amy Baohan, Eric R. Schreiter, et al. 2013. "Ultrasensitive Fluorescent Proteins for Imaging Neuronal Activity." *Nature* 499 (7458): 295–300.
27. Cochilla, A. J., J. K. Angleson, and W. J. Betz. 1999. "Monitoring Secretory Membrane with FM1-43 Fluorescence." *Annual Review of Neuroscience* 22: 1–10.
28. Cohen, Lawrence B. 2011. "Historical Overview and General Methods of Membrane Potential Imaging." In *Membrane Potential Imaging in the Nervous System: Methods and Applications*, edited by Marco Canepari and Dejan Zecevic, 1–11. New York, NY: Springer New York.
29. Cohen, L. B., R. D. Keynes, and B. Hille. 1968. "Light Scattering and Birefringence Changes during Nerve Activity." *Nature* 218 (5140): 438–41.
30. Cole, Kenneth S., and Richard F. Baker. 1941. "LONGITUDINAL IMPEDANCE OF THE SQUID GIANT AXON." *The Journal of General Physiology*.
<https://doi.org/10.1085/jgp.24.6.771>.
31. Cole, K. S. 1982. "Squid Axon Membrane: Impedance Decrease to Voltage Clamp." *Annual Review of Neuroscience* 5: 305–23.
32. Cole, K. S., and H. J. Curtis. 1941. "MEMBRANE POTENTIAL OF THE SQUID GIANT AXON DURING CURRENT FLOW." *The Journal of General Physiology* 24 (4): 551–63.
33. Covarrubias, M., A. A. Wei, and L. Salkoff. 1991. "Shaker, Shal, Shab, and Shaw Express Independent K⁺ Current Systems." *Neuron* 7 (5): 763–73.
34. Crick, F. 1999. "The Impact of Molecular Biology on Neuroscience." *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 354 (1392): 2021–25.

35. Dana, Hod, Yi Sun, Boaz Mohar, Brad K. Hulse, Aaron M. Kerlin, Jeremy P. Hasseman, Getahun Tsegaye, et al. 2019. "High-Performance Calcium Sensors for Imaging Activity in Neuronal Populations and Microcompartments." *Nature Methods* 16 (7): 649–57.
36. Davila, H. V., Brian M. Salzberg, L. B. Cohen, and A. S. Waggoner. 1973. "A Large Change in Axon Fluorescence That Provides a Promising Method for Measuring Membrane Potential." *Nature*, January. <http://www.nature.com/nature-newbio/journal/v241/n109/abs/newbio241159a0.html>.
37. Deisseroth, Karl, Guoping Feng, Ania K. Majewska, Gero Miesenböck, Alice Ting, and Mark J. Schnitzer. 2006. "Next-Generation Optical Technologies for Illuminating Genetically Targeted Brain Circuits." *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 26 (41): 10380–86.
38. Deiters, Vera S., and R. W. Guillery. 2013. "Otto Friedrich Karl Deiters (1834-1863)." *The Journal of Comparative Neurology* 521 (9): 1929–53.
39. Dixit, Ram, and Richard Cyr. 2003. "Cell Damage and Reactive Oxygen Species Production Induced by Fluorescence Microscopy: Effect on Mitosis and Guidelines for Non-Invasive Fluorescence Microscopy." *The Plant Journal: For Cell and Molecular Biology* 36 (2): 280–90.
40. Dunn, Rob. 2015. *The Man Who Touched His Own Heart: True Tales of Science, Surgery, and Mystery*. Little, Brown.
41. Fahrenbach, John P., Rafael Mejia-Alvarez, and Kathrin Banach. 2007. "The Relevance of Non-Excitable Cells for Cardiac Pacemaker Function." *The Journal of Physiology* 585 (Pt 2): 565–78.
42. Faller, Larry D. 2008. "Mechanistic Studies of Sodium Pump." *Archives of Biochemistry and Biophysics* 476 (1): 12–21.
43. Finn, Emily, and News Office. n.d. "Gobind Khorana, MIT Professor Emeritus, Dies at 89." MIT News. Accessed April 29, 2020. <https://news.mit.edu/2011/obit-khorana-1110>.
44. Galvani, Luigi. 1791. "Aloysii Galvani De Viribus Electricitatis in Motu Musculari Commentarius." <https://doi.org/10.5479/sil.324681.39088000932442>.
45. Gasser, H. S., and Joseph Erlanger. 1922. "A STUDY OF THE ACTION CURRENTS OF NERVE WITH THE CATHODE RAY OSCILLOGRAPH." *American Journal of Physiology-Legacy Content* 62 (3): 496–524.

46. Ghosh, Souparno, Peter Harvey, Jacob C. Simon, and Alan Jasanoff. 2018. "Probing the Brain with Molecular fMRI." *Current Opinion in Neurobiology* 50 (June): 201–10.
47. Giorgi, Carlotta, Saverio Marchi, and Paolo Pinton. 2018. "The Machineries, Regulation and Cellular Functions of Mitochondrial Calcium." *Nature Reviews. Molecular Cell Biology* 19 (11): 713–30.
48. Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. "Improved Patch-Clamp Techniques for High-Resolution Current Recording from Cells and Cell-Free Membrane Patches." *Pflugers Archiv: European Journal of Physiology* 391 (2): 85–100.
49. Helassa, Nordine, Borbala Podor, Alan Fine, and Katalin Török. 2016. "Design and Mechanistic Insight into Ultrafast Calcium Indicators for Monitoring Intracellular Calcium Dynamics." *Scientific Reports* 6 (December): 38276.
50. Heuser, J. E., and T. S. Reese. 1973. "Evidence for Recycling of Synaptic Vesicle Membrane during Transmitter Release at the Frog Neuromuscular Junction." *The Journal of Cell Biology* 57 (2): 315–44.
51. Hildebrandt, V., K. Fendler, J. Heberle, A. Hoffmann, E. Bamberg, and G. Büldt. 1993. "Bacteriorhodopsin Expressed in *Schizosaccharomyces Pombe* Pumps Protons through the Plasma Membrane." *Proceedings of the National Academy of Sciences of the United States of America* 90 (8): 3578–82.
52. Hildebrandt, V., M. Ramezani-Rad, U. Swida, P. Wrede, S. Grzesiek, M. Primke, and G. Büldt. 1989. "Genetic Transfer of the Pigment Bacteriorhodopsin into the Eukaryote *Schizosaccharomyces Pombe*." *FEBS Letters* 243 (2): 137–40.
53. Hill, D. K., and R. D. Keynes. 1949. "Opacity Changes in Stimulated Nerve." *The Journal of Physiology* 108 (3): 278–81.
54. Hille, Bertil. 2001. *Ionic Channels of Excitable Membranes*. Sinauer.
55. Hirase, Hajime, Volodymyr Nikolenko, Jesse H. Goldberg, and Rafael Yuste. 2002. "Multiphoton Stimulation of Neurons." *Journal of Neurobiology* 51 (3): 237–47.
56. Hodgkin, A. L., and A. F. Huxley. 1952a. "Currents Carried by Sodium and Potassium Ions through the Membrane of the Giant Axon of *Loligo*." *The Journal of Physiology* 116 (4): 449–72.
57. ———. 1952b. "The Components of Membrane Conductance in the Giant Axon of *Loligo*." *The Journal of Physiology* 116 (4): 473–96.

58. ———. 1952c. “The Dual Effect of Membrane Potential on Sodium Conductance in the Giant Axon of Loligo.” *The Journal of Physiology* 116 (4): 497–506.
59. ———. 1952d. “A Quantitative Description of Membrane Current and Its Application to Conduction and Excitation in Nerve.” *The Journal of Physiology* 117 (4): 500–544.
60. Hodgkin, A. L., A. F. Huxley, and B. Katz. 1952. “Measurement of Current-voltage Relations in the Membrane of the Giant Axon of Loligo.” *The Journal of Physiology* 116 (4): 424–48.
61. Hodgkin, A. L., and B. Katz. 1949. “The Effect of Sodium Ions on the Electrical Activity of Giant Axon of the Squid.” *The Journal of Physiology* 108 (1): 37–77.
62. Holtzman, E., A. R. Freeman, and L. A. Kashner. 1971. “Stimulation-Dependent Alterations in Peroxidase Uptake at Lobster Neuromuscular Junctions.” *Science* 173 (3998): 733–36.
63. Hsu, H., E. Huang, X. C. Yang, A. Karschin, C. Labarca, A. Figl, B. Ho, N. Davidson, and H. A. Lester. 1993. “Slow and Incomplete Inactivations of Voltage-Gated Channels Dominate Encoding in Synthetic Neurons.” *Biophysical Journal* 65 (3): 1196–1206.
64. Huang, Lawrence, Ulf Knoblich, Peter Ledochowitsch, Jérôme Lecoq, R. Clay Reid, Saskia E. J. de Vries, Michael A. Buice, et al. 2019. “Relationship between Spiking Activity and Simultaneously Recorded Fluorescence Signals in Transgenic Mice Expressing GCaMP6.” *bioRxiv*. <https://doi.org/10.1101/788802>.
65. Hug, Daniel H. 1978. “The Activation of Enzymes with Light.” In *Photochemical and Photobiological Reviews: Volume 3*, edited by Kendric C. Smith, 1–33. Boston, MA: Springer US.
66. “Index of Review, 1921.” 1921. *American Physical Education Review* 26 (9): 3–6.
67. Jacobson, D. A., and L. H. Philipson. 2007. “Action Potentials and Insulin Secretion: New Insights into the Role of Kv Channels.” *Diabetes, Obesity & Metabolism* 9 Suppl 2 (November): 89–98.
68. KATZ, and B. 1969. “The Release of Neural Transmitter Substances.” *Liverpool University Press*, 5–39.
69. Katz, B., and R. Miledi. 1972. “The Statistical Nature of the Acetylcholine Potential and Its Molecular Components.” *The Journal of Physiology* 224 (3): 665–99.

70. Kaufman, H., S. M. Vratsanos, and B. F. Erlanger. 1968. "Photoregulation of an Enzymic Process by Means of a Light-Sensitive Ligand." *Science* 162 (3861): 1487–89.
71. Kerruth, Silke, Catherine Coates, Céline D. Dürst, Thomas G. Oertner, and Katalin Török. 2019. "The Kinetic Mechanisms of Fast-Decay Red-Fluorescent Genetically Encoded Calcium Indicators." *The Journal of Biological Chemistry* 294 (11): 3934–46.
72. Khan, Kishwar Hayat. 2013. "Gene Expression in Mammalian Cells and Its Applications." *Advanced Pharmaceutical Bulletin* 3 (2): 257–63.
73. Kim, Douglas, Karel Svoboda, Loren Looger, and Eric Schreier. 2019. Genetically encoded calcium indicators and methods of use. USPTO 20190153067:A1. *US Patent*, filed November 21, 2018, and issued May 23, 2019.
<https://patentimages.storage.googleapis.com/48/48/53/5bb27ffa25d100/US20190153067A1.pdf>.
74. Kim, Jong-Myoung, John Hwa, Pere Garriga, Philip J. Reeves, Uttam L. RajBhandary, and H. Gobind Khorana. 2005. "Light-Driven Activation of Beta 2-Adrenergic Receptor Signaling by a Chimeric Rhodopsin Containing the Beta 2-Adrenergic Receptor Cytoplasmic Loops." *Biochemistry* 44 (7): 2284–92.
75. Kirkton, Robert D., Nima Badie, and Nenad Bursac. 2013. "Spatial Profiles of Electrical Mismatch Determine Vulnerability to Conduction Failure across a Host--Donor Cell Interface." *Circulation. Arrhythmia and Electrophysiology* 6 (6): 1200–1207.
76. Kirkton, Robert D., and Nenad Bursac. 2011. "Engineering Biosynthetic Excitable Tissues from Unexcitable Cells for Electrophysiological and Cell Therapy Studies." *Nature Communications* 2: 300.
77. ———. 2012. "Genetic Engineering of Somatic Cells to Study and Improve Cardiac Function." *Europace: European Pacing, Arrhythmias, and Cardiac Electrophysiology: Journal of the Working Groups on Cardiac Pacing, Arrhythmias, and Cardiac Cellular Electrophysiology of the European Society of Cardiology* 14 Suppl 5 (November): v40–49.
78. Koishi, Ryuta, Haoxing Xu, Dejian Ren, Betsy Navarro, Benjamin W. Spiller, Qing Shi, and David E. Clapham. 2004. "A Superfamily of Voltage-Gated Sodium Channels in Bacteria." *The Journal of Biological Chemistry* 279 (10): 9532–38.
79. Kralj, Joel M., Daniel R. Hochbaum, Adam D. Douglass, and Adam E. Cohen. 2011. "Electrical Spiking in Escherichia Coli Probed with a Fluorescent Voltage-Indicating Protein." *Science* 333. <https://doi.org/10.1126/science.1204831>.
80. Kuhn, Thomas S. 2012. *The Structure of Scientific Revolutions: 50th Anniversary Edition*. University of Chicago Press.

81. Laird, Dale W. 2006. "Life Cycle of Connexins in Health and Disease." *Biochemical Journal* 394 (Pt 3): 527–43.
82. Lima, Susana Q., and Gero Miesenböck. 2005. "Remote Control of Behavior through Genetically Targeted Photostimulation of Neurons." *Cell* 121 (1): 141–52.
83. Littleton, J. T., and B. Ganetzky. 2000. "Ion Channels and Synaptic Organization: Analysis of the *Drosophila* Genome." *Neuron* 26 (1): 35–43.
84. Looger, Loren L., and Oliver Griesbeck. 2012. "Genetically Encoded Neural Activity Indicators." *Current Opinion in Neurobiology* 22 (1): 18–23.
85. MacRae, Edith K. 1967. "Nerve, Muscle and Synapse Bernard Katz." *The American Biology Teacher*. <https://doi.org/10.2307/4441678>.
86. Mammucari, Cristina, Anna Raffaello, Denis Vecellio Reane, and Rosario Rizzuto. 2016. "Molecular Structure and Pathophysiological Roles of the Mitochondrial Calcium Uniporter." *Biochimica et Biophysica Acta* 1863 (10): 2457–64.
87. Mank, Marco, and Oliver Griesbeck. 2008. "Genetically Encoded Calcium Indicators." *Chemical Reviews* 108 (5): 1550–64.
88. Marmont, George. 1940. "Action Potential Artifacts from Single Nerve Fibers." *American Journal of Physiology-Legacy Content* 130 (2): 392–402.
89. Marvin, Jonathan S. 2012. "A Single-Wavelength Genetically Encoded Glutamate Sensor for in Vivo Neurotransmitter Observation." *Journal of Biosensors & Bioelectronics*. <https://doi.org/10.4172/2155-6210.s1.07>.
90. Marvin, Jonathan S., Yoshiteru Shimoda, Vincent Magloire, Marco Leite, Takashi Kawashima, Thomas P. Jensen, Ilya Kolb, et al. 2019. "A Genetically Encoded Fluorescent Sensor for in Vivo Imaging of GABA." *Nature Methods* 16 (8): 763–70.
91. Mazzarello, P. 1999. "A Unifying Concept: The History of Cell Theory." *Nature Cell Biology* 1 (1): E13–15.
92. McCusker, Emily C., Claire Bagn  ris, Claire E. Naylor, Ambrose R. Cole, Nazzareno D'Avanzo, Colin G. Nichols, and B. A. Wallace. 2012. "Structure of a Bacterial Voltage-Gated Sodium Channel Pore Reveals Mechanisms of Opening and Closing." *Nature Communications* 3: 1102.

93. Miesenböck, G., D. A. De Angelis, and J. E. Rothman. 1998. "Visualizing Secretion and Synaptic Transmission with pH-Sensitive Green Fluorescent Proteins." *Nature* 394 (6689): 192–95.
94. Miesenböck, G., and J. E. Rothman. 1997. "Patterns of Synaptic Activity in Neural Networks Recorded by Light Emission from Synaptolucins." *Proceedings of the National Academy of Sciences of the United States of America* 94 (7): 3402–7.
95. Mikoshiba, Katsuhiko. 2007. "IP3 receptor/Ca²⁺ Channel: From Discovery to New Signaling Concepts." *Journal of Neurochemistry* 102 (5): 1426–46.
96. Miller, Christopher. 1992. "Ionic Channels of Excitable Membranes.: By Bertil Hille. Sunderland, Massachusetts: Sinauer.(1991). 607 Pp. \$46.95." Cell Press.
[https://www.cell.com/cell/pdf/0092-8674\(92\)90220-7.pdf](https://www.cell.com/cell/pdf/0092-8674(92)90220-7.pdf).
97. Milstein, Michelle L., Hassan Musa, Daniela Ponce Balbuena, Justus M. B. Anumonwo, David S. Auerbach, Philip B. Furspan, Luqia Hou, et al. 2012. "Dynamic Reciprocity of Sodium and Potassium Channel Expression in a Macromolecular Complex Controls Cardiac Excitability and Arrhythmia." *Proceedings of the National Academy of Sciences of the United States of America* 109 (31): E2134–43.
98. Mohanty, Samarendra K., Rainer K. Reinscheid, Xiaobin Liu, Naoe Okamura, Tatiana B. Krasieva, and Michael W. Berns. 2008. "In-Depth Activation of Channelrhodopsin 2-Sensitized Excitable Cells with High Spatial Resolution Using Two-Photon Excitation with a near-Infrared Laser Microbeam." *Biophysical Journal* 95 (8): 3916–26.
99. Montagnoli, G., S. Monti, L. Nannicini, and R. Felicioli. 1976. "Azoaldolase Photosensitivity." *Photochemistry and Photobiology* 23 (1): 29–32.
100. Nagel, Georg, Tanjef Szellas, Wolfram Huhn, Suneel Kateriya, Nona Adeishvili, Peter Berthold, Doris Ollig, Peter Hegemann, and Ernst Bamberg. 2003. "Channelrhodopsin-2, a Directly Light-Gated Cation-Selective Membrane Channel." *Proceedings of the National Academy of Sciences of the United States of America* 100 (24): 13940–45.
101. Nagel, G., B. Möckel, G. Büldt, and E. Bamberg. 1995. "Functional Expression of Bacteriorhodopsin in Oocytes Allows Direct Measurement of Voltage Dependence of Light Induced H⁺ Pumping." *FEBS Letters* 377 (2): 263–66.
102. Neher, Erwin, and J. H. Steinbach. 1978. "Local Anaesthetics Transiently Block Currents through Single Acetylcholine-Receptor Channels." *The Journal of Physiology* 277 (1): 153–76.
103. Neher, E., and B. Sakmann. 1976. "Single-Channel Currents Recorded from Membrane of Denervated Frog Muscle Fibres." *Nature* 260 (5554): 799–802.

104. ———. 1992. “The Patch Clamp Technique.” *Scientific American* 266 (3): 44–51.
105. Nguyen, Hung X., and Nenad Bursac. 2019. “Ion Channel Engineering for Modulation and de Novo Generation of Electrical Excitability.” *Current Opinion in Biotechnology* 58 (August): 100–107.
106. Nguyen, Hung X., Robert D. Kirkton, and Nenad Bursac. 2016. “Engineering Prokaryotic Channels for Control of Mammalian Tissue Excitability.” *Nature Communications* 7 (October): 13132.
107. Nicholls, D. G., and M. W. Ward. 2000. “Mitochondrial Membrane Potential and Neuronal Glutamate Excitotoxicity: Mortality and Millivolts.” *Trends in Neurosciences* 23 (4): 166–74.
108. “Nobel Prize for Physiology and Medicine for 1944: Prof. J. Erlanger and Dr. H. S. Gasser.” 1944. *Nature* 154 (3914): 572–572.
109. Oesterhelt, D., and W. Stoeckenius. 1973. “Functions of a New Photoreceptor Membrane.” *Proceedings of the National Academy of Sciences of the United States of America* 70 (10): 2853–57.
110. Ouzounov, Dimitre G., Tianyu Wang, Mengran Wang, Danielle D. Feng, Nicholas G. Horton, Jean C. Cruz-Hernández, Yu-Ting Cheng, et al. 2017. “In Vivo Three-Photon Imaging of Activity of GCaMP6-Labeled Neurons Deep in Intact Mouse Brain.” *Nature Methods* 14 (4): 388–90.
111. Papazian, D. M., T. L. Schwarz, B. L. Tempel, Y. N. Jan, and L. Y. Jan. 1987. “Cloning of Genomic and Complementary DNA from Shaker, a Putative Potassium Channel Gene from *Drosophila*.” *Science* 237 (4816): 749–53.
112. Park, Jeehae, Christopher A. Werley, Veena Venkatachalam, Joel M. Kralj, Sulayman D. Dib-Hajj, Stephen G. Waxman, and Adam E. Cohen. 2013. “Screening Fluorescent Voltage Indicators with Spontaneously Spiking HEK Cells.” *PloS One* 8 (12): e85221.
113. Patriarchi, Tommaso, Jounhong Ryan Cho, Katharina Merten, Mark W. Howe, Aaron Marley, Wei-Hong Xiong, Robert W. Folk, et al. 2018. “Ultrafast Neuronal Imaging of Dopamine Dynamics with Designed Genetically Encoded Sensors.” *Science*. <https://doi.org/10.1126/science.aat4422>.
114. Payandeh, Jian, Tamer M. Gamal El-Din, Todd Scheuer, Ning Zheng, and William A. Catterall. 2012. “Crystal Structure of a Voltage-Gated Sodium Channel in Two Potentially Inactivated States.” *Nature* 486 (7401): 135–39.

115. Payandeh, Jian, and Daniel L. Minor Jr. 2015. "Bacterial Voltage-Gated Sodium Channels (BacNa(V)s) from the Soil, Sea, and Salt Lakes Enlighten Molecular Mechanisms of Electrical Signaling and Pharmacology in the Brain and Heart." *Journal of Molecular Biology* 427 (1): 3–30.
116. Payandeh, Jian, Todd Scheuer, Ning Zheng, and William A. Catterall. 2011. "The Crystal Structure of a Voltage-Gated Sodium Channel." *Nature* 475 (7356): 353–58.
117. Peron, Simon P., Jeremy Freeman, Vijay Iyer, Caiying Guo, and Karel Svoboda. 2015. "A Cellular Resolution Map of Barrel Cortex Activity during Tactile Behavior." *Neuron* 86 (3): 783–99.
118. Piccolino, Marco. 2006. "Luigi Galvani's Path to Animal Electricity." *Comptes Rendus Biologies* 329 (5-6): 303–18.
119. Primeau, Joseph O., Gareth P. Armanious, M'lynn E. Fisher, and Howard S. Young. 2018. "The SarcoEndoplasmic Reticulum Calcium ATPase." *Sub-Cellular Biochemistry* 87: 229–58.
120. Probst, A., and D. Langui. 1994. "[The neuron theory: one of the main scientific achievements of the 19th century]." *Schweizerische Rundschau fur Medizin Praxis = Revue suisse de medecine Praxis* 83 (16): 462–69.
121. Ren, D., B. Navarro, H. Xu, L. Yue, Q. Shi, and D. E. Clapham. 2001. "A Prokaryotic Voltage-Gated Sodium Channel." *Science* 294 (5550): 2372–75.
122. Richardson, Arthur. 1893. "LXXXII.—The Action of Light in Preventing Putrefactive Decomposition; and in Inducing the Formation of Hydrogen Peroxide in Organic Liquids." *Journal of the Chemical Society, Transactions* 63 (0): 1109–30.
123. Rodin, Sergei N., and Arthur D. Riggs. 2003. "Epigenetic Silencing May Aid Evolution by Gene Duplication." *Journal of Molecular Evolution* 56 (6): 718–29.
124. Rogero, Oscar, Barbara Hämmerle, and Francisco J. Tejedor. 1997. "Diverse Expression and Distribution of Shaker Potassium Channels during the Development of the Drosophila Nervous System." *The Journal of Neuroscience*. <https://doi.org/10.1523/jneurosci.17-13-05108.1997>.
125. Rosenmund, C., and C. F. Stevens. 1996. "Definition of the Readily Releasable Pool of Vesicles at Hippocampal Synapses." *Neuron* 16 (6): 1197–1207.

126. Ryan, T. A., H. Reuter, B. Wendland, F. E. Schweizer, R. W. Tsien, and S. J. Smith. 1993. "The Kinetics of Synaptic Vesicle Recycling Measured at Single Presynaptic Boutons." *Neuron* 11 (4): 713–24.
127. Ryan, Timothy A., Harald Reuter, and Stephen J. Smith. 1997. "Optical Detection of a Quantal Presynaptic Membrane Turnover." *Nature*. <https://doi.org/10.1038/41335>.
128. Sakmann, B. 1992. "Nobel Lecture. Elementary Steps in Synaptic Transmission Revealed by Currents through Single Ion Channels." *The EMBO Journal* 11 (6): 2002–16.
129. Seyfarth, Ernst-August. 2006. "Julius Bernstein (1839-1917): Pioneer Neurobiologist and Biophysicist." *Biological Cybernetics* 94 (1): 2–8.
130. Shah, Bhaval S., Anthony M. Rush, Shujun Liu, Lynda Tyrrell, Joel A. Black, Sulayman D. Dib-Hajj, and Stephen G. Waxman. 2004. "Contactin Associates with Sodium Channel Nav1.3 in Native Tissues and Increases Channel Density at the Cell Surface." *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 24 (33): 7387–99.
131. Shaya, David, Felix Findeisen, Fayal Abderemane-Ali, Cristina Arrigoni, Stephanie Wong, Shailika Reddy Nurva, Gildas Loussouarn, and Daniel L. Minor Jr. 2014. "Structure of a Prokaryotic Sodium Channel Pore Reveals Essential Gating Elements and an Outer Ion Binding Site Common to Eukaryotic Channels." *Journal of Molecular Biology* 426 (2): 467–83.
132. Shen, Huaizong, Qiang Zhou, Xiaojing Pan, Zhangqiang Li, Jianping Wu, and Nieng Yan. 2017. "Structure of a Eukaryotic Voltage-Gated Sodium Channel at near-Atomic Resolution." *Science* 355 (6328). <https://doi.org/10.1126/science.aal4326>.
133. Siegel, M. S., and E. Y. Isacoff. 1997. "A Genetically Encoded Optical Probe of Membrane Voltage." *Neuron* 19 (4): 735–41.
134. Sigworth, F. J., and E. Neher. 1980. "Single Na⁺ Channel Currents Observed in Cultured Rat Muscle Cells." *Nature* 287 (5781): 447–49.
135. Stierl, Manuela, Patrick Stumpf, Daniel Udvari, Ronnie Gueta, Rolf Hagedorn, Aba Losi, Wolfgang Gärtner, et al. 2011. "Light Modulation of Cellular cAMP by a Small Bacterial Photoactivated Adenylyl Cyclase, bPAC, of the Soil Bacterium *Beggiatoa*." *The Journal of Biological Chemistry* 286 (2): 1181–88.
136. Stockley, John H., Kimberley Evans, Moritz Matthey, Katrin Volbracht, Sylvia Agathou, Jana Mukanowa, Juan Burrone, and Ragnhildur T. Káradóttir. 2017. "Surpassing Light-Induced Cell Damage in Vitro with Novel Cell Culture Media." *Scientific Reports* 7 (1): 849.
137. Stoien, J. D., and R. J. Wang. 1974. "Effect of near-Ultraviolet and Visible Light on Mammalian Cells in Culture II. Formation of Toxic Photoproducts in Tissue Culture Medium by

Blacklight.” *Proceedings of the National Academy of Sciences of the United States of America* 71 (10): 3961–65.

138. “The Nobel Prize in Physiology or Medicine 1906.” n.d. NobelPrize.org. Accessed April 17, 2020. <https://www.nobelprize.org/prizes/medicine/1906/cajal/biographical/>.

139. [Thomas, Merrilee and Thom Hughes. 2020. “Design for Fast Optogenetic Screen In Mammalian Cells For Next Gen Ca²⁺ Sensors.” <https://doi.org/10.1101/2020.02.03.932426>.](#)

140. Tyssowski, Kelsey M., and Jesse M. Gray. 2019. “Blue Light Increases Neuronal Activity-Regulated Gene Expression in the Absence of Optogenetic Proteins.” *eNeuro* 6 (5). <https://doi.org/10.1523/ENEURO.0085-19.2019>.

141. Wainberg, M. A., and B. F. Erlanger. 1971. “Investigation of the Active Center of Trypsin Using Photochromic Substrates.” *Biochemistry* 10 (21): 3816–19.

142. Wang, S. S., and G. J. Augustine. 1995. “Confocal Imaging and Local Photolysis of Caged Compounds: Dual Probes of Synaptic Function.” *Neuron* 15 (4): 755–60.

143. Woehler, Andrew, Jakub Wlodarczyk, and Erwin Neher. 2010. “Signal/noise Analysis of FRET-Based Sensors.” *Biophysical Journal* 99 (7): 2344–54.

144. Yuste, Rafael, Rebecca B. Miller, Knut Holthoff, Shifang Zhang, and Gero Miesenböck. 2000. “Synapto-pHluorins: Chimeras between pH-Sensitive Mutants of Green Fluorescent Protein and Synaptic Vesicle Membrane Proteins as Reporters of Neurotransmitter Release.” In *Methods in Enzymology*, edited by Jeremy Thorner, Scott D. Emr, and John N. Abelson, 327:522–46. Academic Press.

145. Zemelman, Boris V., Georgia A. Lee, Minna Ng, and Gero Miesenböck. 2002. “Selective Photostimulation of Genetically chARGed Neurons.” *Neuron* 33 (1): 15–22.

146. Zemelman, Boris V., Nasri Nesnas, Georgia A. Lee, and Gero Miesenböck. 2003. “Photochemical Gating of Heterologous Ion Channels: Remote Control over Genetically Designated Populations of Neurons.” *Proceedings of the National Academy of Sciences of the United States of America* 100 (3): 1352–57.

147. Zhang, Hongkang, and Adam E. Cohen. 2017. “Optogenetic Approaches to Drug Discovery in Neuroscience and Beyond.” *Trends in Biotechnology* 35 (7): 625–39.

148. [Zhang, Hongkang, Elaine Reichert, and Adam E. Cohen. 2016. “Optical Electrophysiology for Probing Function and Pharmacology of Voltage-Gated Ion Channels.” *eLife* 5 \(May\). <https://doi.org/10.7554/eLife.15202>.](#)

CHAPTER THREE

OPTICALLY ACTIVATED, CUSTOMIZABLE, EXCITABLE CELLS

KUHL PLATFORM FOR EVOLVING NEXT GEN BIOSENSORS

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

Author: Merrilee A. Thomas

Contributions: Designed and conducted research; analyzed data; wrote the manuscript; revised the manuscript.

Co-Author: Thomas E. Hughes

Contributions: Conducted and supervised research; revised the manuscript; procured funding.

Manuscript Information

Merrilee A. Thomas and Thomas E. Hughes

PLOS One

Status of Manuscript:

- ☐ Prepared for submission to a peer-reviewed journal
- ☒ Officially submitted to a peer-reviewed journal
- ☐ Accepted by a peer-reviewed journal
- ☐ Published in a peer-reviewed journal

PLOS One

February 2020

Optically Activated, Customizable, Excitable Cells
Kuhl Platform for Evolving Next Gen Biosensors

Merrilee A. Thomas¹ and Thomas Hughes^{1,2}

¹Department of Neuroscience and Cell Biology,
Montana State University, Bozeman, Montana, United States of America

²Montana Molecular, Bozeman, Montana, United States of America

Corresponding Author

Email: Merrileethomas1@gmail.com

Abstract

Genetically encoded fluorescent biosensors are powerful tools for studying complex signaling in the nervous system, and now both Ca^{2+} and voltage sensors are available to study the signaling behavior of entire neural circuits. There is a pressing need for improved sensors to properly interrogate these systems. Improving them is challenging because testing them involves low throughput, labor-intensive processes. Our goal was to create a live cell system in HEK293 cells that use a simple, reproducible, optogenetic process for testing prototypes of genetically encoded biosensors.

In this live cell system, blue light activates an adenylyl cyclase enzyme (bPAC) that increases intracellular cAMP [1]. In turn, the cAMP opens a cAMP gated ion channel (olfactory cyclic nucleotide-gated channel, CNG, or the hyperpolarization-activated cyclic nucleotide-gated channel, HCN2). This produces slow, whole-cell Ca^{2+} transients and voltage changes. To increase the speed of these transients, we added the inwardly rectifying potassium channel Kir2.1, the bacterial voltage-gated sodium channel NAVROSD, and Connexin-43. This is a modular system in which the types of channels, and their relative amounts, can be tuned to produce the cellular behavior that is crucial for screening biosensors. The result is a highly reproducible, high-throughput live cell system that can be used to screen voltage and Ca^{2+} sensors in multiple fluorescent wavelengths simultaneously.

Introduction

Why are Biosensors Important?

Genetically encoded, fluorescent biosensors are powerful tools for studying cell signaling in real-time [2,3]. They are minimally invasive, and since they are genetically encoded their expression can be exquisitely targeted to specific cell types and tissues [4–6]. However, the short wavelengths of light needed to image green biosensors can heat and damage the brain, decreasing the duration of imaging [7]. There is a need for better biosensors that emit red [8] or near-infrared [9] light because these longer wavelengths enable investigators to image deeper into thick tissues. Furthermore, a better signal to noise ratio can help increase imaging speeds and increase the field of view. Brighter sensors can be detected with lower expression levels potentially decreasing overexpression which can lead to epileptiform activity [10].

A proven approach to expanding the fluorescent biosensor toolbox involves screening thousands of prototypes, evolving for optimal function [11–14]. Ca^{2+} sensors have been highly optimized in *E. coli*. One study used a microfluidic sorter (FACs) where it was possible to screen up to 10^6 colonies per round of evolution in *E. coli* [8][15]. A more recent study optimized sensors by picking *E. coli* expressing sensors with the greatest fluorescence intensity. As a second screen, the proteins were cultured and the lysate was placed into Ca^{2+} and Ca^{2+} free buffers and read using a microplate reader [16]. A drawback to this approach is that a sensor cannot be screened for its kinetics, there are only two steady states.

A different approach to screen activity-based biosensors is in mammalian excitable cells. Screening in primary cultures of differentiated, excitable cells is time consuming, expensive, and low throughput([Storace et al. 2016](#)). Another approach is to create excitable cells de novo using

transformed cells lines compatible with high throughput screening. One of the first de novo excitable cells was made by expressing rat brain IIA Na^+ channel and the Drosophila Shaker K^+ channel into Chinese hamster ovary cells via vaccinia virus [17]. Additionally, one study stably expressed three ion channels, Kir2.1, Nav1.5, and Cx-43 in HEK293 cells. Using electrophysiology they found that with all three channels expressed fast, uniform action potential propagation could be created. However, these particular cells cannot be used to test fluorescent biosensors, because each exogenous channel was marked with a fluorescent protein spanning the visible spectrum [18].

Current methods to screen voltage sensors, in particular, include using excitable HEK293 cells that combine Kir2.1 and a voltage-gated sodium channel (NAV1.3) with field stimulation to produce rhythmic depolarization and hyperpolarization in HEK cells [12,19]; [20]. Another option is to use a stable cell line that expresses Kir2.1, Nav1.7, and CheRiff, a channelrhodopsin. This can be photoactivated thereby creating a system that produces oscillating membrane voltage [21]. Yet, there are inherent problems with stable cell lines, such as genetic drift and gene silencing. Often stable cell lines will regulate the expression of exogenous genes, and mutations in the exogenous genes will occur after several cell culture passages [22,23]. In addition, the level of expression of each of the channels cannot be easily changed and currently, only wavelengths outside of the rhodopsin's excitation spectrum can be used for screening.

What if we could screen thousands of variants in mammalian cells for sensitivity, speed, and dynamic range? We hypothesized that we could use similar channels as those used previously to create cells that (1) can be optically stimulated (2) produce action potentials and whole-cell Ca^{2+} transients and (3) can be imaged with at least two wavelengths simultaneously.

We wanted the system to be automatable, inexpensive, modular, and useful in many different cell types. While it could be incorporated into a stable cell line we opted for a more modular route by using baculovirus [24]. While viral transduction involves more work than a stable cell line, it gives us the ability to optimize each component independently, something that cannot be done in stable cells.

Our goal was to create a screening platform for biosensors that is consistent, reliable, and capable of quickly screening thousands of prototypes. We first created a minimal cellular system with a light-activated enzyme, we used an adenylyl cyclase, to establish a “Kuhl” cell. Next, we added a cAMP-gated ion channel, either a CNG or HCN channel, to produce “Kuhl-C, and Kuhl-H” cells respectively. We then added additional channels and proteins to tune the responses. To improve the responses we added the Kir2.1 potassium channel, to create Kuhl-CK or Kuhl-HK cells. To speed up the action potentials we added the bacterial sodium channel NavD to design Kuhl-CKNa and Kuhl-HKNa cells. Finally, we found that connexin-43 improved the coordination of the signaling, producing Kuhl-CKNaCx and Kuhl-HKNaCx cells.

Results

Establishing an Optical Actuator

How can we activate a biosynthetic cell line using light? To develop an optogenetically controlled screen, we chose to use bPAC (blue photo-activated cyclase), a blue light activated adenylyl cyclase from the soil bacteria *Beggiatoia* [1]. In theory, coupling a light activated enzyme to large conductance channels, should produce larger whole cell currents with less protein expression than the current optogenetic approaches that rely upon channel rhodopsins.

The bPAC enzyme is activated with 480 nm light and converts ATP into cyclic adenosine monophosphate (cAMP). We began by examining whether blue light stimulation can produce a measurable change in cAMP levels in bPAC-expressing HEK293 cells as seen with R-cADDIs, a red fluorescent cAMP sensor [25].

We transduced HEK293 cells with a BacMam virus that expresses bPAC and R-cADDIs (S2 Table). The following day, we stimulated cells with 20 milliseconds of blue light and then collected images continuously with 561 nm excitation that does not activate bPAC (Fig 1A). Upon the blue light stimulation, the R-cADDIs red fluorescence increases (Fig 1B). cAMP levels within the cell rise over the time frame of ~100 seconds (Fig 1C). Control cells with no bPAC did not show an increase in red fluorescence. The system response is remarkably reproducible and the same cells can be repeatedly stimulated. Indeed the only limitation is that too much blue light given over a short time will create too much cAMP and saturate the sensor. The slow decrease in fluorescence of R-cADDIs is most likely due to the phosphodiesterases present in the cell which work to eliminate cAMP [26].

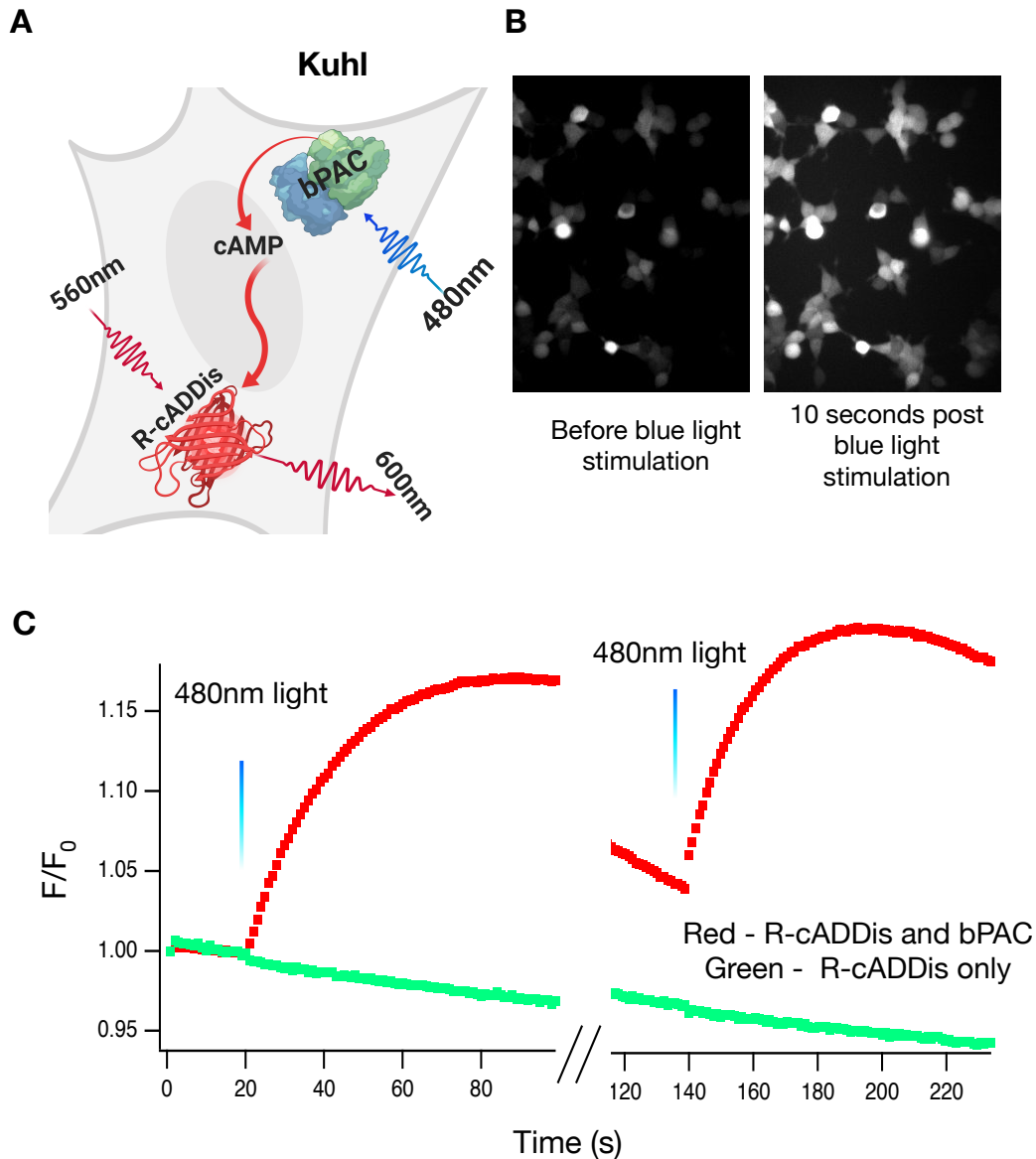


Fig 1. 20ms of Blue Light Repeatedly Activates bPAC. A) HEK-293 cells were transduced with bPAC (the actuator) and R-cADDIs, the red fluorescence cAMP sensor (S2 Table). B) The cells were illuminated with 20ms of 480nm light. This activation of bPAC increases cAMP levels which increases R-cADDIs fluorescence. Brightness and contrast are the same for both images. C) The traces are a discontinuous recording of R-cADDIs with 560nm excitation from the same cell (100s on, 20s off, 100s on). 20 ms of blue light at 20s and 140s activates bPAC which produces bursts of cAMP for 100s. This is a stereotyped response that is repeatable with multiple doses of blue light.

Dose Dependence

To examine the dose-response relationship between blue light and cAMP production, we systematically varied the intensity of the blue light during stimulation while quantifying the R-cADDIs fluorescence. HEK293 cells were transduced with bPAC and R-cADDIs, and each well in a 96-well plate was stimulated with a 15ms dose of blue light. For each well, LED 480nm light power was increased stepwise from 0 to 70 mW/cm² in increments of five %power using a LED controller. At 100% power, the light intensity at the specimen plane measured 70mW/cm². Figure 2 reveals a dose response in which increasing levels of blue light produces increases in cAMP levels. Lowering the level of bPAC expression by decreasing the total viral load may make it possible to create a system with a greater range of tunability before saturating the sensor with cAMP. The fluorescence change, ΔF , was used to determine the cAMP level and all points in the dose response are an average of multiple regions of interest (ROIs) within a trial (Fig 2). This is consistent with a similar study that found bPAC had a linear dose response to light when cAMP was recorded [27]

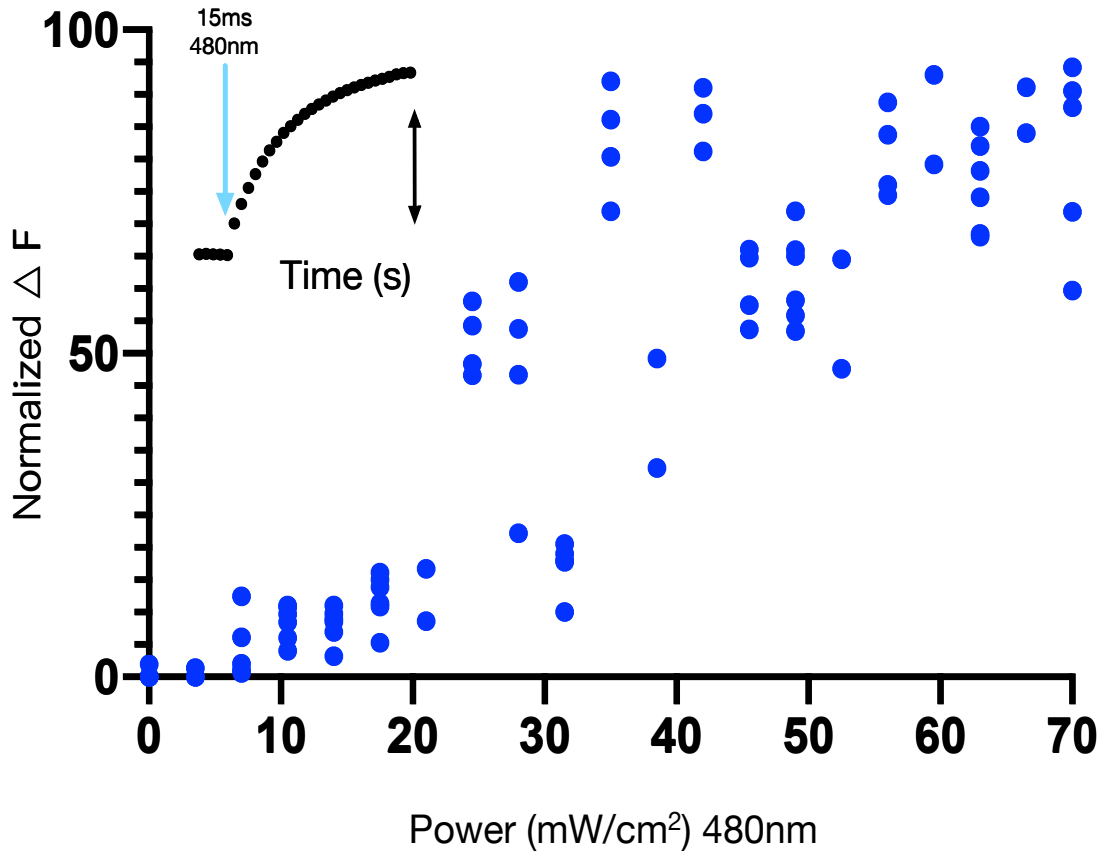


Fig 2. There is a dose/response relationship between the intensity of blue excitation of bPAC and the fluorescence response of R-cADDIs. As the blue light intensity increases so does the cAMP production. The ΔF is a measure between the baseline fluorescence and the maximum of the change in fluorescence (inset). Each data point represents an average of ~ 100 ROIs within a trial.

Coupling the Actuator with an Ion Channel

To couple cAMP production with channel activation, we expressed a cyclic nucleotide-gated ion channel (the rat olfactory CNG or human heart HCN2 channel) (S2 Table). The rat olfactory CNG channel is normally gated by cyclic guanosine monophosphate (cGMP) [28]. However, several mutations (61–90/C460W/E583M) can be introduced [29] that renders it sensitive to cAMP. In theory, this mutant channel should couple bPAC activation to a current

that depolarizes the cell, creating Kuhl-C cells. To create Kuhl-H cells, we expressed the human HCN2 pacemaker channel which is also cAMP-gated and is hyperpolarization-activated (S2 Table) [30]. Once the cell is hyperpolarized, the HCN2 channel can open when bound to cAMP.

We activated the Kuhl-C or Kuhl-H cells with blue light thereby increasing cAMP.

Figure 3 illustrates that 20ms of blue light activation of bPAC, produces an increase in the red fluorescence of R-GECO1, demonstrating a gradual increase in whole-cell Ca^{2+} over several minutes. Opening either the CNG or HCN2 channel increased the red fluorescence of R-GECO1, consistent with increased cytosolic Ca^{2+} levels (Figs 3B and 3F). Interestingly, blue light stimulation also produced bright subcellular flashes of fluorescence in both the Kuhl-C and Kuhl-H cells. The source of this Ca^{2+} is not clear, but one possibility is that increases in cAMP could be causing brief IP_3 channel opening, or perhaps mitochondrial release [31] (Figs 3C and 3G). The subcellular flashes are small flashes of Ca^{2+} that last ~20 seconds (Figs 3D and 3H). They are far more frequent in cells without NavD but can be seen in all Kuhl cells (S1 Movie). The control cells without bPAC showed no such responses (S1 Fig).

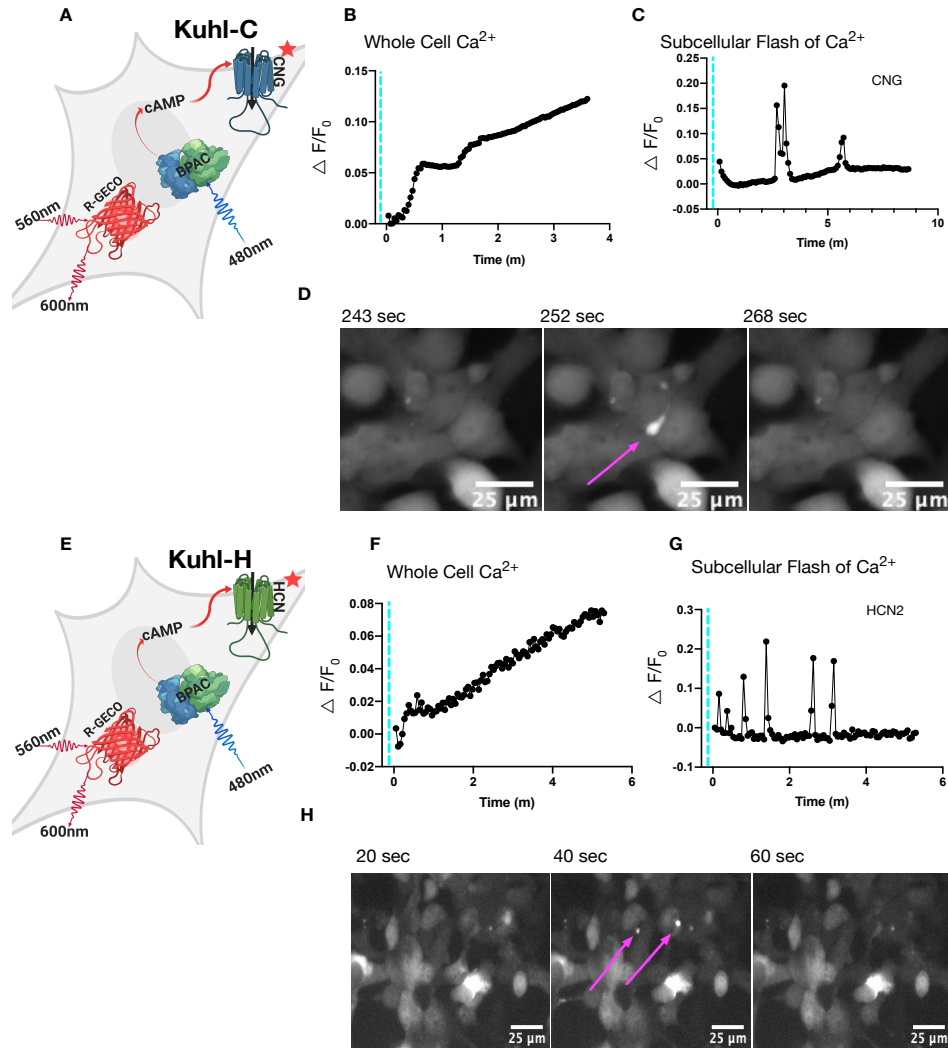


Fig 3. Blue light activation of bPAC slowly increases whole-cell Ca^{2+} when CNG or HCN2 is expressed. There were two types of increases in Ca^{2+} activity: a slow whole-cell Ca^{2+} increase and bright subcellular flashes of Ca^{2+} . These can be seen when bPAC and a cyclic nucleotide-gated channel are expressed. A, E) Cartoon depicting all the components in the Kuhl-C or Kuhl-H cell system (S2 Table). Blue light stimulation of bPAC produces cAMP that in turn opens a cAMP gated CNG (delta61–90/C460W/E583M) channel or HCN2 channel. B) The black trace is the R-GECO1 response to 30 ms of blue light activation in cells with both CNG and bPAC, F) or with HCN2 and bPAC. C) The black trace depicts small brief subcellular flashes of Ca^{2+} that can be seen in cells with CNG and bPAC, G) or HCN2 and bPAC. D) The localized subcellular flash

of Ca^{2+} seen in the Kuhl-C cell system in a series of three frames at 243s, 252s, 268s, and H) in Kuhl-H cells at 20s, 40s, 60s.

Addition of Kir2.1 Coupled with bPAC

The bPAC and CNG, or HCN2, expression produced very reliable Ca^{2+} transients, but these were long-lived events with slow kinetics. We hypothesized that introducing a voltage-regulated inward-rectifying potassium channel could speed up, and increase the amplitude, of these transients by creating a greater driving force on Ca^{2+} to enter the cell. Wild type HEK293 cells have a resting membrane potential of only -25 mV, but Kir2.1 expression shifts their resting potential to -70mV [18,32,33]; [34].

We expressed Kir2.1, bPAC, R-GECO1, and a cyclic nucleotide-gated channel in HEK293 cells to produce Kuhl-CK and Kuhl-HK cells (S2 Table). In the Kuhl-C/HK cell system, there is some degree of spontaneous activity before blue light stimulation, but when we activate bPAC with 20ms of blue light there are robust responses in all of the wells. There are Ca^{2+} transients that occur in 85% of the cells, but the activity is largely uncoordinated. Each cell has its own “signature” change in Ca^{2+} levels, as seen in the R-GECO1 fluorescence traces (Fig 4B). Ca^{2+} transients can be seen for up to 30 minutes following the blue light stimulus (S2 Fig). The histogram shows the average cumulative number of fluorescence peaks per cell over 340s (Fig 4G). HCN2 produces a slightly greater cumulative average frequency of Ca^{2+} fluorescence peaks than CNG.

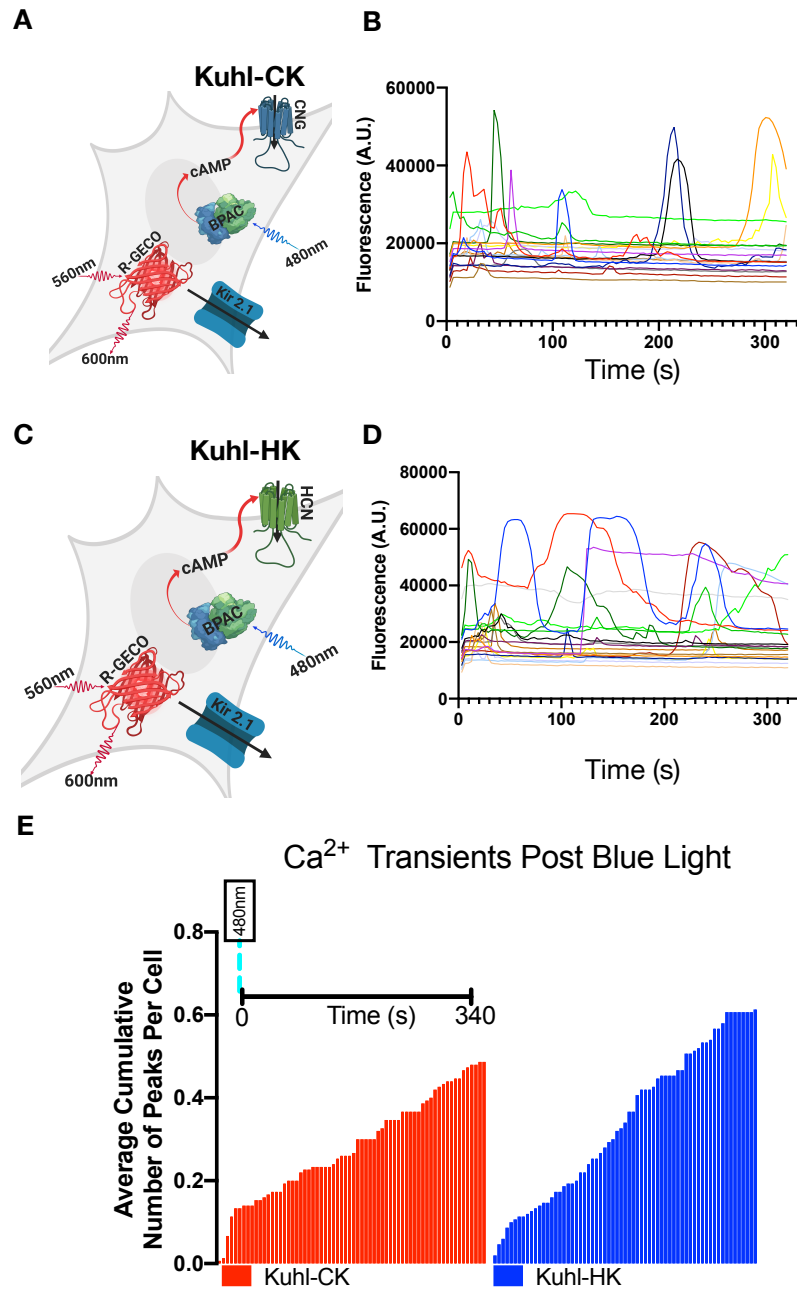


Fig 4 . The addition of Kir2.1 leads to fluctuating whole-cell Ca²⁺ transients in the Kuhl-H/CK system. A, C) Cartoon depicting all the components transduced into the experimental Kuhl-H/CK cell system (S2 Table). Blue light stimulates bPAC which will produce cAMP that opens the (CNG or HCN2) channel, and Kir2.1 should hyperpolarize the cells, creating a greater driving force on Ca²⁺ to enter the cell. B) R-GECO1 fluorescence response to 20ms of blue light in Kuhl-CK or D) 20ms of blue light stimulating Kuhl-HK cells. The traces show a subset of the

total responses (S2 Fig). G) Average cumulative number of Ca^{2+} peaks in Kuhl-HK and Kuhl-CK system (all peaks detected across 150 cells for each condition). Kuhl-HK cells have a higher average cumulative number of peaks per cell. Each cell has a unique fluctuating Ca^{2+} transient response after the blue light stimulation of bPAC.

Optimizing the Activity

To quantify the Ca^{2+} imaging data we analyzed it using a custom MATLAB program that harnessed MATLAB's built-in peak finder (Fig 5A) (methods). We wanted to break down each Ca^{2+} transient into quantifiable components that could be compared across different conditions. We defined a transient as a change in fluorescence that exceeded the standard deviation of the variability in the baseline by a factor of 26. In other words, we only analyzed events where the SNR exceeded 26 [35]. For each fluorescent peak, we quantified the total cumulative peaks (Fig 5b) and the total number of peaks per ROI (Fig 5D). The normalized ΔF of the peak (amplitude) (Fig 5E), the full width of the peak at half maximum (FWHM) (Fig 5F), and the inter-peak interval (Fig 5G). Also, we examined the total number of Ca^{2+} transients recorded per experiment and recorded the time it took for a Ca^{2+} transient to occur post blue light stimulation.

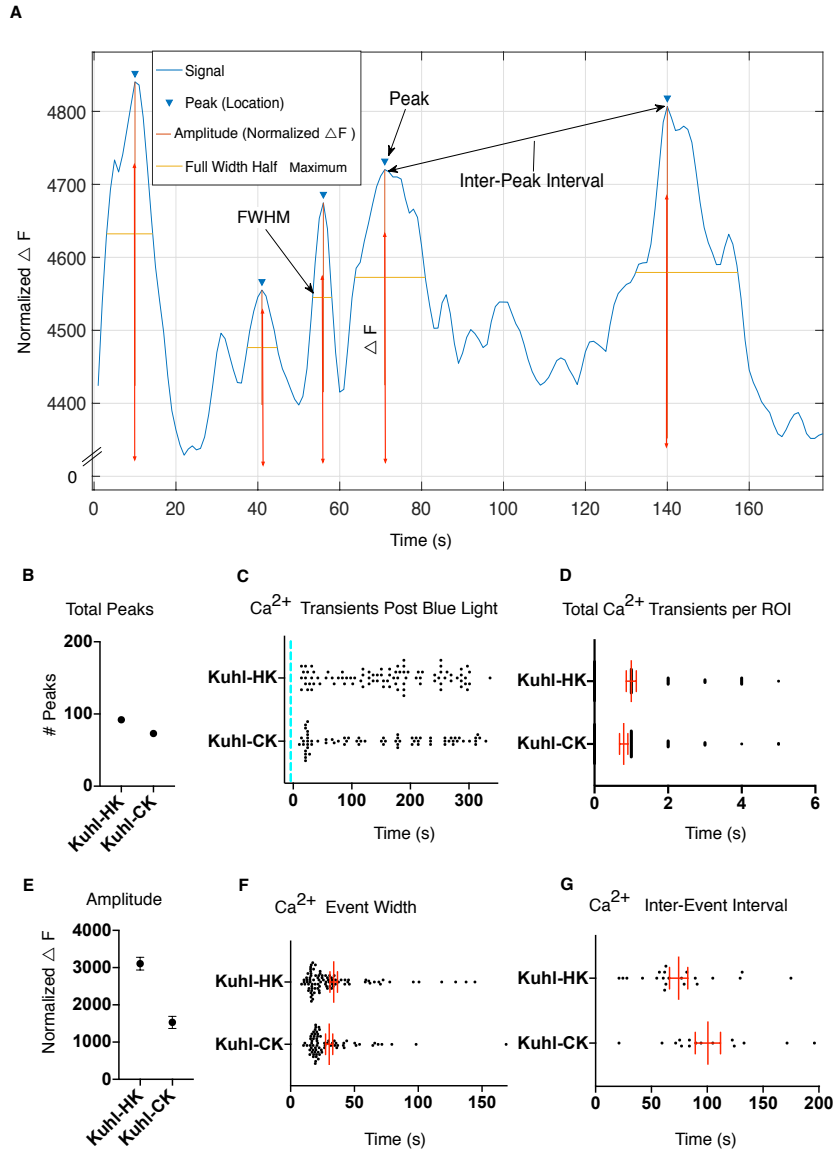


Fig 5. Features of the prolonged Ca^{2+} transients in Kuhl cells shown in Figure 4 differ depending on the presence of CNG or HCN2. A) Diagram of the MATLAB analysis of the Ca^{2+} transients. Peak intensity, the full width of the peak at half maximum (FWHM), and interpeak intervals were analyzed. B) The total Ca^{2+} peaks ($n = 150$ cells per condition) for Kuhl-H/C were analyzed. The Kuhl-HK system had more Ca^{2+} peaks in 350s than the Kuhl-CK system. C) Blue line indicates a point of 20s blue light stimulus. The time of each fluorescence peak was recorded and is indicated by a black dot. D) The number of Ca^{2+} transients per cell (ROI) for both HCN2 and CNG. E) The normalized ΔF is the difference between the baseline fluorescence and the maximum fluorescence of each peak. The mean and S.E.M of the Ca^{2+} transients in

Kuhl-H/CK cells. F) Representative duration of elevated R-GECO1 fluorescence overtime per Ca^{2+} transient. FWHM is an estimate of the length of time that the fluorescence stays elevated for each defined peak. G) The Inter-event interval, which is the difference in time between two consecutive peaks. The time between Ca^{2+} transient events per cell. Bars indicate mean and $\pm$ S.E.M for 150 cells.

We found that in the Kuhl-HK cell system ($n = 150$ cells) there is a 62% chance that any given cell produces a Ca^{2+} peak (Fig 4G) and when imaged for 340s a 52% chance (methods) that it will produce a second peak within ~ 70 s (Fig 5G) of the initial response. The Kuhl-CK system has a 20% chance that a cell will produce a significant Ca^{2+} peak and out of the cells that were suprathreshold there is a 63% chance that there will be a second response in that cell ~ 100 s (Fig 5G) following.

Voltage-gated Sodium Channel

NavD Creates Faster Ca^{2+} Transients

The bacterial sodium channel, NavRosDg217a (NavD) is characterized by a rapid activation state that depends on the cell depolarizing to -30mV , and a slow inactivation state (hundreds of milliseconds) that starts during the depolarization [32]. Co-expressing the NavD channel with Kir2.1 can potentially create a faster excitable system because the Kir2.1 hyperpolarizes the cell after each spontaneous depolarization caused by the sodium channel. We chose NavD because the mutant is slower than the mammalian sodium channels and in theory, we could create a system with depolarizations such that membrane depolarization events could be captured with slower imaging speeds (10Hz).

A recent paper by Chen et al. found that varying the levels of Kir2.1, and Cav1.3 or Nav1.5 affected the membrane oscillations as recorded by electrophysiology [30]. We

hypothesized that different levels of the Kir2.1 and NavD would lead to different corresponding Ca^{2+} responses.

To test this we systematically transduced cells with varying ratios of Kir2.1 and NavD while holding the viral concentrations of bPAC, CNG and R-GECO1 constant. The Kuhl-CKNa cells were transduced (S2 Table) and ~48 hours later the cells were stimulated with 20ms of blue light and images were collected with 561nm excitation. Upon activation of bPAC there were varying responses in each condition that are quantified (S4 Fig).

The Kuhl-CKNa cells with a viral titer of (Kir2.1 $1\text{e}8$):(NavD $5\text{e}8$) VG (Viral Genomes)/ μL had the greatest cumulative peaks(S4a Fig) over 210s. To understand which (Kir2.1):(NavD) ratio had the greatest cumulative number of Ca^{2+} transients divided by the total number of cells (Fig 6b). Using the data from the total number of peaks per ROI (S4d Fig) we ran a Dunnett's test (S1 Table) to examine if there was a statistical significance between the conditions. (Kir2.1 $1\text{e}8$):(NavD $5\text{e}8$) VG/ μL had a significant increase ($****p \leq .0001$) in the average cumulative number of peaks per cell (Fig 6b). In the Kuhl-CKNa system, there is a 35% chance that a cell will produce a significant Ca^{2+} peak and out of the cells that were suprathreshold there is a 43% chance of a second peak within 66s of the initial fluorescence peak (S4f Fig). Introducing NavD created significantly faster Ca^{2+} transients (S3 Fig). In S3 Figure the differences in the mean duration that Ca^{2+} stayed elevated in the Kuhl-CKNa system with and without NavD are shown. Further optimizing connexin-43 and CNG concentrations significantly decreased the time that Ca^{2+} transients stayed elevated (S3 Fig).

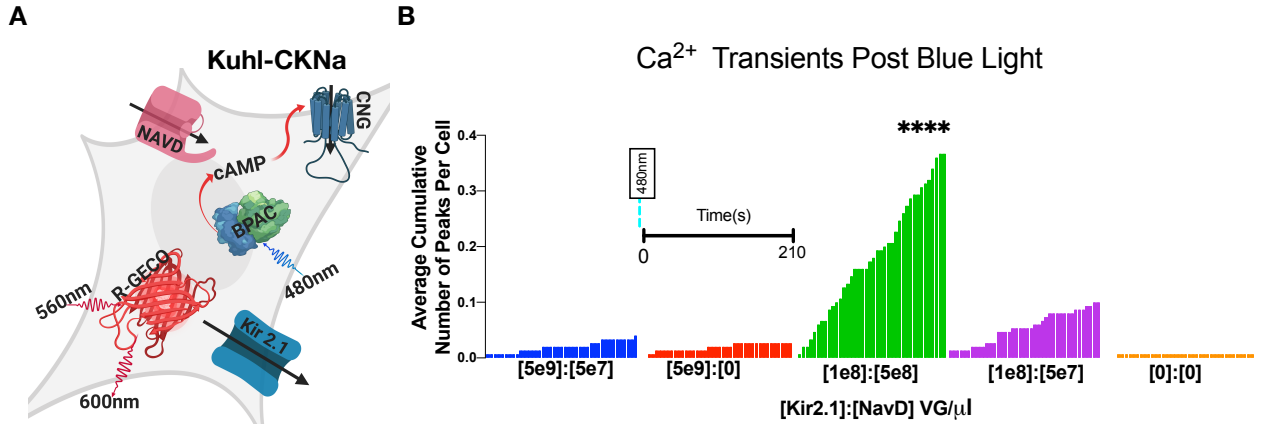


Fig 6. The Kir2.1 and NavD concentration were optimized. NavD was introduced to the Kuhl-CKNa cell system. Introducing NavD created significantly faster Ca^{2+} transients (S3 Fig). A) Cartoon depicting all the components transduced into the experimental Kuhl-CKNa cell (S2 Table). B) Shown is the average cumulative fluorescence peaks per cell across 210s for each indicated viral concentration of Kir2.1 and NavD ($n = 150$ cells per condition). Differences were analyzed using Dunnett's multiple comparisons test on the Ca^{2+} transient frequency (S4d Fig). (Kir2.1 1e8):(NavD 5e8) VG/ μL was significantly greater (**** $p \leq .0001$) than the control (Kir2.1 0):(NavD 0) VG/ μL .

Further Optimization with Connexin-43

and Kir2.1 and NavD

HEK293 cells have endogenous connexin proteins, connexin-45, which create gap junctions that connect the cytoplasm of the cells [36,37]. However, Kirkton and Bursac [18] found that when a confluent layer of HEK293 cells expressing Kir2.1 and Nav1.5 had action potentials that did not propagate uniformly through the monolayer. When they added Connexin-43 Cx-43 they recorded rapid and robust action potentials. In addition, several other studies have shown that including the Cx-43 protein increases the electrical gap junction coupling of HEK293 cells [18,32,38].

The concentrations for (Kir2.1):(NavD) VG/ μ L were varied for each condition and the following concentrations were held constant for bPAC, CNG, Cx-43, and R-GECO1 (S2 Table). The Kuhl-CKNaCx cells were transduced (S2 Table) and 48 hours later the cells were stimulated with 20ms of blue light and images were collected with 561nm excitation. Upon activation of bPAC there were varying responses in each condition that are fully quantified (S5 Fig). Interestingly there seems to be a significant effect on the responses in the Kuhl-CKNaCx cells (Fig 7B) depending on the amount of (Kir2.1):(NavD) VG/ μ L applied.

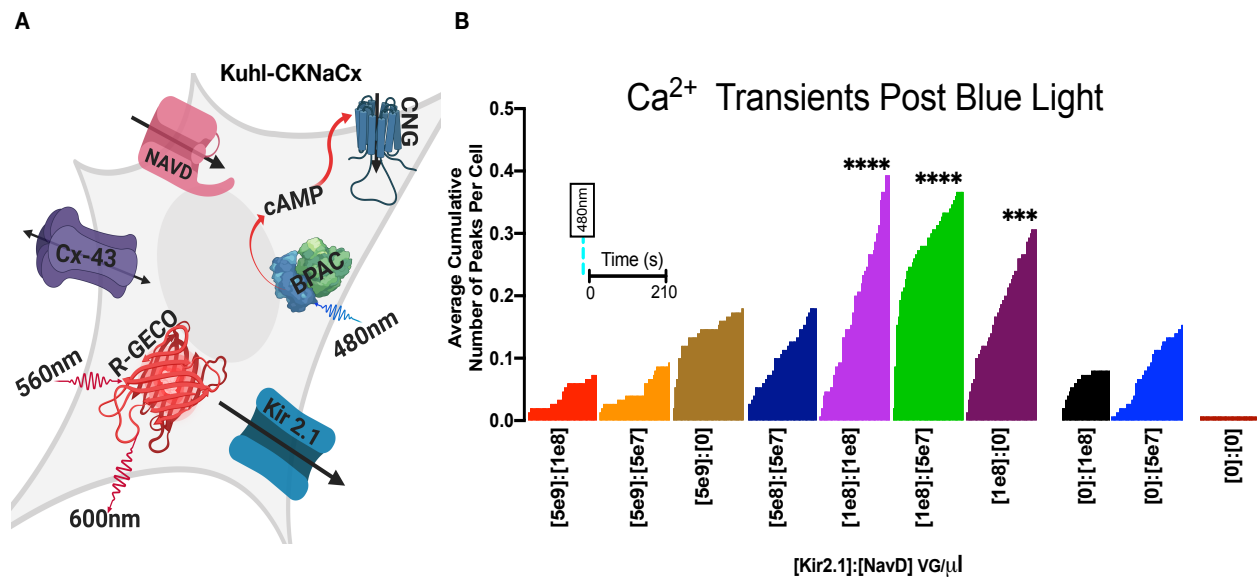


Fig 7. Optimizing Kir2.1 and NavD with Connexin-43 increases the average number of Ca²⁺ transients. Introducing the NavD and Kir2.1 channel is expected to create faster oscillating Ca²⁺ transients in the cell while introducing the Cx-43 will increase coordinated activity between cells. Further optimization of Kir2.1 to NavD is shown. Cx-43 was introduced to the Kuhl-CKNaCx system. A) Cartoon depicting all the components transduced into the experimental Kuhl-CKNaCx cell (S2 Table). B) Shown is the average cumulative fluorescence peaks per cell across 210s for each indicated viral concentration of Kir2.1 and NavD ($n = 150$ cells per condition). Differences were analyzed using Dunnett's multiple comparison test on the Ca²⁺ transient frequency (S5d Fig) compared to the control (Kir2.1 0):(NavD 0) VG/ μ L. The experimental trials that were statistically greater were marked (**** $p \leq .0001$) and (***) $p \leq .0002$).

The total cumulative peaks (S5A Fig) were greatest in the condition with a viral titer of (Kir2.1 1e8):(NavD 1e8) VG/ μ L. To understand which viral titer impacted the cells we examined the cumulative number of Ca²⁺ transients divided by the total number of cells (Fig 7B). Using the data from the total number of peaks per ROI (S5d Fig) we ran Dunnett's test (S1 Table) to examine if there was a statistical significance between the conditions. The experimental trials that were statistically greater than the control were marked (**** $p \leq .0001$, *** $p \leq .0002$) on the graph (Fig 7B).

It appears that using comparable amounts of the two channels (Kir2.1 1e8) and (NavD 1e8) VG/ μ L evoked a significant response because there is a 40% chance that out of 150 cells a significant peak will occur in any given cell in 210s. Of the cells that were suprathreshold (S5d Fig) there is a 47% chance of a second peak within ~50s (S5F Fig) after the initial peak. The mean event width decreased significantly in Kuhl-CKNa cells with (1e8 Kir2.1):(1e8 NavD) VG/ μ L and Cx-43 (S3 fig).

Optimizing Activity with Connexin-43

To further improve activity connexin-43 was optimized by varying the viral titer. The following concentrations were held constant for Kir2.1, NavD, bPAC, CNG, R-GECO (S2 Table). The Kuhl-CKNaCx cells were transduced and ~48 hours later the cells were stimulated with 20ms of blue light at 0s, 130s, and 240s. Images were collected with 561nm excitation.

The total cumulative peaks (S6A Fig) was greatest in the condition with a viral titer of (CNG 1e5) VG/ μ L. To understand which viral titer impacted the chance of a cell firing we

examined the cumulative number of Ca^{2+} transients divided by the total number of cells (Fig 8). Using the data from the total number of peaks per ROI (S6D Fig) we ran Dunnett's test (S1 Table) to examine if there was a statistical significance between the conditions compared to the control (Cx-43 0) $\text{VG}/\mu\text{l}$. The experimental trials (Cx 1e5) ($***p \leq .0003$) and (Cx 1e1) ($**p \leq .0062$) had a 100% chance that out of 150 cells they would fire at least twice in 400s. Further, there is a 36% chance that the cells that were suprathreshold (S6D Fig) are likely to produce another Ca^{2+} peak $\sim 112\text{s}$ after the initial fluorescence peak (S6F Fig). While (Cx 1e1) was significant we chose to use (Cx 1e5) because it is 20% more likely to produce a significant fluorescence peak.

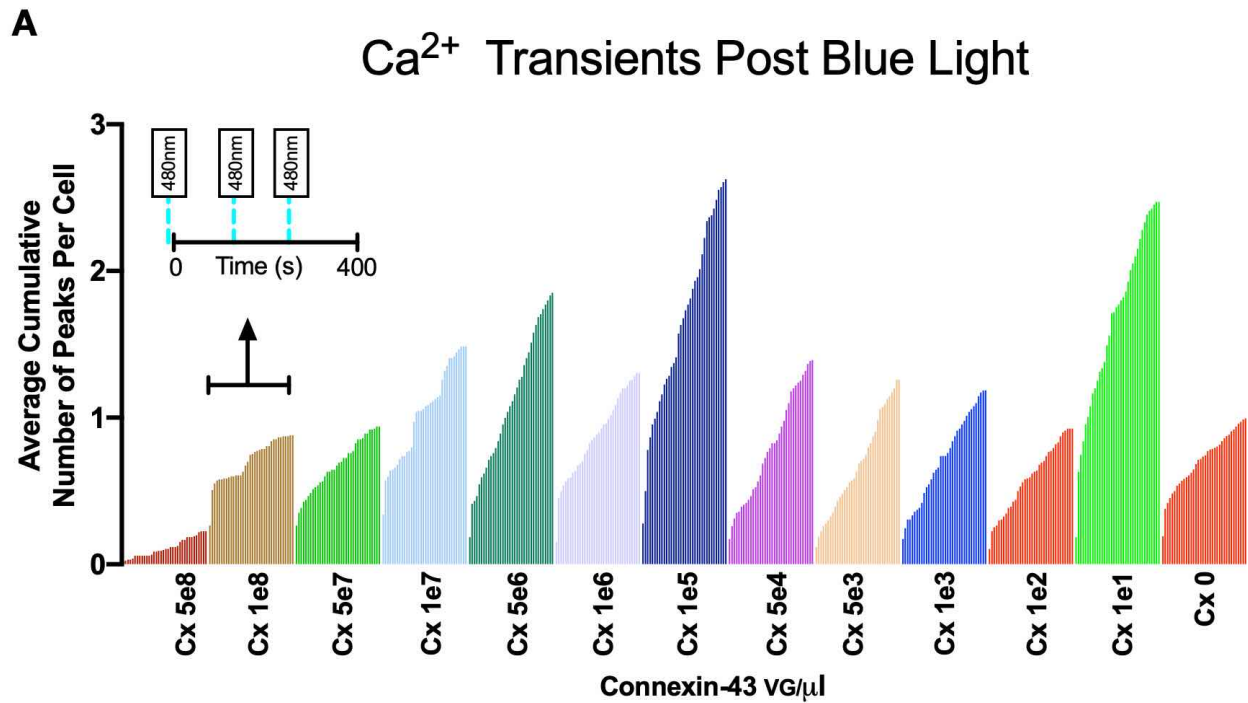


Fig 8. The gap junctions between cells using connexin-43 was optimized by varying levels of viral titer. The representative cartoon (Fig 7A) depicts all the components transduced into the experimental Kuhl-CKNaCx system (S2 Table). A) Shown are the average cumulative fluorescence peaks per cell across 400s for each indicated viral concentration of Cx-43 ($n = 150$ cells per condition).

Optimizing Activity with CNG

The addition of NavD and connexin-43 had such a profound effect on the activity that we decided to re-optimize the CNG and HCN2 channel. To optimize activity with CNG the viral titer was varied and the following concentrations were held constant for Kir2.1, NavD, bPAC, Cx-43, and R-GECO1 (S2 Table). The Kuhl-CKNaCx cells were transduced and the following day the cells were stimulated with 20ms of blue light at 130s and 240s. Images were collected with 561nm excitation before and following each blue light stimulus.

Upon activation of bPAC there were various responses in each condition that are fully quantified in (S7 Fig). The total cumulative peaks (S7A Fig) was greatest in the condition with a viral titer of (CNG 1e5) VG/ μ L. To understand which viral titer impacted the chance of a cell firing we examined the average cumulative number of Ca^{2+} transients divided by the total number of cells (Fig 9A). Using the data from the total number of peaks per ROI (S7A Fig) we used Dunnett's test (S1 Table) to examine if there was a statistical significance between the conditions. The experimental trials (CNG 1e2 to 1e5) VG/ μ L (**** $p \leq .0001$) were significantly greater than the control (CNG 0) VG/ μ L and had a 100% chance that out of 150 cells they would fire at least once in 360s. Further, in Kuhl-CKNaCx (CNG 1e3) there is a 40% chance that of the cells that fired (S7D Fig) are likely to fire again ~80s after the initial fluorescence peak (S7F Fig).

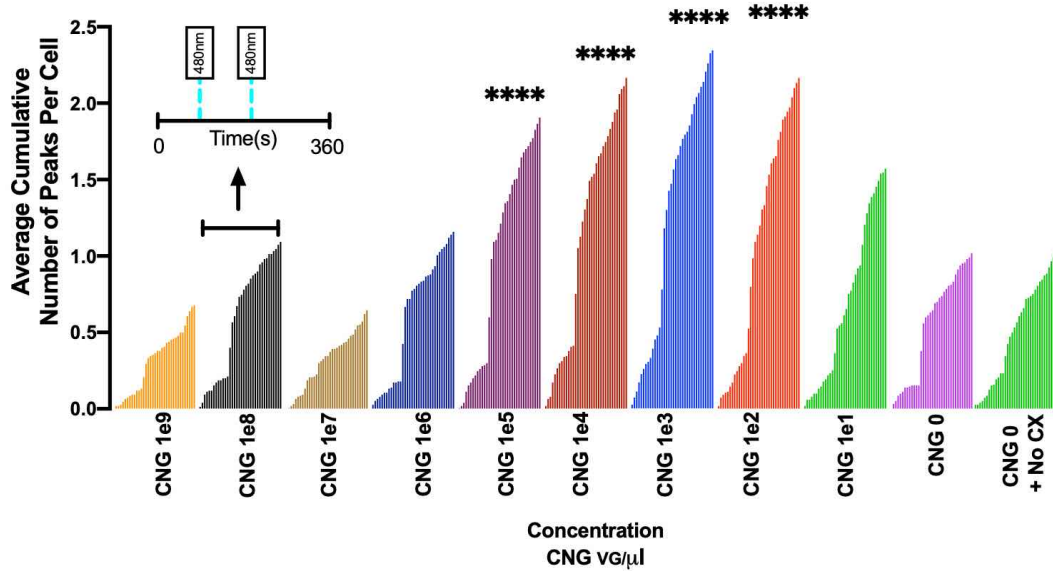
A**Ca²⁺ Transients Post Blue Light**

Fig 9. The amount of CNG channel was optimized. The representative cartoon (Fig 7A) depicts all the components transduced into the experimental Kuhl-KCNaCx system (S2 Table). A) Shown are the average cumulative fluorescence peaks per cell for each indicated viral concentration of CNG ($n = 150$ cells per condition). Differences were analyzed using Dunnett's multiple comparison test on the Ca²⁺ transient frequency (S7D Fig) compared to the control (CNG 0) VG/μl. The experimental trials that were statistically greater than the control were marked (**** $p \leq .0001$) on the graph.

Optimizing Activity with HCN2

The HCN2 ion channel can be exchanged for the modified CNG channel. To optimize activity the viral titer for HCN2 was varied and the following concentrations held constant for Kir2.1, NavD, bPAC, Cx-43 and R-GECO1 (S2 Table). The Kuhl-HKNaCx cells were transduced and ~48 hours following the cells were stimulated with 20ms of blue light at 120s and 200s. Images were collected before and following each blue light stimulus with 561nm excitation.

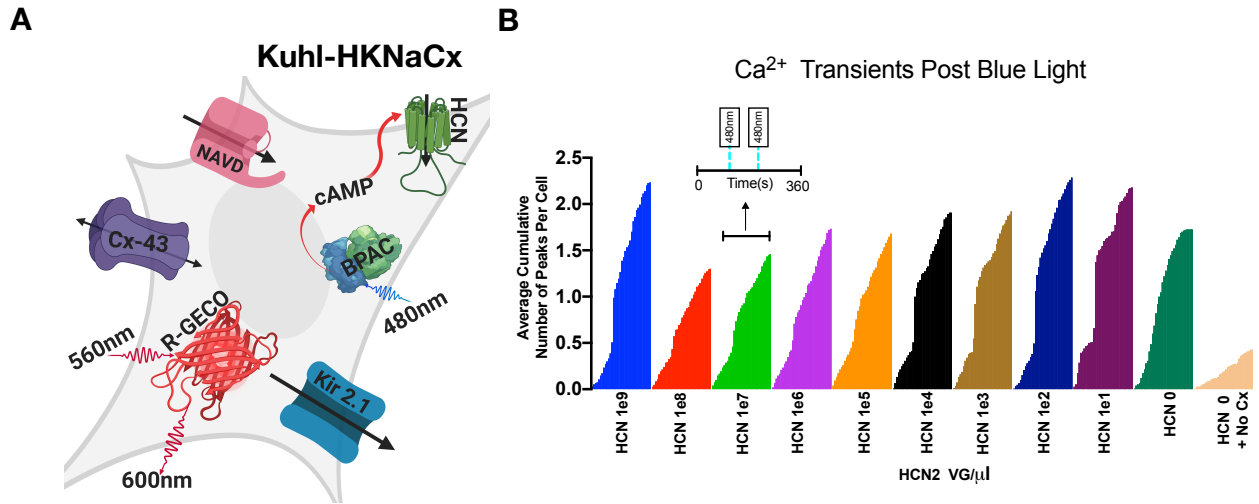


Fig 10. HCN2 can be exchanged for the modified CNG channel. A) Cartoon depicting all the components transduced into the experimental Kuhl-HKNaCx cell (S2 Table). B) Shown are the average cumulative fluorescence peaks per cell across 360s for each indicated viral concentration of HCN2 ($n = 150$ cells per condition).

Upon activation of bPAC there were somewhat varied responses in each condition that are fully quantified in (S8 Fig). The total cumulative peaks (S8A Fig) was greatest in the condition with a viral titer of (HCN2 1e2) VG/μL. To understand which viral titer impacted the chance of a cell firing we examined the average cumulative number of Ca²⁺ transients divided by the total number of cells (Fig 10B).

Using the data from the total number of peaks per ROI (S8 Fig) we used Dunnett's test (S1 Table) to examine if there was a statistical significance between the conditions. The experimental trials (HCN2 1e1, 1e2, and 1e9) VG/μL ($*p \leq .0001$) were significantly greater than the control (HCN2 0) VG/μl. Further in (HCN2 1e2) Kuhl-HKNaCx cells there is a 41%

chance that out of 150 cells a significant peak will occur and of the cells that were above threshold (S8D Fig) are likely to fire ~77s after the initial fluorescence peak (S8F Fig).

It appears that the optimal concentration is (HCN2 1e2) VG/ μ L but there appeared to be a range between (HCN2 1e1 to 1e2) VG/ μ L (Fig 10B). The controls still display some random activity even without the HCN2 channel due to having the Kir2.1, NavD, and Cx-43 channel expressed. However, unlike the CNG control data (Fig 9A) the activity seems to be dependent on the Cx-43 being present as well as the Kir2.1 and NavD channel.

The behavior of HCN2 was visually different from CNG. While both wells have random activity, there is a brief somewhat coordinated activity after a blue light stimulus (S7C and S8C Figs). S2 and S3 Movies show the movement of CNG and HCN2. CNG has a Ca^{2+} bursting rosette pattern following a stimulus, while HCN2 seems to have a spreading wave-like pattern.

A Kuhl-KCNaCx

The Kuhl-KCNaCx cells show remarkable, consistent activity when stimulated with blue light. One can image using them to screen prototype activity sensors, new GECI and GEVI biosensors. Such a screen, however, would depend critically on consistent well to well activity. To measure how consistent the activity is we measured the activity in 14 wells of cells transduced with an optimized amount of each channel. The representative cartoon (Fig 11A) depicts all the components transduced into the Kuhl-KCNaCx cell. The Kuhl-KCNaCx cells (S2 Table) were transduced and ~48 hours later the cells were imaged for 25 minutes and stimulated with 20ms of blue light at 500s and 1000s. Images were collected before and after each blue light stimulus with 561nm excitation for 4 wells over 25 minutes (Fig 11A) or 10 wells for 15 minutes (S4 Movie).

The well to well average cumulative peak activity (Fig 11A) has a mean of 2.4 .4. Taking the mean number of total peaks per trial by the mean total number of peaks that in Kuhl-CKNaCx cells 100% of the 600 cells will produce a significant peak and of the cells that were above threshold (S9F Fig) 50.8% are likely to produce a significant peak ~94s 32 following.

Another 10 wells (Fig 11B) were imaged for 15 minutes and stimulated with 20ms of blue light at 198s and 400s. There appears to be increased coordinated activity between the cells following blue light stimulus (S10C Fig). The cells have an oscillating firing pattern (S10C Fig) that occurs and is consistent with the mean inter-event interval (S10F Fig) for each well. The well to well activity when measuring the average cumulative number of peaks per cell is fairly consistent with a mean of 1.8.4. There is a 100% chance that out of 1,500 cells a significant peak will occur 1,500 times when imaged for 15 minutes and of the cells that were above threshold (S10F Fig) 29% are likely to produce a significant peak ~148s89 following the initial peak. Tukey's multiple comparison test found that there wasn't a significant difference between the average cumulative number of peaks per cell for the 14 trials with Kuhl-CKNaCx.

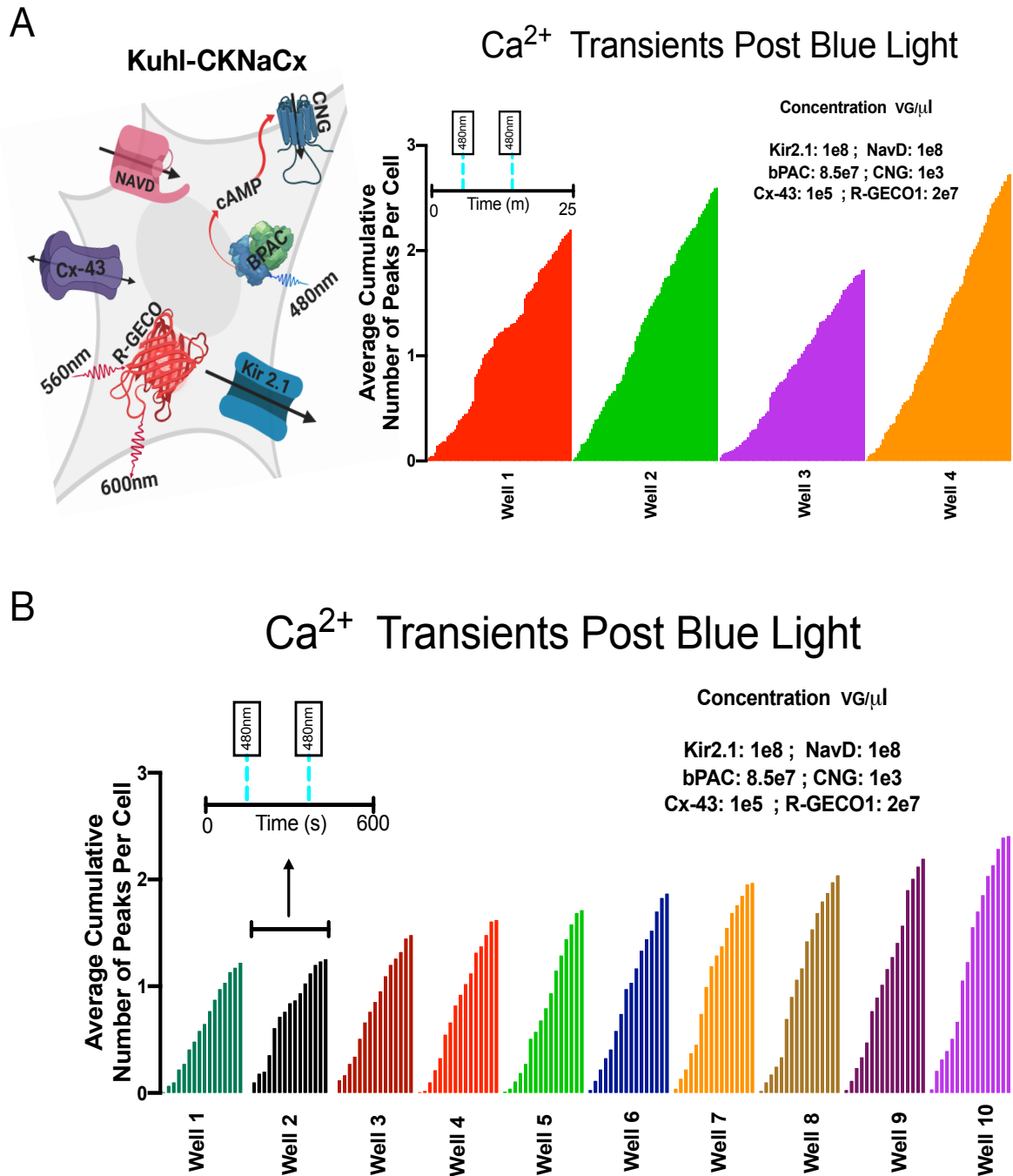


Fig 11. A Kuhl-CKNaCx - Consistent activity with stable viral concentration. Well to well average cumulative peak activity is fairly consistent. A) The representative cartoon depicts all the components transduced into the Kuhl-CKNaCx cell (S2 Table). The average cumulative number of peaks per cell over four trials in 25m. B) The average cumulative number of peaks per

cell over 10 trials in 15m. Tukey's multiple comparison test was run on the Kuhl-CKNaCx trials and found no significant difference between the wells.

Comparing Green and Red Sensors in the Same Cell

While slow and laborious, an advantage of patch clamp fluorometry is that whole-cell voltage changes can be directly compared with fluorescence changes. The all-optical Kuhl cell approach does not have the advantage of such an independent measurement. However, we reasoned that different colored sensors could be simultaneously measured to arrive at a comparative measure. For example, a green sensor of known properties could be compared to a new one with red fluorescence. To test the feasibility of this approach, we used an optical splitter to compare the responses of R-GECO1 and G-GECO1 within the same cell.

The Kuhl-KCNaCx cells were transduced and 48 hours following the cells were imaged. In figure 12 an image created by the OptoSplit II is shown where the green and red emission from both sensors are split and imaged on two different portions of the EMCCD camera(S5 Movie). The fluorescence response from both sensors is plotted in figure 12 (Fig 12B). Previous photophysical characterization of G-GECO1 and R-GECO1 show that there are relatively large differences between the two sensors. G-GECO1 has a faster K_{on} and R-GECO1 has a faster K_{off} [8]. G-GECO1 has a greater K_d , kinetic with a difference of 347(nm)¹. G-GECO1 also is ~150x brighter than R-GECO1 [8]. This simple analysis shows that differences can be easily measured in Figure 12 (Fig 12B) where the $\tau_{on/off}$ of both signals was measured. R-GECO1 τ_{on} was consistently slower than G-GECO1 and R-GECO1 τ_{off} was consistently faster than G-GECO1 τ_{off} .

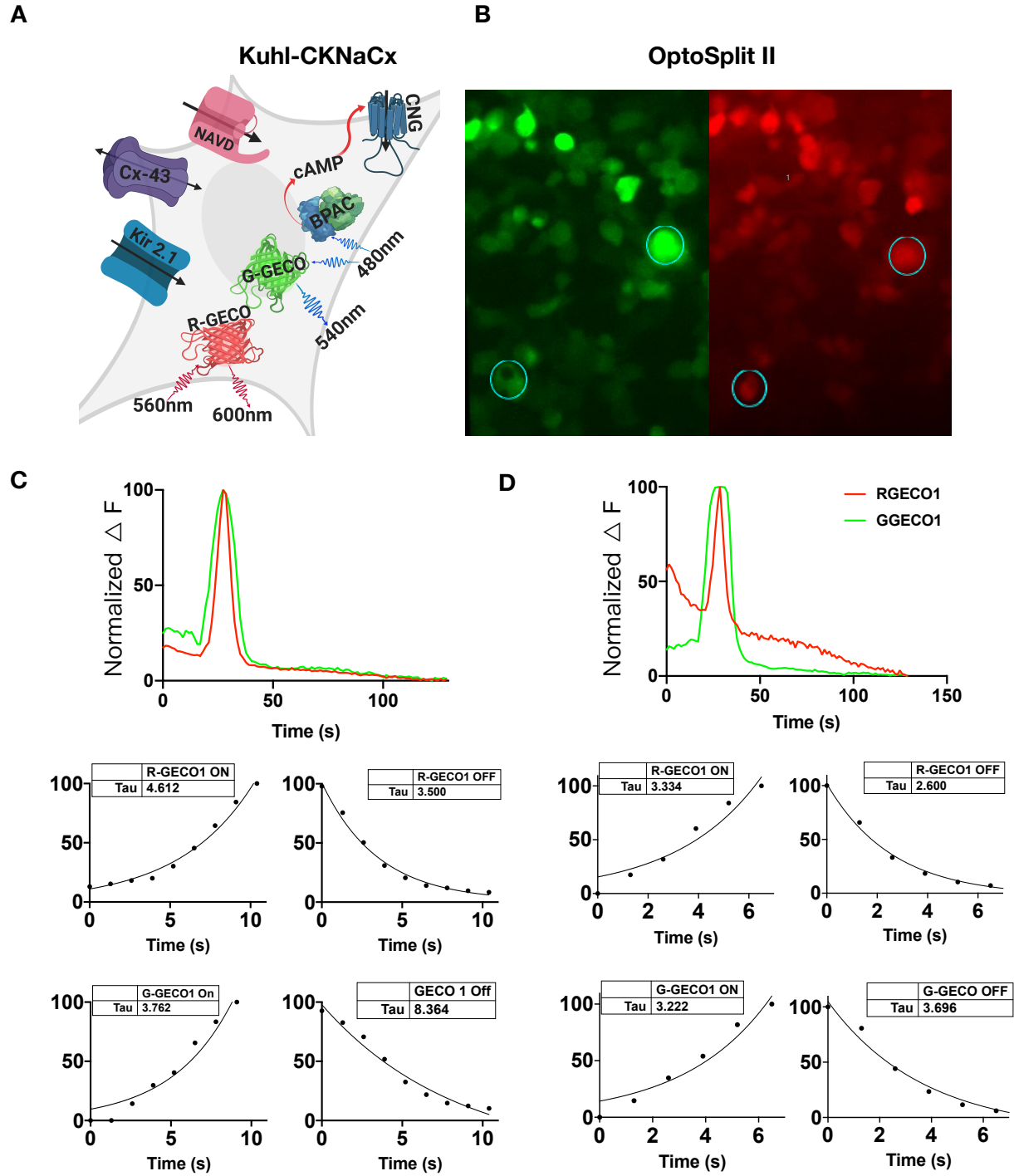


Fig 12. Both red and green Ca^{2+} sensors can be recorded from the same cell and imaged simultaneously using an OptoSplit II. A) Cartoon depicting all the components transduced into

the Kuhl-CKNaCx cell. bPAC was transfected at a low level so that the cells could be imaged with green light continuously. B) The cells were simultaneously imaged with green and red wavelengths for 150s. The image is of the same cells in two different wavelengths and ROIs from both the G-GECO1 and R-GECO1 were obtained (circles and numbers). Representative traces from the ROIs show that both G-GECO1 and R-GECO1 fluorescence are visible and their respective responses can be compared and plotted over time. The difference between G-GECO1 and R-GECO1 can be seen because it has a greater intensity change (150x) in fluorescence to Ca^{2+} concentrations and has a ~2 fold increase in the speed of its kinetics (K_d).

Green Voltage Sensors Can be Screened in Kuhl-CKNaCx Cells

ArcLight is a downward going voltage sensor, meaning that it gets brighter upon the hyperpolarization of the membrane and dimmer at depolarization. It is pH sensitive and has been demonstrated to work in single-cell fly neurons but requires high-intensity illumination to achieve an adequate signal-to-noise ratio [39].

The following concentrations were held constant for Kir2.1, NavD, bPAC, Cx-43, CNG, ArcLight (S2 Table). The Kuhl-CKNaCx cells were transduced and ~48 hours following the cells were imaged with continuous 480nm excitation. Slow depolarizations can be seen in the Kuhl-CKNaCx cell system (Fig 13) with a reasonable SNR of 5.5 4 averaged from 15 representative traces. We believe that the Kuhl-CKNaCx system shows promise for screening voltage sensor prototypes(S6-S8 Movie).

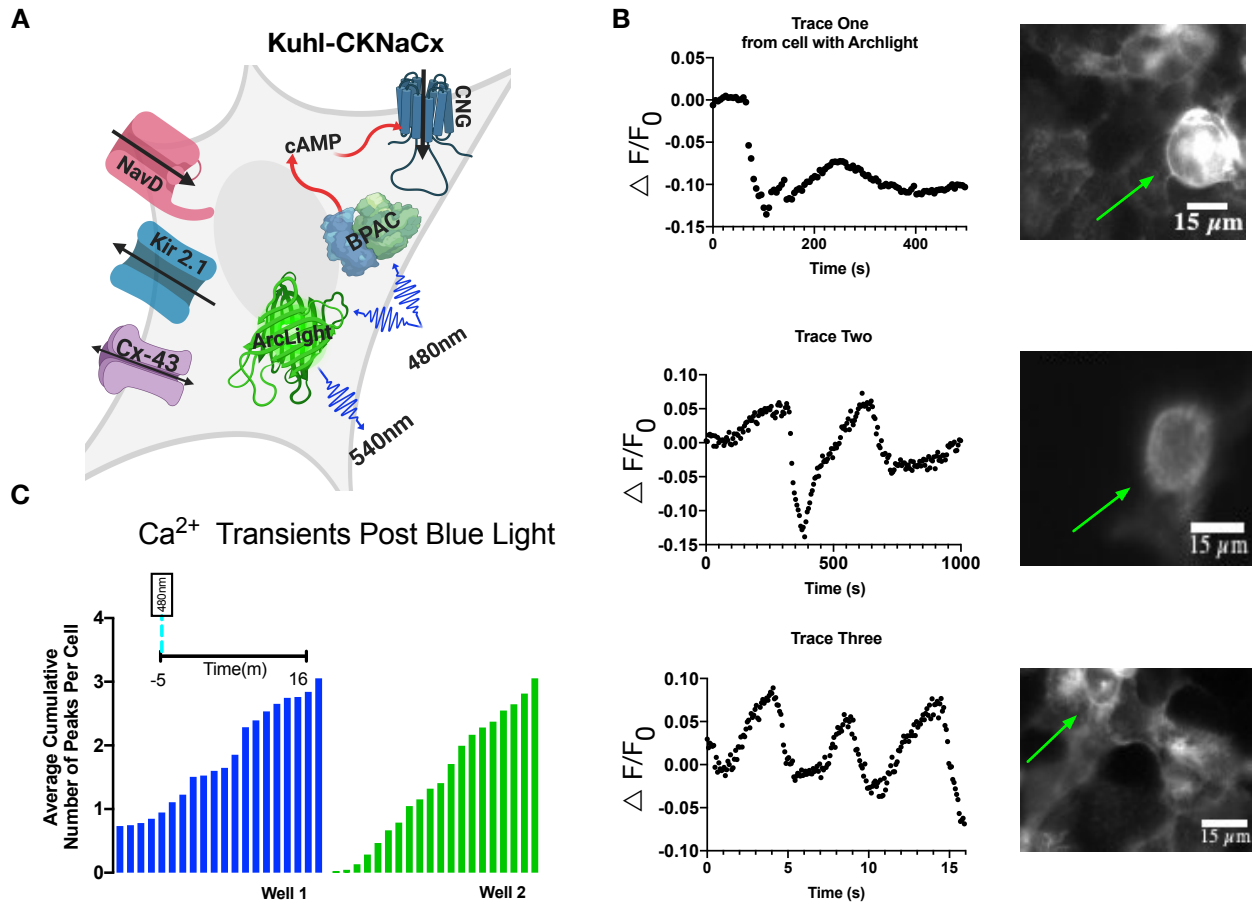


Fig 13. The Kuhl-CKNaCx system undergoes voltage changes that are visible with ArcLight. A) Cartoon depicting all the components transduced into the Kuhl-CKNaCx cell. bPAC was stimulated continuously while exciting ArcLight. B) Blue light activation of bPAC caused a slow depolarization that occurred over a few seconds. Representative traces from the ROIs show that voltage changes are visible using ArcLight. Images of ArcLight fluorescence response over time. The green arrows annotate which cells were used for the fluorescence traces. C) Two separate wells that were imaged are shown.

Discussion

This Kuhl cell system was designed to be customizable and there are several important considerations to take into account when customizing for a particular activity sensor. Multiple channels could be exchanged for the ones used in this study. For example, a faster voltage-gated sodium channel would most likely create a faster action potential in the cells.

The Kuhl cells can potentially be used in high throughput screens for better activity biosensors. The throughput of any screen, however, depends on the time budget. Here we arbitrarily picked fluorescence peaks that exceeded the SNR of 26. That is, the peak was 26 times larger than the standard deviation of the baseline noise. Using this threshold, we found that out of 150 cells, imaging for 80s30 was sufficient to detect 100 cells that would produce a Ca^{2+} peak above threshold. Recording the activity in a well for 80s would eliminate false negatives. The imaging time, plus the plate translation time, should result in a time budget of roughly 100 minutes per 96 well plate. While 400 to 500 wells a day is low throughput, it is considerably better than the manual patch clamp fluorometry used today.

We have shown that screening both red and green GECIs simultaneously in the Kuhl-CKNaCx system is possible, and that one sensor can be used to benchmark the other. Individual cells' dual GECI response can be measured for its brightness, kinetics (Tau), SNR, and sensitivity. In theory, if a lower objective lens was used, an average of the field of view for the mutant sensor's fluorescence response could be easily compared to the average response of the benchmark sensor in that same well.

The improvement of the genetically encoded voltage indicators - GEVIs - has been slow due to limitations in the throughput involved in manual, whole-cell patch clamp fluorometry [12,40]. The system described here will enable investigators to screen without field stimulation or any manual intervention. Voltage changes along the membrane of the HEK293 cells are visible and can be tracked in the Kuhl-CKNaCx cells using the GEVI ArcLight, since the action potential in these cells is quite slow, lasting seconds ~ 4 s. We believe that because we were able to screen and benchmark Ca^{2+} indicators simultaneously that this should also work for voltage

sensors. The prototype sensors could be expressed in Kuhl cells that also express a benchmark sensor such as GCAMP7f or Voltron [2,41].

Methods

Growth conditions for HEK293 cells

A line of HEK293 were cultured in DMEM, 10% FBS, and 1% Penicillin (100 U/mL). For experiments, HE293 cells were plated in 96 well plastic plates at 100ml per well ($\sim 3\text{-}5 \times 10^4$ cells/mL).

Plasmids

The HCN2 plasmid was kindly donated by Joan Lemire at Tufts University from Dr. Michael Levin's lab. The NavRosDg217a was reverse translated using a human codon preference and synthesized as a gBblock by IDT (Coralville, Iowa). The cyclic nucleotide-gated olfactory channel (from *Rattus norvegicus*) has the following mutations, 61–90/C460W/E583M and was synthesized as a gBblock by IDT. [42]. pGEMTEZ-Kir2.1 was a gift from Richard Axel, Joseph Gogos & Ron Yu (Addgene plasmid #32641)

Baculovirus

The channels Kir2.1, CNG, HCN2 and NavRosDg217a were packaged in BacMam (Montana Molecular, Bozeman, MT). The virus titers were: Kir2.1 3.6×10^{10} VG/mL, bPAC 8.59×10^{10} VG/mL, CNG 3.55×10^{10} VG/mL, and NavRosDg217a 7.26×10^{10} VG/mL. The following bio-sensors in baculovirus were obtained from Montana Molecular: R-cADDIs (titer 5.06×10^{10} VG/mL); R-GECO (2×10^{10} VG/mL); G-GECO (9.17×10^{10} VG/mL); and Arclight

(2×10^{10} VG/mL). Supplemental figure 12 depicts each experimental condition and the final viral concentrations used.

Transduction

HEK-293 cells were prepared and transduced following the manufacturer's recommended protocol. Briefly, HEK293 cells were plated in 96 well plastic plates (48×10^3 cells/well) and transduced with the appropriate mix of viruses and the HDAC inhibitor sodium butyrate (2mM final concentration). Two days later, the media was exchanged with PBS, tyrodes buffer, or Fluorobrite. The best results were consistently obtained with PBS.

An ASI modular microscope

The experiments (Figs 1-11 and 13) were conducted using wide field imaging on an ASI-XYZ stage fitted with a modular infinity microscope. The objective imaged onto the detector chip of a Hamamatsu ORCA-Flash 4.0 scientific-CMOS camera. The images were collected using either MATLAB scripts that controlled the camera, ASI stage, SH1-Thorlabs shutter, and ThorLabs DC4100 Four Channel-LED Driver, or by using μ Manager [43]. A custom illumination system was arranged. Briefly, a dichroic mirror was positioned at the entrance of the microscope to combine blue light LED illumination with 561 laser illumination. An SH1-Thorlabs shutter was used to create brief illumination from a blue ThorLABS LED (488nm) for rapid stimulation of bPAC. At 100% the blue light illumination was 70 mW/cm^2 . The yellow illumination was provided with a Sapphire laser (561nm, 50mW, Coherent). The laser beam was steered with two mirrors (arranged in a periscope) into an entrance aperture of the ASI microscope. Before entering the microscope, the laser beam passed through a 50° diffuser (ED1

C50 MD, Thorlabs) placed in the focus of an $f = 20$ mm aspheric lens (ACL2520U, Thorlabs) that collimated the beam for further traveling into the microscope.

Optosplitter

To simultaneously collect images of both G-GECO and R-GECO, we used an optical splitter (CAIRN optosplit II, Faversham Kent ME13 8UP). This makes it possible to simultaneously collect both the green and red images on a single camera. An Olympus IX81 was fitted with LED illumination (470 nm, 565 nm, ThorLabs) where the power to the LEDs can be independently adjusted. A Pinkel filter set was used to simultaneously image both G-GECO and R-GECO (GFP/DsRed-2X-A-000, Semrock, Rochester, New York). The CAIRN splitter was positioned in between the microscope and EMCCD camera such that a dichroic filter (561 nm, Semrock, Rochester, New York) split the green and red emission into two optical paths. The red emission passed through an additional band pass filter (617/70, Semrock, Rochester, New York) to eliminate any potential green emission from the G-GECO.

Analysis

Image data was stored in a Z-stack tiff file and loaded into the FIJI distribution of the ImageJ software [44]. The initial frame of the stack was eliminated from all analysis to correct for photoactivation of R-GECO1 upon bluelight stimulation of bPAC [45]. The cells were selected using a freehand ROI surrounding the cell of interest. The average total intensity pixel value within the ROI for each frame was collected using the time series analyzer plugin and saved as a .csv file. The raw fluorescence trace data was loaded into MATLAB.

The analysis was done in MATLAB using the Find Peaks function in the Signal Processing Toolbox. The raw fluorescence traces were rescaled from zero to 10,000. The data was smoothed using 'sgolay' function. The main peak prominence threshold was set to 50.5 with an SNR of 2610. The Signal-to-noise ratio was defined as the average of the peaks amplitudes that were defined using the peak finder threshold by the standard deviation (S.D.) of the signal before blue light stimulus. We examined the SNR for Kuhl-CKNaCx cells over 5 individual trials which were averaged over 15 trials. The FindPeaks function returns a vector with the local maxima (peaks) from the ROI trace data. The Peak Finder in MATLAB marks each fluorescence peak by its location in time and records the number of peaks per ROI. In addition, it returns the widths of the peak and the prominence of the peak. The time that a fluorescence peak occurred during imaging was recorded. The inter-event interval is the absolute difference between each consecutive peak. The FWHM and inter-event interval time was adjusted for exposure times. The total cumulative number of peaks for an entire trial was measured. Each fluorescence peak was measured from zero to the maximum peak intensity (normalized F) using the MATLAB Peak Finder. The time and total quantity of a fluorescence peak in a ROI were recorded. To determine the chance that a cell will fire twice was determined by (total number of peaks for a well/total number of points collected from Inter-Peak interval)

Acknowledgements

The authors thank the following people: Paul Tewson for HEK293 cells. Elsa Roush for multiple 96 well plates of transduced HEK293 cells. Scott Martinka for excellent high titer virus. Anne Marie. Rosana Molina. Steven Hoffman, Dr. Jamie Mazer, Dr. Kaspar Podgorski, and Nadav Oakes for MATLAB assistance. Joan Lemire at Tufts University for HCN2 plasmid. Mikhail Drobizhev was a constant source of advice and optical expertise. The reviewers for their invaluable suggestions.

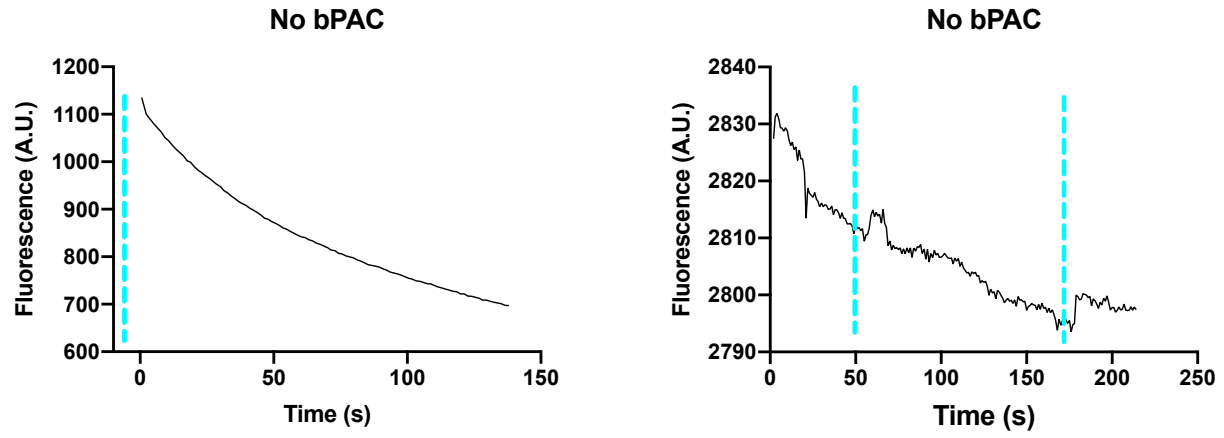
REFERENCES CITED

1. Stierl M, Stumpf P, Udvari D, Gueta R, Hagedorn R, Losi A, et al. Light modulation of cellular cAMP by a small bacterial photoactivated adenylyl cyclase, bPAC, of the soil bacterium *Beggiatoa*. *J Biol Chem*. 2011;286: 1181–1188.
2. Kerruth S, Coates C, Dürst CD, Oertner TG, Török K. The kinetic mechanisms of fast-decay red-fluorescent genetically encoded calcium indicators. *J Biol Chem*. 2019;294: 3934–3946.
3. Huang L, Knoblich U, Ledochowitsch P, Lecoq J, Clay Reid R, de Vries SEJ, et al. Relationship between spiking activity and simultaneously recorded fluorescence signals in transgenic mice expressing GCaMP6. *bioRxiv*. 2019. p. 788802. doi:10.1101/788802
4. Agetsuma M, Matsuda T, Nagai T. Methods for monitoring signaling molecules in cellular compartments. *Cell Calcium*. 2017;64: 12–19.
5. Bruton J, Cheng AJ, Westerblad H. Measuring Ca²⁺ in Living Cells. *Adv Exp Med Biol*. 2020;1131: 7–26.
6. Kim D, Svoboda K, Looger L, Schreier E. Genetically encoded calcium indicators and methods of use. US Patent. 20190153067:A1, 2019. Available: <https://patentimages.storage.googleapis.com/48/48/53/5bb27ffa25d100/US20190153067A1.pdf>
7. Podgorski K, Ranganathan G. Brain heating induced by near-infrared lasers during multiphoton microscopy. *J Neurophysiol*. 2016;116: 1012–1023.
8. Zhao Y, Araki S, Wu J, Teramoto T, Chang Y-F, Nakano M, et al. An expanded palette of genetically encoded Ca²⁺ indicators. *Science*. 2011;333: 1888–1891.
9. Qian Y, Piatkevich KD, Mc Larney B, Abdelfattah AS, Mehta S, Murdock MH, et al. A genetically encoded near-infrared fluorescent calcium ion indicator. *Nat Methods*. 2019;16: 171–174.
10. Dana H, Sun Y, Mohar B, Hulse BK, Kerlin AM, Hasseman JP, et al. High-performance calcium sensors for imaging activity in neuronal populations and microcompartments. *Nat Methods*. 2019;16: 649–657.
11. Ibraheem A, Yap H, Ding Y, Campbell RE. A bacteria colony-based screen for optimal linker combinations in genetically encoded biosensors. *BMC Biotechnol*. 2011;11: 105.
12. Park J, Werley CA, Venkatachalam V, Kralj JM, Dib-Hajj SD, Waxman SG, et al. Screening fluorescent voltage indicators with spontaneously spiking HEK cells. *PLoS One*. 2013;8: e85221.

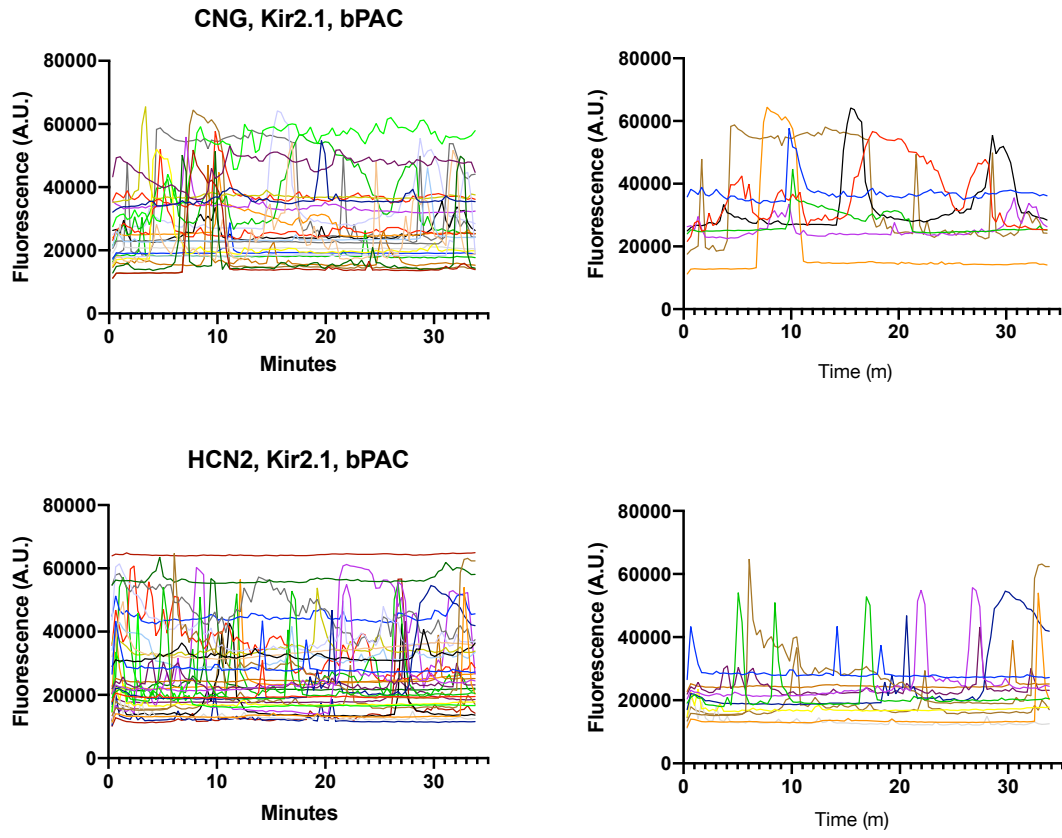
13. Ding Y, Li J, Enterina JR, Shen Y, Zhang I, Tewson PH, et al. Ratiometric biosensors based on dimerization-dependent fluorescent protein exchange. *Nat Methods*. 2015;12: 195–198.
14. Alford SC, Abdelfattah AS, Ding Y, Campbell RE. A fluorogenic red fluorescent protein heterodimer. *Chem Biol*. 2012;19: 353–360.
15. Zhao Y, Abdelfattah AS, Zhao Y, Ruangkittisakul A, Ballanyi K, Campbell RE, et al. Microfluidic cell sorter-aided directed evolution of a protein-based calcium ion indicator with an inverted fluorescent response. *Integr Biol*. 2014;6: 714–725.
16. Shen Y, Dana H, Abdelfattah AS, Patel R, Shea J, Molina RS, et al. A genetically encoded Ca²⁺ indicator based on circularly permuted sea anemone red fluorescent protein eqFP578. *BMC Biol*. 2018;16: 9.
17. Hsu H, Huang E, Yang XC, Karschin A, Labarca C, Figl A, et al. Slow and incomplete inactivations of voltage-gated channels dominate encoding in synthetic neurons. *Biophys J*. 1993;65: 1196–1206.
18. Kirkton RD, Bursac N. Engineering biosynthetic excitable tissues from unexcitable cells for electrophysiological and cell therapy studies. *Nat Commun*. 2011;2: 300.
19. Zhang H, Reichert E, Cohen AE. Optical electrophysiology for probing function and pharmacology of voltage-gated ion channels. *Elife*. 2016;5. doi:10.7554/eLife.15202
20. Shah BS, Rush AM, Liu S, Tyrrell L, Black JA, Dib-Hajj SD, et al. Contactin associates with sodium channel Nav1.3 in native tissues and increases channel density at the cell surface. *J Neurosci*. 2004;24: 7387–7399.
21. Zhang H, Cohen AE. Optogenetic Approaches to Drug Discovery in Neuroscience and Beyond. *Trends Biotechnol*. 2017;35: 625–639.
22. Khan KH. Gene expression in Mammalian cells and its applications. *Adv Pharm Bull*. 2013;3: 257–263.
23. Rodin SN, Riggs AD. Epigenetic silencing may aid evolution by gene duplication. *J Mol Evol*. 2003;56: 718–729.
24. Kost TA, Condreay JP, Ames RS, Rees S, Romanos MA. Implementation of BacMam virus gene delivery technology in a drug discovery setting. *Drug Discov Today*. 2007;12: 396–403.
25. Bernal Sierra YA, Rost BR, Pofahl M, Fernandes AM, Kopton RA, Moser S, et al. Potassium channel-based optogenetic silencing. *Nat Commun*. 2018;9: 4611.

26. Tewson PH, Martinka S, Shaner NC, Hughes TE, Quinn AM. New DAG and cAMP Sensors Optimized for Live-Cell Assays in Automated Laboratories. *J Biomol Screen*. 2016;21: 298–305.
27. Hirano M, Takebe M, Ishido T, Ide T, Matsunaga S. The C-terminal region affects the activity of photoactivated adenylyl cyclase from *Oscillatoria acuminata*. *Sci Rep*. 2019;9: 20262.
28. Fesenko EE, Kolesnikov SS, Lyubarsky AL. Induction by cyclic GMP of cationic conductance in plasma membrane of retinal rod outer segment. *Nature*. 1985;313: 310–313.
29. Rich TC, Fagan KA, Nakata H, Schaack J, Cooper DMF, Karpen JW. Cyclic Nucleotide-Gated Channels Colocalize with Adenylyl Cyclase in Regions of Restricted Camp Diffusion. *J Gen Physiol*. 2000;116: 147–162.
30. Chen K, Zuo D, Wang S-Y, Chen H. Kir2 inward rectification-controlled precise and dynamic balances between Kir2 and HCN currents initiate pacemaking activity. *FASEB J*. 2018;32: 3047–3057.
31. Capel RA, Collins TP, Bose SJ, Rajasundaram S, Ayagama T, Zaccolo M, et al. cAMP signalling is required for the actions of IP3 on Ca²⁺-transients in cardiac atria and beating rate in sino-atrial node. *bioRxiv*. 2019. p. 694349. doi:10.1101/694349
32. Nguyen HX, Kirkton RD, Bursac N. Engineering prokaryotic channels for control of mammalian tissue excitability. *Nat Commun*. 2016;7: 13132.
33. Hille B. *Ionic Channels of Excitable Membranes*. Sinauer; 2001.
34. Yu CR, Power J, Barnea G, O'Donnell S, Brown HEV, Osborne J, et al. Spontaneous neural activity is required for the establishment and maintenance of the olfactory sensory map. *Neuron*. 2004;42: 553–566.
35. Woehler A, Wlodarczyk J, Neher E. Signal/noise analysis of FRET-based sensors. *Biophys J*. 2010;99: 2344–2354.
36. Laird DW. Life cycle of connexins in health and disease. *Biochem J*. 2006;394: 527–543.
37. Butterweck A, Gergs U, Elfgang C, Willecke K, Traub O. Immunochemical characterization of the gap junction protein connexin45 in mouse kidney and transfected human HeLa cells. *J Membr Biol*. 1994;141: 247–256.
38. Fahrenbach JP, Mejia-Alvarez R, Banach K. The relevance of non-excitable cells for cardiac pacemaker function. *J Physiol*. 2007;585: 565–578.
39. Cao G, Platasa J, Pieribone VA, Raccuglia D, Kunst M, Nitabach MN. Genetically Targeted Optical Electrophysiology in Intact Neural Circuits. *Cell*. 2013;154: 904–913.

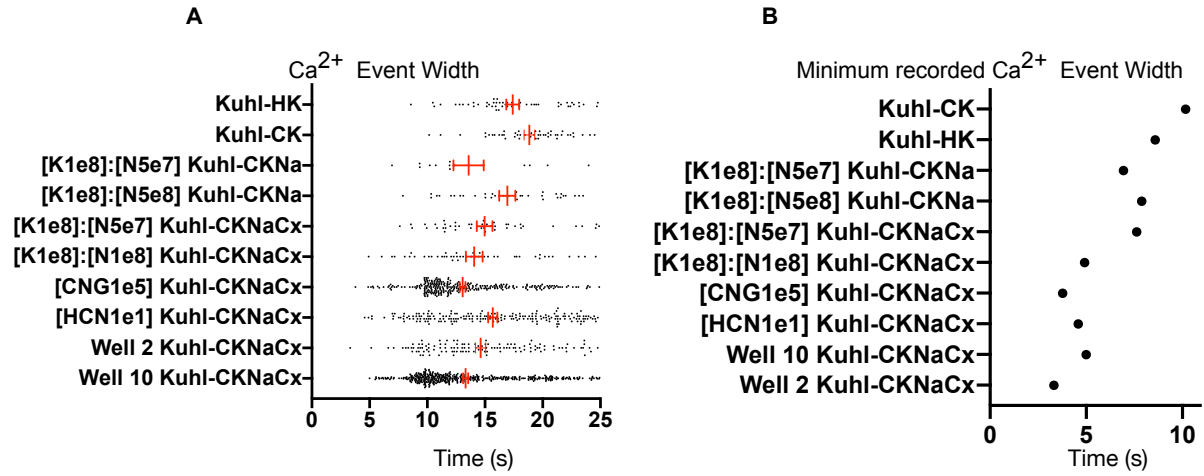
40. Storace D, Sepehri Rad M, Kang B, Cohen LB, Hughes T, Baker BJ. Toward Better Genetically Encoded Sensors of Membrane Potential. *Trends Neurosci.* 2016;39: 277–289.
41. Abdelfattah AS, Kawashima T, Singh A, Novak O, Liu H, Shuai Y, et al. Bright and photostable chemigenetic indicators for extended in vivo voltage imaging. *Science.* 2019;365: 699–704.
42. Rich TC, Tse TE, Rohan JG, Schaack J, Karpen JW. In Vivo Assessment of Local Phosphodiesterase Activity Using Tailored Cyclic Nucleotide–Gated Channels as Camp Sensors. *J Gen Physiol.* 2001;118: 63–78.
43. Edelstein AD, Tsuchida MA, Amodaj N, Pinkard H, Vale RD, Stuurman N. Advanced methods of microscope control using µManager software. *J Biol Methods.* 2014;1. doi:10.14440/jbm.2014.36
44. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods.* 2012;9: 676–682.
45. Wu J, Liu L, Matsuda T, Zhao Y, Rebane A, Drobizhev M, et al. Improved orange and red Ca^{2±} indicators and photophysical considerations for optogenetic applications. *ACS Chem Neurosci.* 2013;4: 963–972.

Supplemental Material

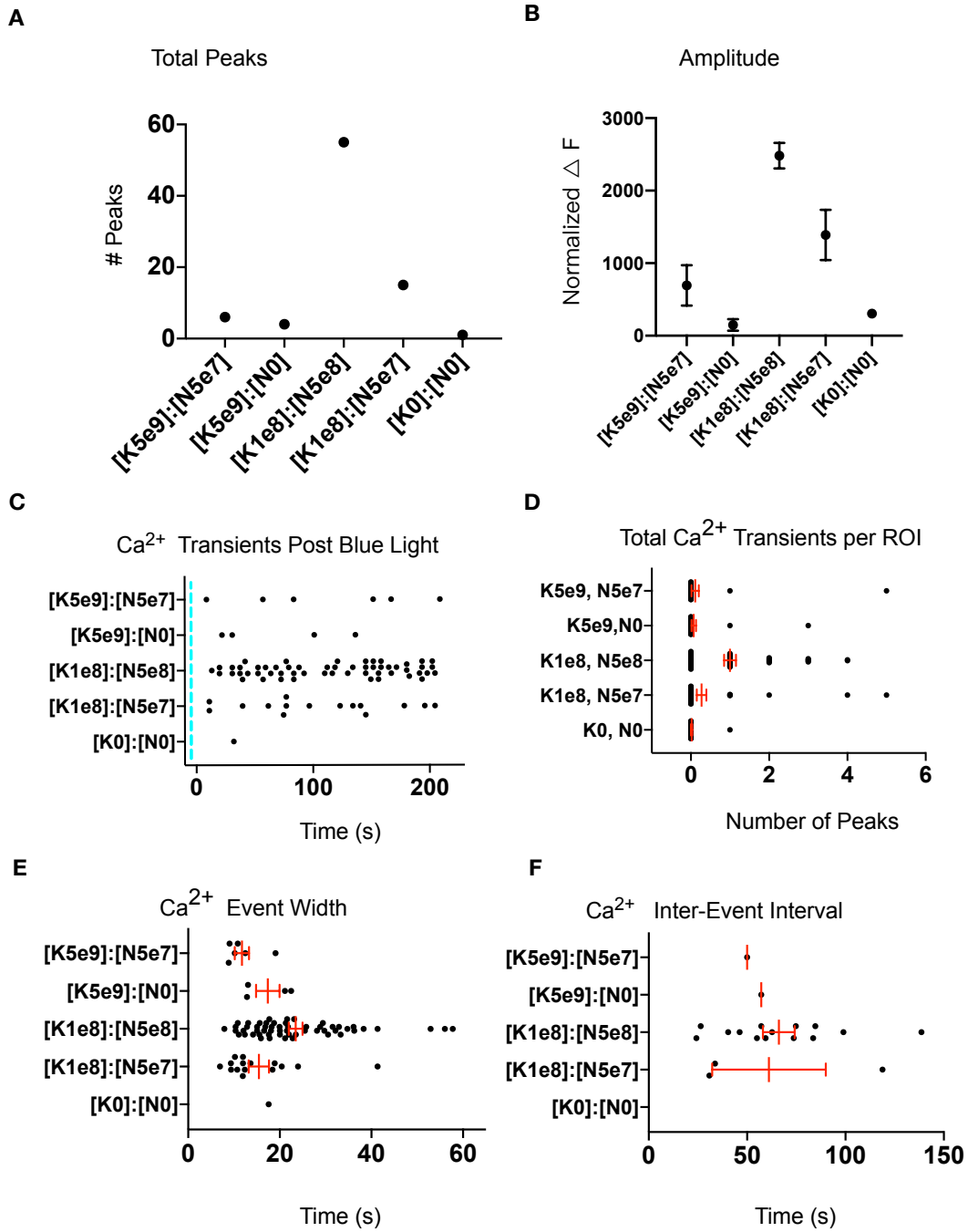
S1 Fig. Fluorescence trace of cells with Kir2.1, CNG, R-GECO1 (Kuhl-CK). Without bPAC blue light stimulus is ineffective at creating a response within cells.



S2 Fig. Representative traces. Raw fluorescence traces from Kuhl-CK AND Kuhl-HK. 20s of blue light stimulus leads to varied fluctuating responses in each cell.

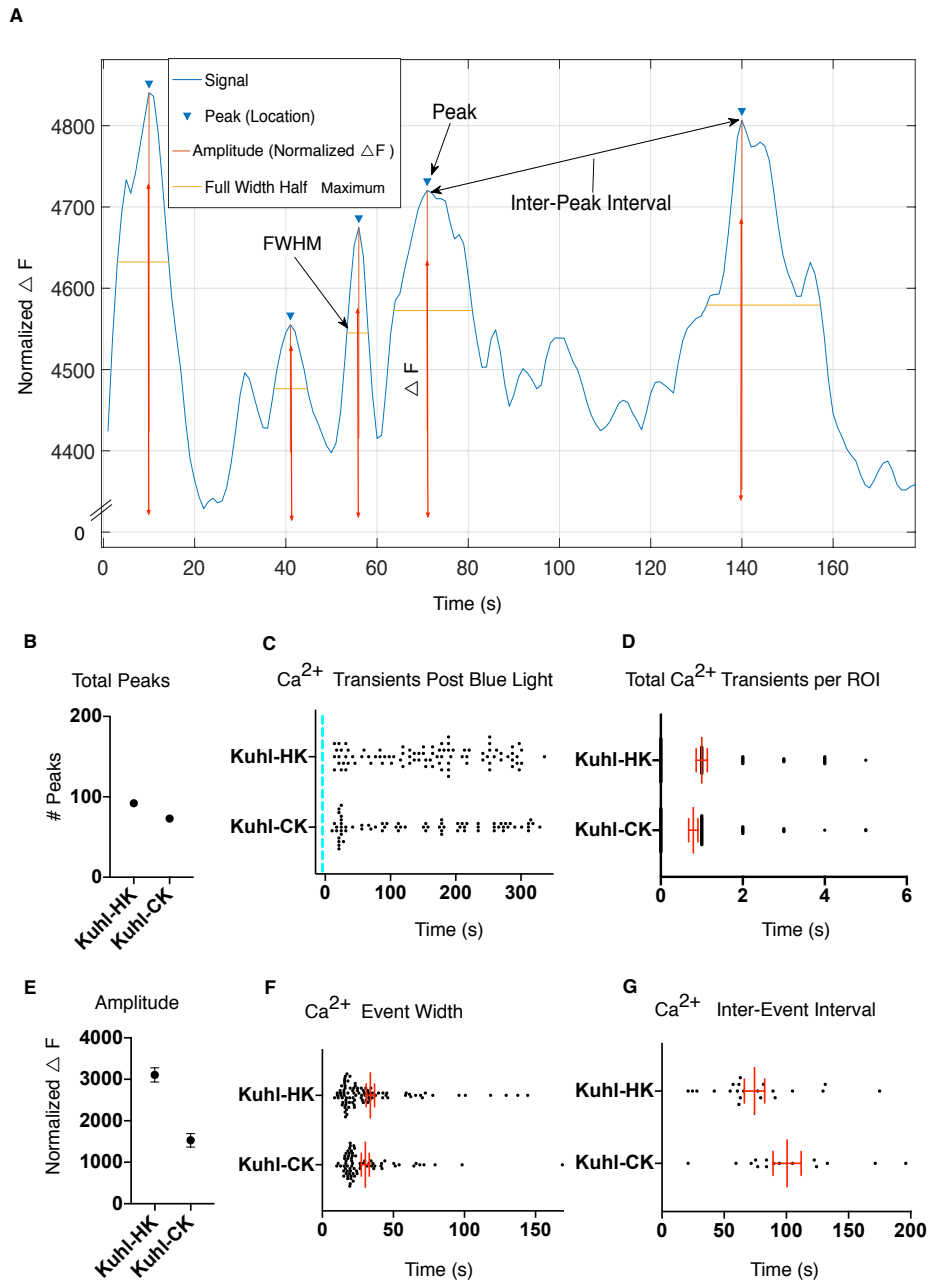


S3 Fig. Introduction of NavD to Kuhl and Cx-43 increases the speed of the Ca²⁺ transients. A) The most significant wavelengths from each optimized set was included in the data to show the increase in Ca²⁺ transient speed.

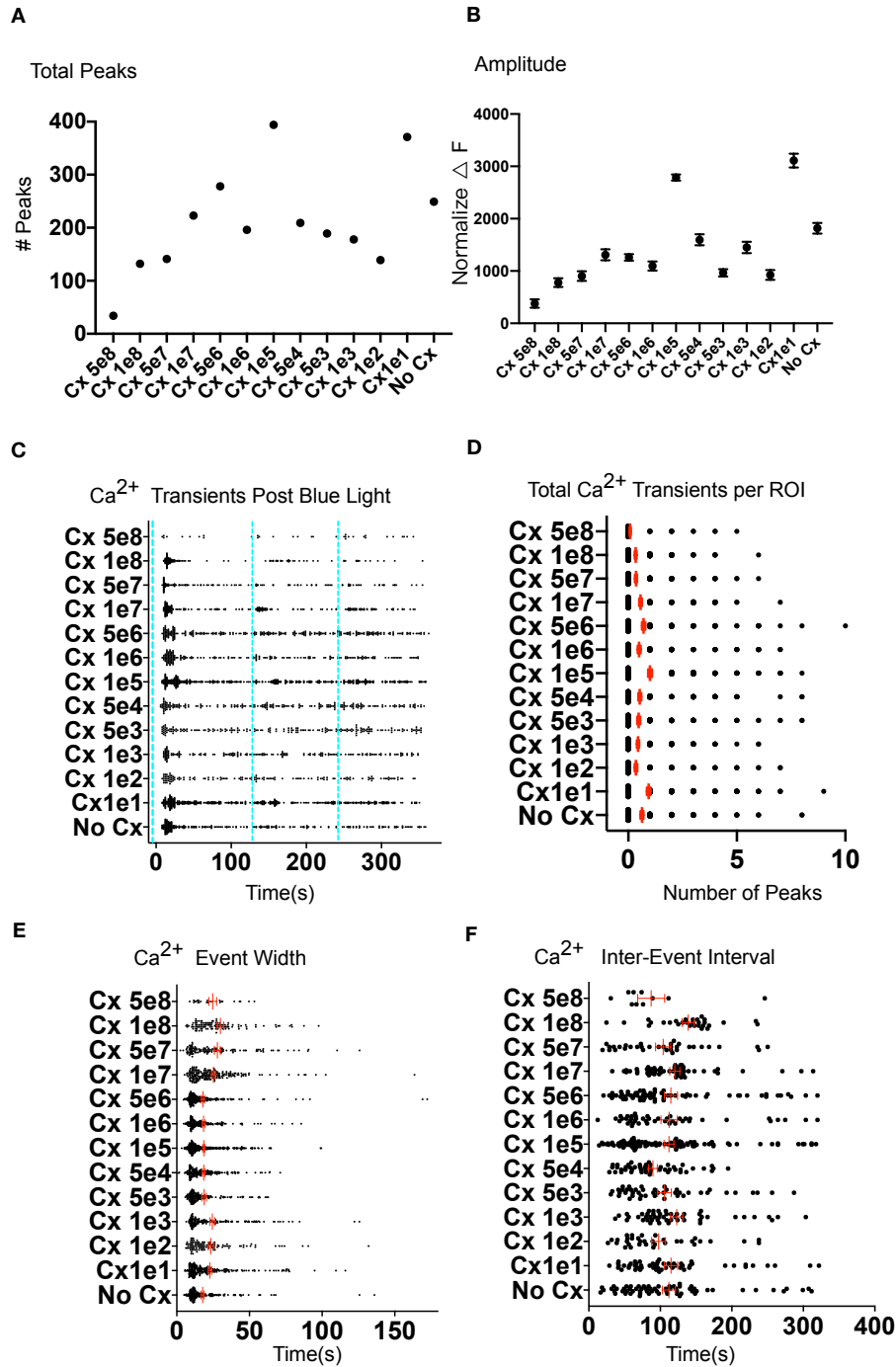


S4 Fig. Features of the [Kir2.1]:[NavD] optimized Kuhl-CKNa cells and their Ca^{2+} transients. A) The total peaks for each Ca^{2+} transient within a trial was recorded, and the experiment with [1e8 Kir2.1]:[5e8 NavD] VG/ μ L had the greatest recorded peaks. B) The ΔF is the difference between the baseline fluorescence and the maximum fluorescence of each peak. The mean and S.E.M of the Ca^{2+} transients in [Kir2.1]:[NavD] optimized Kuhl-CKNa cells C)

Blue line indicates point of 20s blue light stimulus. The time of each fluorescence peak was recorded and is indicated by a black dot. D) The total number of Ca^{2+} transients per cell (ROI) for experimental [Kir2.1]:[NavD] optimized Kuhl-CKNa cells. E) Representative duration of elevated R-GECO1 fluorescence over time per Ca^{2+} transient. Black dot indicates the Ca^{2+} transients FWHM for each peak. F) Black dot indicates the time between Ca^{2+} transient events per cell. Red bars indicate mean and $\pm$ S.E.M ($n = 150$ cells per condition).

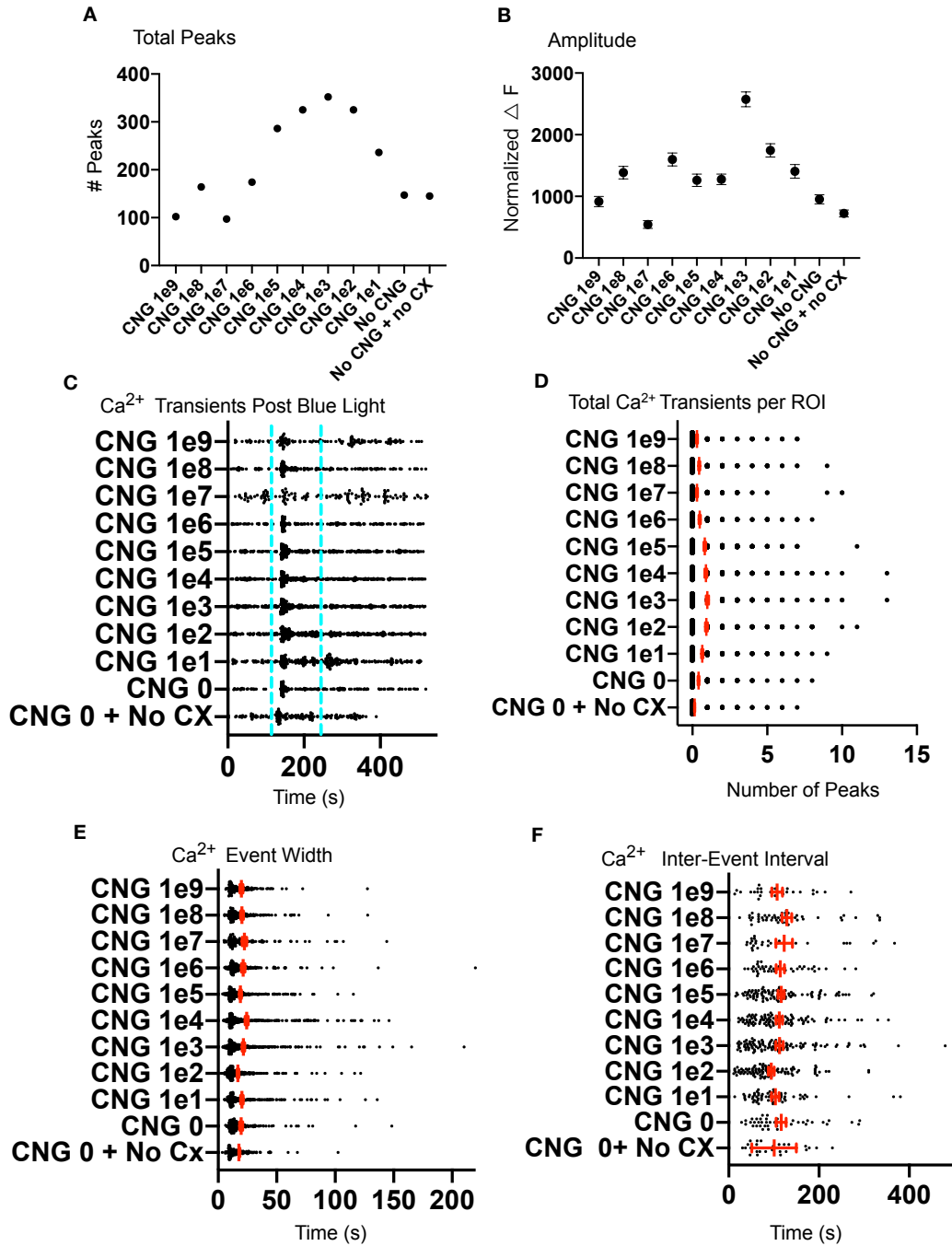


S5 Fig. Features of the [Kir2.1]:[NavD] optimized Kuhl-CKNaCx cells and their Ca²⁺ transients. A) The total peaks for each Ca²⁺ transient within a trial was recorded, and the experiment with [1e8 Kir2.1]:[1e8 NavD] VG/ μ L had the greatest recorded peaks. B) The ΔF is the difference between the baseline fluorescence and the maximum fluorescence of each peak. The mean and S.E.M of the Ca²⁺ transients in [Kir2.1]:[NavD] optimized Kuhl-CKNaCx cells C) Blue line indicates point of 20s blue light stimulus. The time of each fluorescence peak was recorded and is indicated by a black dot. D) The total number of Ca²⁺ transients per cell (ROI) for experimental [Kir2.1]:[NavD] optimized Kuhl-CKNaCx. E) Representative duration of elevated R-GECO1 fluorescence over time per Ca²⁺ transient. Black dot indicates the Ca²⁺ transients FWHM for each peak. F) Black dot indicates the time between Ca²⁺ transient events per cell. Red bars indicate mean and $\pm$ S.E.M (n = 150 cells per condition).



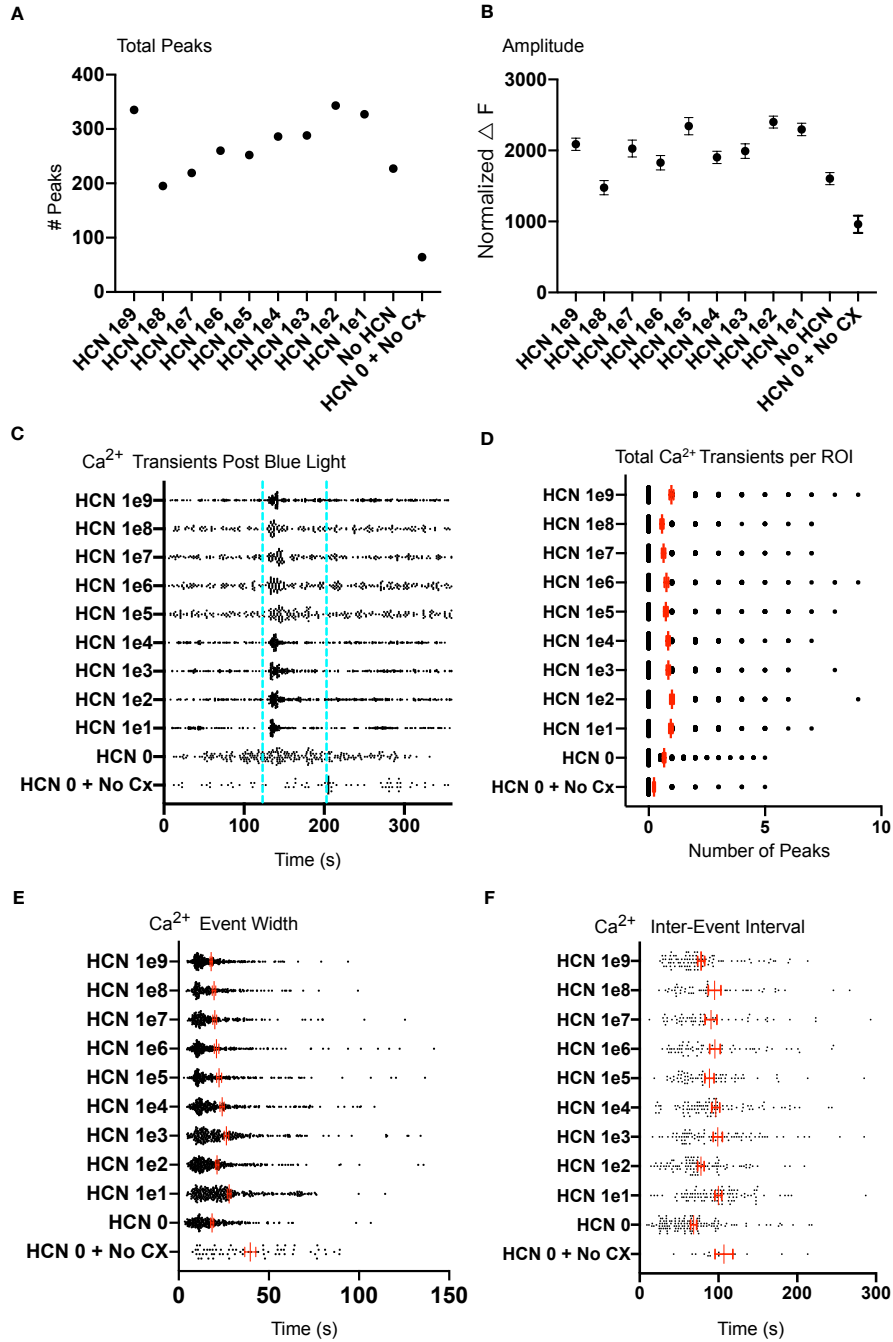
S6 Fig. Features of the Cx-43 optimized Kuhl-CKNaCx cells and their Ca^{2+} transients. Corresponding figure 8. A) The total peaks for each Ca^{2+} transient within a trial was recorded, and the experiment with [Cx 1e5] VG/ μL had the greatest recorded peaks. B) The ΔF is the difference between the baseline fluorescence and the maximum fluorescence of each peak. The

mean and S.E.M of the Ca^{2+} transients in Cx-43 optimized Kuhl-CKNaCx cells. C) Blue line indicates point of 20s blue light stimulus. The time of each fluorescence peak was recorded and is indicated by a black dot. D) The number of Ca^{2+} transients per cell (ROI) for Cx-43 optimized Kuhl-CKNaCx cells. E) Representative duration of elevated R-GECO1 fluorescence over time per Ca^{2+} transient. Black dot indicates the Ca^{2+} transients FWHM for each peak. F) Black dot indicates the time between Ca^{2+} transient events per cell. Red bars indicate mean and $\pm$ S.E.M (n = 150 cells per condition).



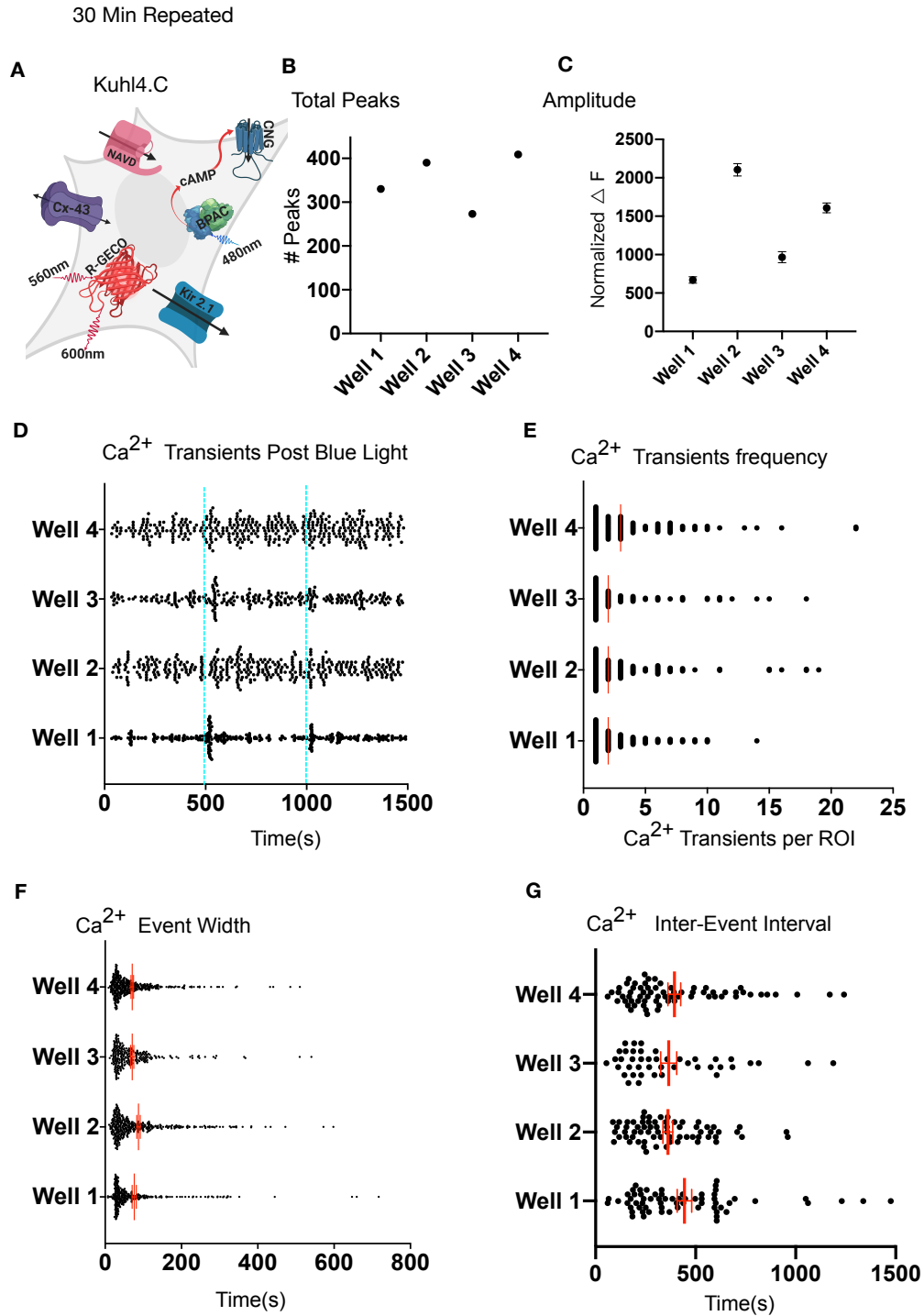
S7 Fig: Features of the CNG optimized Kuhl-CKNaCx cells and their Ca^{2+} transients. Corresponding figure 9. A) The total peaks for each Ca^{2+} transient within a trial was recorded, and the experiment with [CNG 1e3] VG/ μL had the greatest recorded peaks. B) The ΔF is the difference between the baseline fluorescence and the maximum fluorescence of each peak. The mean and S.E.M of the Ca^{2+} transients in CNG optimized Kuhl-CKNaCx cells C) Blue line

indicates point of 20s blue light stimulus. The time of each fluorescence peak was recorded and is indicated by a black dot. The wells without CNG and Cx were very interesting because it appears that without blue light there is a D) The total number of Ca^{2+} transients per cell (ROI) for experimental CNG optimized Kuhl-CKNaCx cells. E) Representative duration of elevated R-GECO1 fluorescence over time per Ca^{2+} transient. Black dot indicates the Ca^{2+} transients FWHM for each peak. F) Black dot indicates the time between Ca^{2+} transient events per cell. Red bars indicate mean and $\pm$ S.E.M ($n = 150$ cells per condition).



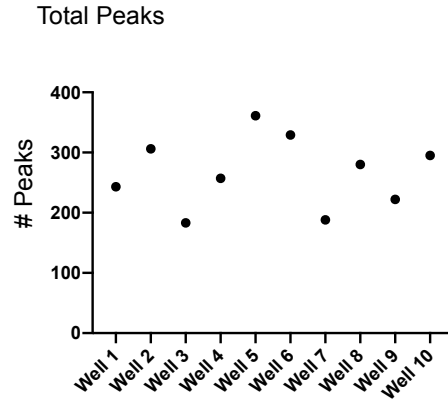
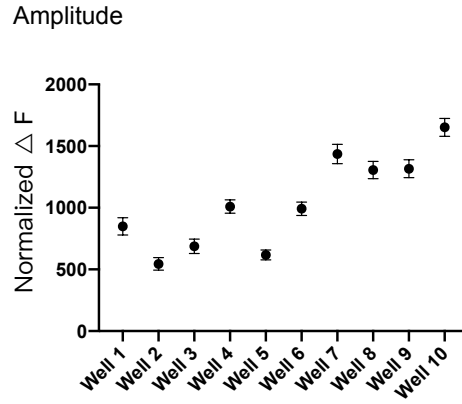
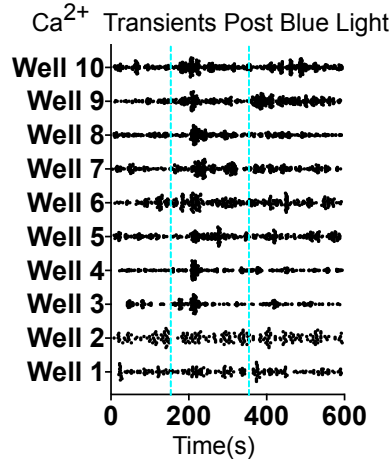
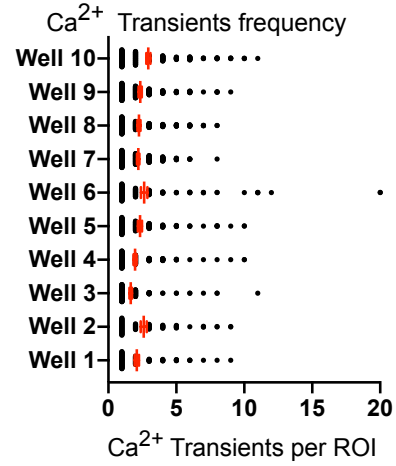
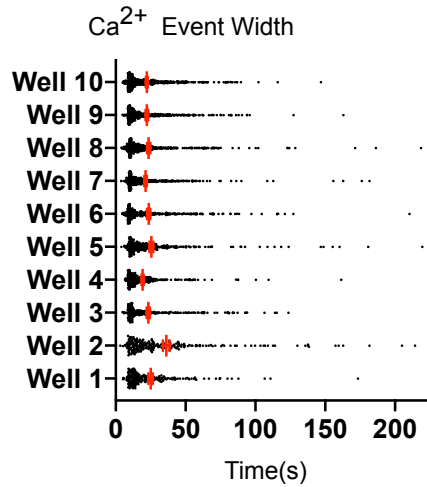
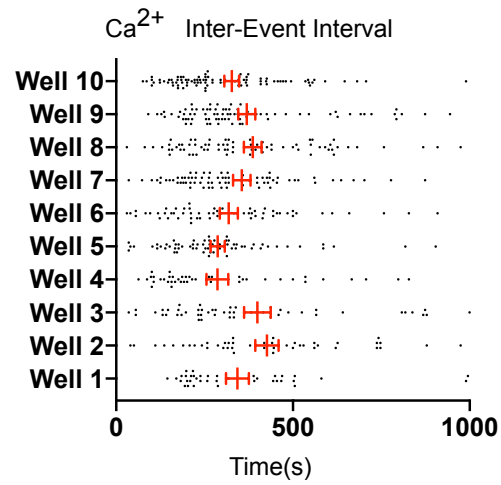
S8 Fig. Features of the HCN2 optimized Kuhl-HKNaCx cells and their Ca^{2+} transients. Corresponding figure 10. A) The total peaks for each Ca^{2+} transient within a trial ($T=300s$) was recorded. The experiment with [HCN2 1e2] VG/ μL had the greatest recorded peaks. B) The ΔF is the difference between the baseline fluorescence and the maximum fluorescence of each peak. The mean and S.E.M of the Ca^{2+} transients in HCN2 optimized Kuhl-HKNaCx cells C) Blue

line indicates point of 20s blue light stimulus. The time of each fluorescence peak was recorded and is indicated by a black dot. D) The total number of Ca^{2+} transients per cell (ROI) for experimental HCN2 optimized Kuhl-HKNaCx . E) Representative duration of elevated R-GECO1 fluorescence over time per Ca^{2+} transient. Black dot indicates the Ca^{2+} transients FWHM for each peak. F) Black dot indicates the time between Ca^{2+} transient events per cell. Red bars indicate mean and $\pm$ S.E.M ($n = 150$ cells per condition).



S9 Fig. Features of the optimized Kuhl-CKNaCx cells and their Ca^{2+} transients. Corresponding figure 11a. A) The total peaks for each Ca^{2+} transient within a trial ($T=25m$) was recorded. B) The ΔF is the difference between the baseline fluorescence and the maximum

fluorescence of each peak. The mean and S.E.M of the Ca^{2+} transients in optimized Kuhl-CKNaCx cells C) Blue line indicates point of 20s blue light stimulus. The time of each fluorescence peak was recorded and is indicated by a black dot. D) The total number of Ca^{2+} transients per cell (ROI) for optimized Kuhl-CKNaCx cells. Representative duration of elevated R-GECO1 fluorescence over time per Ca^{2+} transient. Black dot indicates the Ca^{2+} transients FWHM for each peak. F) Black dot indicates the time between Ca^{2+} transient events per cell. Red bars indicate mean and $\pm$ S.E.M ($n = 150$ cells per condition).

A**B****C****D****E****F**

S10 Fig. Features of the optimized Kuhl-CKNaCx cells and their Ca²⁺ transients. Corresponding figure 11b. A) The total peaks for each Ca²⁺ transient within a trial (T=25m) was recorded. B) The ΔF is the difference between the baseline fluorescence and the maximum fluorescence of each peak. The mean and S.E.M of the Ca²⁺ transients in optimized Kuhl-CKNaCx cells C) Blue line indicates point of 20s blue light stimulus. The time of each fluorescence peak was recorded and is indicated by a black dot. D) The total number of Ca²⁺ transients per cell (ROI) for optimized Kuhl-CKNaCx. Representative duration of elevated R-GECO1 fluorescence over time per Ca²⁺ transient. Black dot indicates the Ca²⁺ transients FWHM for each peak. F) Black dot indicates the time between Ca²⁺ transient events per cell. Red bars indicate mean and $\pm$ S.E.M (n = 150 cells per condition).

Figure 6						Figure 7					
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant ?	Summary	Adjusted P Value	Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant ?	Summary	Adjusted P Value
K0, N0 vs. K1e8, N5e8	-0.9818	-1.330 to -0.6339	Yes	****	<0.0001	K0, N0 vs. K1e8 N 5e7	-0.8983	-1.365 to -0.4320	Yes	****	<0.0001
K0, N0 vs. K1e8, N5e7	-0.2545	-0.6025 to 0.09337	No	ns	0.2191	K0, N0 vs. K1e8 N 1e8	-0.9661	-1.432 to -0.4998	Yes	****	<0.0001
K0, N0 vs. K5e9, N5e7	-0.09091	-0.4388 to 0.2570	No	ns	0.9183	K0, N0 vs. K5e9 N5e7	-0.2034	-0.6697 to 0.2629	No	ns	0.8100
K0, N0 vs. K5e9, N0	-0.05455	-0.4025 to 0.2934	No	ns	0.9863	K0, N0 vs. K5e9 N 0	-0.4237	-0.8900 to 0.04256	No	ns	0.0940
						K0, N0 vs. K5e9 N 1e8	-0.1525	-0.6188 to 0.3137	No	ns	0.9532
						K0, N0 vs. K0, N5e7	-0.3559	-0.8222 to 0.1104	No	ns	0.2242
						K0, N0 vs. K5e8, N 5e7	-0.4237	-0.8900 to 0.04256	No	ns	0.0940
						K0, N0 vs. K1e8, N 0	-0.7458	-1.212 to -0.2795	Yes	***	0.0002
						K0, N0 vs. K0, N 1e8	-0.1695	-0.6358 to 0.2968	No	ns	0.9175
Figure 8						Figure 9					
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant ?	Summary	Adjusted P Value	Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant ?	Summary	Adjusted P Value
No Cx vs. Cx 5e8	0.5457	0.3016 to 0.7897	Yes	****	<0.0001	CNG 0 vs. CNG 1e1	-0.2471	-0.4933 to -0.0008974	Yes	*	0.0487
No Cx vs. Cx 5e7	0.2741	0.03005 to 0.5182	Yes	*	0.0181	CNG 0 vs. CNG 1e9	0.1026	-0.1436 to 0.3488	No	ns	0.8545
No Cx vs. Cx 5e6	-0.07360	-0.3177 to 0.1705	No	ns	0.9889	CNG 0 vs. CNG 1e8	-0.04429	-0.2905 to 0.2019	No	ns	0.9993
No Cx vs. Cx 5e4	0.1015	-0.1425 to 0.3456	No	ns	0.8977	CNG 0 vs. CNG 1e7	0.1002	-0.1460 to 0.3464	No	ns	0.8693
No Cx vs. Cx 5e3	0.1523	-0.09177 to 0.3963	No	ns	0.4810	CNG 0 vs. CNG 1e6	-0.07925	-0.3254 to 0.1669	No	ns	0.9639
No Cx vs. Cx1e1	-0.3020	-0.5461 to -0.05797	Yes	**	0.0062	CNG 0 vs. CNG 1e5	-0.4172	-0.6634 to -0.1711	Yes	****	<0.0001
No Cx vs. No Cx	-0.1599	-0.4040 to 0.08416	No	ns	0.4192	CNG 0 vs. CNG 1e4	-0.4918	-0.7380 to -0.2457	Yes	****	<0.0001
No Cx vs. Cx 1e8	0.2970	0.05290 to 0.5410	Yes	**	0.0076	CNG 0 vs. CNG 1e3	-0.5921	-0.8383 to -0.3459	Yes	****	<0.0001
No Cx vs. Cx 1e7	0.06599	-0.1781 to 0.3100	No	ns	0.9951	CNG 0 vs. CNG 1e2	-0.5268	-0.7730 to -0.2806	Yes	****	<0.0001
No Cx vs. Cx 1e6	0.1345	-0.1095 to 0.3786	No	ns	0.6374	CNG 0 vs. CNG 0 + No CX	0.2580	0.04882 to 0.4671	Yes	**	0.0072
No Cx vs. Cx 1e5	-0.3680	-0.6121 to -0.1240	Yes	***	0.0003	Fig 11					
No Cx vs. Cx 1e3	0.1802	-0.06386 to 0.4243	No	ns	0.2771	Tukey's multiple comparisons test					
No Cx vs. Cx 1e2	0.2792	0.03513 to 0.5232	Yes	*	0.0150	Well 1 vs. Well 2	-0.09317	-0.3705 to 0.1841	No	ns	0.8236
						Well 1 vs. Well 3	0.08851	-0.1888 to 0.3658	No	ns	0.8448
						Well 1 vs. Well 4	-0.1227	-0.4000 to 0.1546	No	ns	0.6665
						Well 2 vs. Well 3	0.1817	-0.09563 to 0.4590	No	ns	0.3322
						Well 2 vs. Well 4	-0.02950	-0.3068 to 0.2478	No	ns	0.9929
						Well 3 vs. Well 4	-0.2112	-0.4885 to 0.06613	No	ns	0.2045
Figure 10						S3 a					
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant ?	Summary	Adjusted P Value	Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant ?	Summary	Adjusted P Value
HCN 0 vs. HCN 1e1	-0.2915	-0.5747 to -0.008375	Yes	*	0.0399	-NAV Kuhl1.C vs. [K1e8]: [N5e7] Kuhl2.C	5.268	1.539 to 8.997	Yes	**	0.0013
HCN 0 vs. HCN 1e9	-0.3149	-0.5980 to -0.03170	Yes	*	0.0206	-NAV Kuhl1.C vs. [K1e8]: [N5e8] Kuhl2.C	1.914	-0.7989 to 4.626	No	ns	0.3038
HCN 0 vs. HCN 1e8	0.09329	-0.1899 to 0.3765	No	ns	0.9620	-NAV Kuhl1.C vs. [K1e8]: [N5e7] Kuhl3.C	3.878	1.293 to 6.463	Yes	***	0.0005
HCN 0 vs. HCN 1e7	0.02332	-0.2598 to 0.3065	No	ns	0.9997	-NAV Kuhl1.C vs. [K1e8]: [N1e8] Kuhl3.C	4.780	2.240 to 7.320	Yes	****	<0.0001
HCN 0 vs. HCN 1e6	-0.09621	-0.3794 to 0.1870	No	ns	0.9542	-NAV Kuhl1.C vs. [K5e9]: [N5e7] Kuhl3.C	4.962	0.6969 to 9.227	Yes	*	0.0137
HCN 0 vs. HCN 1e5	-0.07289	-0.3561 to 0.2103	No	ns	0.9917	-NAV Kuhl1.C vs. [CNG1e5] Kuhl3.C	5.786	3.861 to 7.712	Yes	****	<0.0001
HCN 0 vs. HCN 1e4	-0.1720	-0.4552 to 0.1112	No	ns	0.4863	-NAV Kuhl1.C vs. [HCN1e1] Kuhl3.H	3.174	1.173 to 5.175	Yes	***	0.0002
HCN 0 vs. HCN 1e3	-0.1778	-0.4610 to 0.1053	No	ns	0.4463	-NAV Kuhl1.C vs. Well 2	4.222	2.114 to 6.330	Yes	****	<0.0001
HCN 0 vs. HCN 1e2	-0.3382	-0.6214 to -0.05502	Yes	*	0.0101	Kuhl4.C	1.437	-1.051 to 3.926	No	ns	0.5290
HCN 0 vs. HCN 0 + No Cx	0.4332	0.1346 to 0.7319	Yes	***	0.0008	-NAV Kuhl1.C vs. Well 10	5.522	3.644 to 7.401	Yes	****	<0.0001

S1 Table. Dunnett's Multiple comparisons test. The table shows all test run using Dunnetts where stated in the manuscript.

Corresponding Figure	Kuhl Cell	Viral Genomes VG/well	Optimized Titer VG/well	Fluorescent Sensor VG/well
Figure 1 & 2	Kuhl	bPAC - 5e7	N/A	R-cADDIS - 2e7
Figure 3A	Kuhl-C	bPAC - 5e7 CNG - 3.5e7	N/A	R-GECO1 - 5e7
Fig 3E	Kuhl-H	bPAC - 5e7 HCN - 3.5 e7	N/A	R-GECO1 - 5e7
Fig 4A & Fig 5	Kuhl-CK	bPAC - 5e7 CNG - 3.5e7 Kir2.1 - 3e6	N/A	R-GECO1 - 5e7
Fig 4C & Fig 5	Kuhl-HK	bPAC - 5e7 HCN - 7.5e7 Kir2.1 - 3e6	N/A	R-GECO1 - 5e7
Fig 6	Kuhl-CKNa	bPAC - 8.6e7 CNG - 3.6e6	Kir2.1 - (0 - 5e9) NavD - (0 - 5e7)	R-GECO1 - 5e7
Fig 7	Kuhl-CKNaCx	bPAC - 8.5e7 CNG - 3.5e7 Cx43 - 3.5e7	Kir2.1 - (0 - 5e9) NavD - (0 - 1e8)	R-GECO1 - 5e7
Fig 8	Kuhl-CKNaCx	bPAC - 8.5e7 CNG - 3.6e7 Kir2.1 - 5e7 NavD - 1e8	Cx43 - (0 - 5e8)	R-GECO1 - 5e7
Fig 9	Kuhl-CKNaCx	bPAC - 1.7e8 Kir2.1 - 5e7 NavD - 1e8 Cx43 - 5e6	CNG - (0 - 1e9)	R-GECO1 - 5e7
Fig 10	Kuhl-HKNaCx	bPAC - 8.6e7 Kir2.1 - 5e7 NavD - 1e8 Cx43 - 5e6	HCN - (0 - 1e9)	R-GECO1 - 5e7
Fig 11	Kuhl - CKNaCx	bPAC - 8.6e7 CNG - 1e3 Kir2.1 - 1e8 NavD - 1e8 Cx43 - 1e6	N/A	R-GECO1 - 5e7
Fig 12	Kuhl - CKNaCx	bPAC - 5e5 CNG - 1e3 Kir2.1 - 1e8 NavD - 1e8 Cx43 - 1e6	N/A	R-GECO1 - 3e7 G-GECO1 - 3e7
Fig 13	Kuhl - CKNaCx	bPAC - 5e5 CNG - 1e3 Kir2.1 - 1e8 NavD - 1e8 Cx - 1e5	N/A	ArcLight - 2e7

S2 Table. Experimental setup for each figure. The table shows the experimental set up, the final viral concentration used, and their corresponding figures.

S1 Movie. Bright Subcellular Flashes of Ca^{2+} . The authors do not know what these come from.

S2 Movie. The temporal/spatial movement of CNG in Kuhl-CKNaCx cells. Rose like patterns.

S3 Movie. The temporal/spatial movement of HCN2 in Kuhl-HKNaCx cells. Wave-like spreading pattern.

S4 Movie. A well from the Kuhl-CKNaCx 10 well experiment. Cells imaged for 15 minutes with robust, repeatable activity.

S5 Movie. G-GEQO1 and R-GEQO1 can be imaged simultaneously. An image created by the OptoSplit II is shown where the green and red emission from both sensors are split and imaged on two different portions of the EMCCD camera

S6 Movie. Whole well image of ArcLight activity. Activity of Kuhl-CKNaCx cells with the GEVI ArcLight.

S7 Movie. Combined images of ArcLight activity. Activity of Kuhl-CKNaCx cells with the GEVI ArcLight from the same well.

S8 Movie. Combined images of ArcLight activity. Activity of Kuhl-CKNaCx cells with the GEVI ArcLight from the same well.

CHAPTER FOUR

FUTURE RESEARCH DIRECTIONS

If a scientist stopped to examine every strange occurrence while performing research, he or she would seldom finish a project and often end up wasting a considerable amount of time paying attention to inconsequential anomalies. It is the refinement of the scientist to know when to dig and continue digging. But what makes an anomaly worth concentrated scrutiny and deemed further investigation? That depends largely on the investigator and their breadth of knowledge and intuition [1].

Further Kuhl Optimization

The Kuhl cell system still needs further optimization. To be used to screen voltage sensors a faster Na⁺ channel should replace the NavD to create faster pseudo action potentials. Also, the repolarization of the cells is not coordinated and relatively slow. Potentially, a confluent layer of Kuhl-CKNaCx HEK293 cells could be co-plated with a small number of cells that only contain a halorhodopsin. In this system, the investigator gains temporal specificity by using different wavelengths that could control the depolarizations and hyperpolarizations.

Or, instead of using cells containing halorhodopsin, a small number of cells could be co-plated and act as a pacemaker using Sinoatrial Node SAN channels, such as HCN2, KCNQ, and a HERG channel. In theory, these pacemaker cells could regulate the depolarization and hyperpolarization of the surrounding Kuhl cells through gap junction coupling. Coordinating the action potential inducing rhythmicity in Kuhl cells with a single pulse of light would be an important next step if this system wants to be used in whole plate fluorescence screening platforms. Potentially, if the initial 100s

following a pulse of blue light was imaged in Kuhl-CKNaCx cells the whole well or whole plate fluorescence could be used to differentiate the biosensors' responses, instead of extracting data cell by cell per well. This may work because the activity immediately following the blue light stimulus is largely coordinated. This could increase throughput when screening biosensors but it would only measure the total fluorescence change. If this can be done, the best ~100 sensors from this initial screen could be moved into a lower throughput screen where each sensor was benchmarked against the standard sensor for sensor kinetics in a cell by cell analysis. Finally, Kir2.1 and NavD should be optimized with the Kuhl-HKNa and Kuhl-HKNaCx system. I believe it should be optimized because HCN2 is a voltage-gated channel and therefore intrinsically acts differently than the CNG channel. It might respond better to higher levels of Kir2.1 because it is hyperpolarization gated dependent.

Screen Activity-Based Biosensors

Traditionally, the throughput of screens for better voltage sensors has been extremely slow [2]. The Kuhl cells described here can be used to screen voltage and Ca^{2+} sensors easily using an optical imaging splitter. This system provides the experimenter with a way to benchmark the novel sensor against the standard, such as Voltron or jGCaMP7 [3,4]. This system can screen novel sensors for their kinetics, however, a lot of work is yet to be done.

The Kuhl-CKNaCx action potential lasts on the order of seconds while the neuronal action potential lasts on the order of milliseconds. Therefore, if screening for faster voltage sensors it would be favorable to increase the speed of the pseudo action potential. Increasing the speed of the action potential will also increase the speed of the throughput if it decreased the inter-event interval between each event. For example, to record 100 fluorescence peaks before moving on to the next well, increasing

the speed at which the cells fire and decreasing the time between firing will decrease the time needed to image. Another way to increase throughput in this system would be to increase the coordination of the cells. Greater coordination of the Ca^{2+} transients/APs and increasing the number of times that a fluorescence peak is produced within 10 seconds would increase the throughput of the system.

Using the Kuhl System to Screen Drugs

While we have examined the use of the Kuhl system to develop novel biosensors it could be used to screen drugs that interact with particular channels. For example, the channels Kir2.1 and NavD could be exchanged for heart voltage-gated ion channels such as $\text{hK}_v1.5$, $\text{hNav}_v1.5$, or HERG [5]. Interestingly, the use of baculovirus in this screen may prove to be increasingly useful because it can overcome the challenge of needing a stronger current from a particular channel depending on the drug target. For example, in human-induced pluripotent stem cell-derived cardiomyocytes hiPSC, the I_{K1} current is weaker than in traditional heart cells. In theory, by using baculovirus the I_{K1} current could be amplified by increasing the total viral load [6]. Furthermore, a similar all-optical cell system using rhodopsin for an actuator has proven useful in creating a high-throughput screen for pain therapy drugs that act on sodium channels such as Nav1.7 [7]. Using an all-optical approach that combines optical reporters and optogenetics obviates the use of electrophysiology and reagents to create a system that can be stimulated repeatedly.

A Need for a Voltage-clamp Protocol Using Electrophysiology

The optogenetic tools available have complex photocurrent kinetics and it can be difficult to counteract the fast gating and high conductivity of voltage-gated channels. The ability to create a light step protocol that could replace voltage-step protocols could be useful when working in-vitro or in-

vivo. It would be interesting to test if the voltage of the Kuhl cells using bPAC can be clamped with varying levels of blue light.

To start, I could use red voltage sensors such as Voltron or BeRST-1 [4,8] in combination with electrophysiology to assign a light intensity of the voltage sensor to the resting membrane potential. Then blue light at small brief doses could be used to activate bPAC and using electrophysiology to determine if the cells membrane potential could be clamped at a certain voltage. While a brief pulse of light to bPAC has a much slower and longer-lasting effect on the cell than a channelrhodopsin, this may stand to benefit experimenters trying to keep a constant voltage for a longer period of time. However, if large and fast positive and negative voltage jumps are needed, halo and channelrhodopsins should be used. For example, a previous study using ArchT and ChR2 has shown that they could create a calibrated light-step protocol. They used one wavelength to depolarize the cell and another to immediately hyperpolarize the cell, however, this doesn't allow for imaging channels within this wavelength and still used electrophysiology to examine the channels currents [5]. 'Optopatch' or 'FloXopatch' (in mice) was a creative design using QuasAr2, an archaerhodopsin-derived voltage indicator, and CheRiff, a blue-shifted channelrhodopsin. This design allows for the activation of Optopatch-expressing neurons to be stimulated with blue light while recording action potentials with the near IR QuasAr2 sensor. They induced and recorded action potentials in different neuronal areas, however in fast-firing neurons the electrical waveforms were not accurate when compared to electrophysiology recordings[9]. Further, they found that when they depolarized the cells with light they increased the SNR of the sensor. This reinforces that there is a need for fluorescent voltage sensors that have improved kinetics as well as SNR.

Subcellular Flashes in the Kuhl Cells

A small but maddening detail is the presence of subcellular flashes in the Kuhl cells. It would be interesting to identify where the Ca^{2+} is coming from. If the subcellular flashes are coming from mitochondrial release a Ca^{2+} sensor could be targeted to the mitochondria. Perhaps the large increase in cAMP or Ca^{2+} is acting on IP3 channels by opening them briefly and dumping Ca^{2+} into the cytosol [10]? If the cells are placed in Ca^{2+} free buffer after stimulation does the phenomena still occur? These questions keep me up at night.

REFERENCES CITED

1. Kuhn TS. *The Structure of Scientific Revolutions: 50th Anniversary Edition*. University of Chicago Press; 2012.
2. O'Keefe J, Bouma H. Complex sensory properties of certain amygdala units in the freely moving cat. *Exp Neurol*. 1969;23: 384–398.
3. O'Keefe J, Dostrovsky J. The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. *Brain Res*. 1971;34: 171–175.
4. Moser MB, Moser EI, Forrest E, Andersen P, Morris RG. Spatial learning with a minislab in the dorsal hippocampus. *Proc Natl Acad Sci U S A*. 1995;92: 9697–9701.
5. Price CJ. A review and synthesis of the first 20 years of PET and fMRI studies of heard speech, spoken language and reading. *Neuroimage*. 2012;62: 816–847.
6. Southern PJ, Berg P. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J Mol Appl Genet*. 1982;1: 327–341.
7. Chalfie M. GFP: Lighting up life. *Proc Natl Acad Sci U S A*. 2009;106: 10073–10080.
8. Boyden ES. A history of optogenetics: the development of tools for controlling brain circuits with light. *F1000 Biol Rep*. 2011;3: 11.
9. Adamantidis AR, Zhang F, Aravanis AM, Deisseroth K, de Lecea L. Neural substrates of awakening probed with optogenetic control of hypocretin neurons. *Nature*. 2007;450: 420–424.
10. Piccolino M. Animal electricity and the birth of electrophysiology: the legacy of Luigi Galvani. *Brain Res Bull*. 1998;46: 381–407.
11. Hsu H, Huang E, Yang XC, Karschin A, Labarca C, Figl A, et al. Slow and incomplete inactivations of voltage-gated channels dominate encoding in synthetic neurons. *Biophys J*. 1993;65: 1196–1206.
12. Park J, Werley CA, Venkatachalam V, Kralj JM, Dib-Hajj SD, Waxman SG, et al. Screening fluorescent voltage indicators with spontaneously spiking HEK cells. *PLoS One*. 2013;8: e85221.
13. Kirkton RD, Bursac N. Engineering biosynthetic excitable tissues from unexcitable cells for electrophysiological and cell therapy studies. *Nat Commun*. 2011;2: 300.

14. Nicholls DG, Ward MW. Mitochondrial membrane potential and neuronal glutamate excitotoxicity: mortality and millivolts. *Trends Neurosci.* 2000;23: 166–174.
15. Abdul Kadir L, Stacey M, Barrett-Jolley R. Emerging Roles of the Membrane Potential: Action Beyond the Action Potential. *Front Physiol.* 2018;9: 1661.
16. Bryson B. *The Body: A Guide for Occupants.* Random House; 2019.
17. Dunn R. *The Man Who Touched His Own Heart: True Tales of Science, Surgery, and Mystery.* Little, Brown; 2015.
18. Milstein ML, Musa H, Balbuena DP, Anumonwo JMB, Auerbach DS, Furspan PB, et al. Dynamic reciprocity of sodium and potassium channel expression in a macromolecular complex controls cardiac excitability and arrhythmia. *Proc Natl Acad Sci U S A.* 2012;109: E2134–43.
19. Jacobson DA, Philipson LH. Action potentials and insulin secretion: new insights into the role of Kv channels. *Diabetes Obes Metab.* 2007;9 Suppl 2: 89–98.
20. Mammucari C, Raffaello A, Vecellio Reane D, Rizzuto R. Molecular structure and pathophysiological roles of the Mitochondrial Calcium Uniporter. *Biochim Biophys Acta.* 2016;1863: 2457–2464.
21. Giorgi C, Marchi S, Pinton P. The machineries, regulation and cellular functions of mitochondrial calcium. *Nat Rev Mol Cell Biol.* 2018;19: 713–730.
22. Deiters VS, Guillery RW. Otto Friedrich Karl Deiters (1834-1863). *J Comp Neurol.* 2013;521: 1929–1953.
23. Mazzarello P. A unifying concept: the history of cell theory. *Nat Cell Biol.* 1999;1: E13–5.
24. Probst A, Langui D. [The neuron theory: one of the main scientific achievements of the 19th century]. *Schweiz Rundsch Med Prax.* 1994;83: 462–469.
25. Piccolino M. Luigi Galvani's path to animal electricity. *C R Biol.* 2006;329: 303–318.
26. Galvani L. Aloysii Galvani De viribus electricitatis in motu musculari commentarius. 1791. doi:10.5479/sil.324681.39088000932442
27. The Nobel Prize in Physiology or Medicine 1906. In: NobelPrize.org [Internet]. [cited 17 Apr 2020]. Available: <https://www.nobelprize.org/prizes/medicine/1906/cajal/biographical/>
28. Seyfarth E-A. Julius Bernstein (1839-1917): pioneer neurobiologist and biophysicist. *Biol Cybern.* 2006;94: 2–8.

29. Gasser HS, Erlanger J. A STUDY OF THE ACTION CURRENTS OF NERVE WITH THE CATHODE RAY OSCILLOGRAPH. *American Journal of Physiology-Legacy Content*. 1922;62: 496–524.
30. Index of Review, 1921. *American Physical Education Review*. 1921;26: 3–6.
31. Nobel Prize for Physiology and Medicine for 1944: Prof. J. Erlanger and Dr. H. S. Gasser. *Nature*. 1944;154: 572–572.
32. Hill DK, Keynes RD. Opacity changes in stimulated nerve. *J Physiol*. 1949;108: 278–281.
33. Cole KS. Squid axon membrane: impedance decrease to voltage clamp. *Annu Rev Neurosci*. 1982;5: 305–323.
34. Marmont G. Action potential artifacts from single nerve fibers. *American Journal of Physiology-Legacy Content*. 1940;130: 392–402.
35. Cole KS, Baker RF. LONGITUDINAL IMPEDANCE OF THE SQUID GIANT AXON. *The Journal of General Physiology*. 1941. pp. 771–788. doi:10.1085/jgp.24.6.771
36. Cole KS, Curtis HJ. MEMBRANE POTENTIAL OF THE SQUID GIANT AXON DURING CURRENT FLOW. *J Gen Physiol*. 1941;24: 551–563.
37. Hodgkin AL, Katz B. The effect of sodium ions on the electrical activity of giant axon of the squid. *J Physiol*. 1949;108: 37–77.
38. Alan Lloyd Hodgkin - Nobel Lecture. Available: <https://www.nobelprize.org/uploads/2018/06/hodgkin-lecture.pdf>
39. Hodgkin AL, Huxley AF. Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*. *J Physiol*. 1952;116: 449–472.
40. Hodgkin AL, Huxley AF. The components of membrane conductance in the giant axon of *Loligo*. *J Physiol*. 1952;116: 473–496.
41. Hodgkin AL, Huxley AF, Katz B. Measurement of current-voltage relations in the membrane of the giant axon of *Loligo*. *J Physiol*. 1952;116: 424–448.
42. Hodgkin AL, Huxley AF. The dual effect of membrane potential on sodium conductance in the giant axon of *Loligo*. *J Physiol*. 1952;116: 497–506.
43. Hodgkin AL, Huxley AF. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J Physiol*. 1952;117: 500–544.

44. Neher E, Sakmann B. The patch clamp technique. *Sci Am.* 1992;266: 44–51.
45. Katz B, Miledi R. The statistical nature of the acetylcholine potential and its molecular components. *J Physiol.* 1972;224: 665–699.
46. Sakmann B. Nobel Lecture. Elementary steps in synaptic transmission revealed by currents through single ion channels. *EMBO J.* 1992;11: 2002–2016.
47. Neher E, Steinbach JH. Local anaesthetics transiently block currents through single acetylcholine-receptor channels. *J Physiol.* 1978;277: 153–176.
48. Sigworth FJ, Neher E. Single Na⁺ channel currents observed in cultured rat muscle cells. *Nature.* 1980;287: 447–449.
49. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* 1981;391: 85–100.
50. Neher E, Sakmann B. Single-channel currents recorded from membrane of denervated frog muscle fibres. *Nature.* 1976;260: 799–802.
51. Bear MF, Connors BW, Paradiso MA. *Neuroscience.* Lippincott Williams & Wilkins; 2007.
52. Miller C. *Ionic channels of excitable membranes.*: By Bertil Hille. Sunderland, Massachusetts: Sinauer.(1991). 607 pp. \$46.95. Cell Press; 1992. Available: [https://www.cell.com/cell/pdf/0092-8674\(92\)90220-7.pdf](https://www.cell.com/cell/pdf/0092-8674(92)90220-7.pdf)
53. Payandeh J, Minor DL Jr. Bacterial voltage-gated sodium channels (BacNa(V)s) from the soil, sea, and salt lakes enlighten molecular mechanisms of electrical signaling and pharmacology in the brain and heart. *J Mol Biol.* 2015;427: 3–30.
54. Catterall WA, Goldin AL, Waxman SG. International Union of Pharmacology. XLVII. Nomenclature and structure-function relationships of voltage-gated sodium channels. *Pharmacol Rev.* 2005;57: 397–409.
55. Ren D, Navarro B, Xu H, Yue L, Shi Q, Clapham DE. A prokaryotic voltage-gated sodium channel. *Science.* 2001;294: 2372–2375.
56. Koishi R, Xu H, Ren D, Navarro B, Spiller BW, Shi Q, et al. A superfamily of voltage-gated sodium channels in bacteria. *J Biol Chem.* 2004;279: 9532–9538.
57. Payandeh J, Scheuer T, Zheng N, Catterall WA. The crystal structure of a voltage-gated sodium channel. *Nature.* 2011;475: 353–358.

58. Amaral C, Carnevale V, Klein ML, Treptow W. Exploring conformational states of the bacterial voltage-gated sodium channel NavAb via molecular dynamics simulations. *Proc Natl Acad Sci U S A*. 2012;109: 21336–21341.
59. Bagn  ris C, Naylor CE, McCusker EC, Wallace BA. Structural model of the open–closed–inactivated cycle of prokaryotic voltage-gated sodium channels. *J Gen Physiol*. 2015;145: 5–16.
60. Payandeh J, Gamal El-Din TM, Scheuer T, Zheng N, Catterall WA. Crystal structure of a voltage-gated sodium channel in two potentially inactivated states. *Nature*. 2012;486: 135–139.
61. Shen H, Zhou Q, Pan X, Li Z, Wu J, Yan N. Structure of a eukaryotic voltage-gated sodium channel at near-atomic resolution. *Science*. 2017;355. doi:10.1126/science.aal4326
62. McCusker EC, Bagn  ris C, Naylor CE, Cole AR, D’Avanzo N, Nichols CG, et al. Structure of a bacterial voltage-gated sodium channel pore reveals mechanisms of opening and closing. *Nat Commun*. 2012;3: 1102.
63. Shaya D, Findeisen F, Abderemane-Ali F, Arrigoni C, Wong S, Nurva SR, et al. Structure of a prokaryotic sodium channel pore reveals essential gating elements and an outer ion binding site common to eukaryotic channels. *J Mol Biol*. 2014;426: 467–483.
64. Nguyen HX, Kirkton RD, Bursac N. Engineering prokaryotic channels for control of mammalian tissue excitability. *Nat Commun*. 2016;7: 13132.
65. Littleton JT, Ganetzky B. Ion channels and synaptic organization: analysis of the *Drosophila* genome. *Neuron*. 2000;26: 35–43.
66. Papazian DM, Schwarz TL, Tempel BL, Jan YN, Jan LY. Cloning of genomic and complementary DNA from Shaker, a putative potassium channel gene from *Drosophila*. *Science*. 1987;237: 749–753.
67. Covarrubias M, Wei AA, Salkoff L. Shaker, Shal, Shab, and Shaw express independent K⁺ current systems. *Neuron*. 1991;7: 763–773.
68. Rogero O, H  mmerle B, Tejedor FJ. Diverse Expression and Distribution of Shaker Potassium Channels during the Development of the *Drosophila* Nervous System. *The Journal of Neuroscience*. 1997. pp. 5108–5118. doi:10.1523/jneurosci.17-13-05108.1997
69. Faller LD. Mechanistic studies of sodium pump. *Arch Biochem Biophys*. 2008;476: 12–21.
70. Primeau JO, Armanious GP, Fisher ME, Young HS. The SarcoEndoplasmic Reticulum Calcium ATPase. *Subcell Biochem*. 2018;87: 229–258.

71. Laird DW. Life cycle of connexins in health and disease. *Biochem J.* 2006;394: 527–543.
72. Atkinson MM, Lampe PD, Lin HH, Kollander R, Li XR, Kiang DT. Cyclic AMP modifies the cellular distribution of connexin43 and induces a persistent increase in the junctional permeability of mouse mammary tumor cells. *J Cell Sci.* 1995;108 (Pt 9): 3079–3090.
73. Cohen LB. Historical Overview and General Methods of Membrane Potential Imaging. In: Canepari M, Zecevic D, editors. *Membrane Potential Imaging in the Nervous System: Methods and Applications.* New York, NY: Springer New York; 2011. pp. 1–11.
74. Cohen LB, Keynes RD, Hille B. Light scattering and birefringence changes during nerve activity. *Nature.* 1968;218: 438–441.
75. Davila HV, Salzberg BM, Cohen LB, Waggoner AS. A large change in axon fluorescence that provides a promising method for measuring membrane potential. *Nature.* 1973. Available: <http://www.nature.com/nature-newbio/journal/v241/n109/abs/newbio241159a0.html>
76. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. *Science.* 1994;263: 802–805.
77. MacRae EK. Nerve, Muscle and Synapse Bernard Katz. *The American Biology Teacher.* 1967. pp. 233–233. doi:10.2307/4441678
78. KATZ, B. The release of neural transmitter substances. Liverpool University Press. 1969; 5–39.
79. Yuste R, Miller RB, Holthoff K, Zhang S, Miesenböck G. Synapto-pHluorins: Chimeras between pH-sensitive mutants of green fluorescent protein and synaptic vesicle membrane proteins as reporters of neurotransmitter release. In: Thorner J, Emr SD, Abelson JN, editors. *Methods in Enzymology.* Academic Press; 2000. pp. 522–546.
80. Rosenmund C, Stevens CF. Definition of the readily releasable pool of vesicles at hippocampal synapses. *Neuron.* 1996;16: 1197–1207.
81. Heuser JE, Reese TS. Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J Cell Biol.* 1973;57: 315–344.
82. Ceccarelli B, Hurlbut WP, Mauro A. Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. *J Cell Biol.* 1973;57: 499–524.
83. Holtzman E, Freeman AR, Kashner LA. Stimulation-dependent alterations in peroxidase uptake at lobster neuromuscular junctions. *Science.* 1971;173: 733–736.
84. Cochilla AJ, Angleson JK, Betz WJ. Monitoring secretory membrane with FM1-43 fluorescence. *Annu Rev Neurosci.* 1999;22: 1–10.

85. Betz WJ, Mao F, Bewick GS. Activity-dependent fluorescent staining and destaining of living vertebrate motor nerve terminals. *J Neurosci*. 1992;12: 363–375.
86. Ryan TA, Reuter H, Wendland B, Schweizer FE, Tsien RW, Smith SJ. The kinetics of synaptic vesicle recycling measured at single presynaptic boutons. *Neuron*. 1993;11: 713–724.
87. Ryan TA, Reuter H, Smith SJ. Optical detection of a quantal presynaptic membrane turnover. *Nature*. 1997. pp. 478–482. doi:10.1038/41335
88. Miesenböck G, De Angelis DA, Rothman JE. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature*. 1998;394: 192–195.
89. Miesenböck G, Rothman JE. Patterns of synaptic activity in neural networks recorded by light emission from synaptolucins. *Proc Natl Acad Sci U S A*. 1997;94: 3402–3407.
90. Richardson A. LXXXII.—The action of light in preventing putrefactive decomposition; and in inducing the formation of hydrogen peroxide in organic liquids. *J Chem Soc, Trans*. 1893;63: 1109–1130.
91. Blum HF. Photodynamic action. *Physiol Rev*. 1932;12: 23–55.
92. Stoien JD, Wang RJ. Effect of near-ultraviolet and visible light on mammalian cells in culture II. Formation of toxic photoproducts in tissue culture medium by blacklight. *Proc Natl Acad Sci U S A*. 1974;71: 3961–3965.
93. Dixit R, Cyr R. Cell damage and reactive oxygen species production induced by fluorescence microscopy: effect on mitosis and guidelines for non-invasive fluorescence microscopy. *Plant J*. 2003;36: 280–290.
94. Stockley JH, Evans K, Matthey M, Volbracht K, Agathou S, Mukanowa J, et al. Surpassing light-induced cell damage in vitro with novel cell culture media. *Sci Rep*. 2017;7: 849.
95. Hirase H, Nikolenko V, Goldberg JH, Yuste R. Multiphoton stimulation of neurons. *J Neurobiol*. 2002;51: 237–247.
96. Tyssowski KM, Gray JM. Blue Light Increases Neuronal Activity-Regulated Gene Expression in the Absence of Optogenetic Proteins. *eNeuro*. 2019;6. doi:10.1523/ENEURO.0085-19.2019
97. Oesterhelt D, Stoeckenius W. Functions of a new photoreceptor membrane. *Proc Natl Acad Sci U S A*. 1973;70: 2853–2857.

98. Nagel G, Möckel B, Büldt G, Bamberg E. Functional expression of bacteriorhodopsin in oocytes allows direct measurement of voltage dependence of light induced H⁺ pumping. *FEBS Lett.* 1995;377: 263–266.
99. Hildebrandt V, Fendler K, Heberle J, Hoffmann A, Bamberg E, Büldt G. Bacteriorhodopsin expressed in *Schizosaccharomyces pombe* pumps protons through the plasma membrane. *Proc Natl Acad Sci U S A.* 1993;90: 3578–3582.
100. Hildebrandt V, Ramezani-Rad M, Swida U, Wrede P, Grzesiek S, Primke M, et al. Genetic transfer of the pigment bacteriorhodopsin into the eukaryote *Schizosaccharomyces pombe*. *FEBS Lett.* 1989;243: 137–140.
101. Adams SR, Tsien RY. Controlling cell chemistry with caged compounds. *Annu Rev Physiol.* 1993;55: 755–784.
102. Wang SS, Augustine GJ. Confocal imaging and local photolysis of caged compounds: dual probes of synaptic function. *Neuron.* 1995;15: 755–760.
103. Crick F. The impact of molecular biology on neuroscience. *Philos Trans R Soc Lond B Biol Sci.* 1999;354: 2021–2025.
104. Alberts B. *Molecular Biology of the Cell.* Garland Science; 2017.
105. Capel RA, Collins TP, Bose SJ, Rajasundaram S, Ayagama T, Zaccolo M, et al. cAMP signalling is required for the actions of IP₃ on Ca²⁺-transients in cardiac atria and beating rate in sino-atrial node. *bioRxiv.* 2019. p. 694349. doi:10.1101/694349
106. Mikoshiba K. IP₃ receptor/Ca²⁺ channel: from discovery to new signaling concepts. *J Neurochem.* 2007;102: 1426–1446.
107. Kaufman H, Vratsanos SM, Erlanger BF. Photoregulation of an enzymic process by means of a light-sensitive ligand. *Science.* 1968;162: 1487–1489.
108. Bieth J, Vratsanos SM, Wassermann N, Erlanger BF. Photoregulation of biological activity by photocromic reagents. II. Inhibitors of acetylcholinesterase. *Proc Natl Acad Sci U S A.* 1969;64: 1103–1106.
109. Wainberg MA, Erlanger BF. Investigation of the active center of trypsin using photochromic substrates. *Biochemistry.* 1971;10: 3816–3819.
110. Montagnoli G, Monti S, Nannicini L, Felicioli R. Azoaldolase photosensitivity. *Photochem Photobiol.* 1976;23: 29–32.
111. Hug DH. The Activation of Enzymes with Light. In: Smith KC, editor. *Photochemical and Photobiological Reviews: Volume 3.* Boston, MA: Springer US; 1978. pp. 1–33.

112. Zemelman BV, Lee GA, Ng M, Miesenböck G. Selective photostimulation of genetically chARGed neurons. *Neuron*. 2002;33: 15–22.
113. Zemelman BV, Nesnas N, Lee GA, Miesenböck G. Photochemical gating of heterologous ion channels: remote control over genetically designated populations of neurons. *Proc Natl Acad Sci U S A*. 2003;100: 1352–1357.
114. Lima SQ, Miesenböck G. Remote control of behavior through genetically targeted photostimulation of neurons. *Cell*. 2005;121: 141–152.
115. Banghart M, Borges K, Isacoff E, Trauner D, Kramer RH. Light-activated ion channels for remote control of neuronal firing. *Nat Neurosci*. 2004;7: 1381–1386.
116. Kim J-M, Hwa J, Garriga P, Reeves PJ, RajBhandary UL, Khorana HG. Light-driven activation of beta 2-adrenergic receptor signaling by a chimeric rhodopsin containing the beta 2-adrenergic receptor cytoplasmic loops. *Biochemistry*. 2005;44: 2284–2292.
117. Finn E, News Office. Gobind Khorana, MIT professor emeritus, dies at 89. In: MIT News [Internet]. [cited 29 Apr 2020]. Available: <https://news.mit.edu/2011/obit-khorana-1110>
118. Boyden ES, Zhang F, Bamberg E. Millisecond-timescale, genetically targeted optical control of neural activity. *Nat. Neurosci*. 8 (9). 2005.
119. Nagel G, Szellas T, Huhn W, Kateriya S, Adeishvili N, Berthold P, et al. Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. *Proc Natl Acad Sci U S A*. 2003;100: 13940–13945.
120. Deisseroth K, Feng G, Majewska AK, Miesenböck G, Ting A, Schnitzer MJ. Next-generation optical technologies for illuminating genetically targeted brain circuits. *J Neurosci*. 2006;26: 10380–10386.
121. Stierl M, Stumpf P, Udvari D, Gueta R, Hagedorn R, Losi A, et al. Light modulation of cellular cAMP by a small bacterial photoactivated adenylyl cyclase, bPAC, of the soil bacterium *Beggiatoa*. *J Biol Chem*. 2011;286: 1181–1188.
122. Kerruth S, Coates C, Dürst CD, Oertner TG, Török K. The kinetic mechanisms of fast-decay red-fluorescent genetically encoded calcium indicators. *J Biol Chem*. 2019;294: 3934–3946.
123. Huang L, Knoblich U, Ledochowitsch P, Lecoq J, Clay Reid R, de Vries SEJ, et al. Relationship between spiking activity and simultaneously recorded fluorescence signals in transgenic mice expressing GCaMP6. *bioRxiv*. 2019. p. 788802. doi:10.1101/788802
124. Looger LL, Griesbeck O. Genetically encoded neural activity indicators. *Curr Opin Neurobiol*. 2012;22: 18–23.

125. Marvin JS, Shimoda Y, Magloire V, Leite M, Kawashima T, Jensen TP, et al. A genetically encoded fluorescent sensor for in vivo imaging of GABA. *Nat Methods*. 2019;16: 763–770.
126. Marvin JS. A single-wavelength genetically encoded glutamate sensor for in vivo neurotransmitter observation. *Journal of Biosensors & Bioelectronics*. 2012. doi:10.4172/2155-6210.s1.07
127. Patriarchi T, Cho JR, Merten K, Howe MW, Marley A, Xiong W-H, et al. Ultrafast neuronal imaging of dopamine dynamics with designed genetically encoded sensors. *Science*. 2018. p. eaat4422. doi:10.1126/science.aat4422
128. Abdelfattah AS, Kawashima T, Singh A, Novak O, Liu H, Shuai Y, et al. Bright and photostable chemigenetic indicators for extended in vivo voltage imaging. *Science*. 2019;365: 699–704.
129. Agetsuma M, Matsuda T, Nagai T. Methods for monitoring signaling molecules in cellular compartments. *Cell Calcium*. 2017;64: 12–19.
130. Bruton J, Cheng AJ, Westerblad H. Measuring Ca²⁺ in Living Cells. *Adv Exp Med Biol*. 2020;1131: 7–26.
131. Kim D, Svoboda K, Looger L, Schreier E. Genetically encoded calcium indicators and methods of use. US Patent. 20190153067:A1, 2019. Available: <https://patentimages.storage.googleapis.com/48/48/53/5bb27ffa25d100/US20190153067A1.pdf>
132. Mank M, Griesbeck O. Genetically encoded calcium indicators. *Chem Rev*. 2008;108: 1550–1564.
133. Chen T-W, Wardill TJ, Sun Y, Pulver SR, Renninger SL, Baohan A, et al. Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature*. 2013;499: 295–300.
134. Peron SP, Freeman J, Iyer V, Guo C, Svoboda K. A Cellular Resolution Map of Barrel Cortex Activity during Tactile Behavior. *Neuron*. 2015;86: 783–799.
135. Ahrens MB, Orger MB, Robson DN, Li JM, Keller PJ. Whole-brain functional imaging at cellular resolution using light-sheet microscopy. *Nat Methods*. 2013;10: 413–420.
136. Barandov A, Bartelle BB, Williamson CG, Loucks ES, Lippard SJ, Jasanoff A. Sensing intracellular calcium ions using a manganese-based MRI contrast agent. *Nat Commun*. 2019;10: 897.
137. Ghosh S, Harvey P, Simon JC, Jasanoff A. Probing the brain with molecular fMRI. *Curr Opin Neurobiol*. 2018;50: 201–210.

138. Ouzounov DG, Wang T, Wang M, Feng DD, Horton NG, Cruz-Hernández JC, et al. In vivo three-photon imaging of activity of GCaMP6-labeled neurons deep in intact mouse brain. *Nat Methods*. 2017;14: 388–390.
139. Helassa N, Podor B, Fine A, Török K. Design and mechanistic insight into ultrafast calcium indicators for monitoring intracellular calcium dynamics. *Sci Rep*. 2016;6: 38276.
140. Dana H, Sun Y, Mohar B, Hulse BK, Kerlin AM, Hasseman JP, et al. High-performance calcium sensors for imaging activity in neuronal populations and microcompartments. *Nat Methods*. 2019;16: 649–657.
141. Siegel MS, Isacoff EY. A genetically encoded optical probe of membrane voltage. *Neuron*. 1997;19: 735–741.
142. Woehler A, Wlodarczyk J, Neher E. Signal/noise analysis of FRET-based sensors. *Biophys J*. 2010;99: 2344–2354.
143. Nguyen HX, Bursac N. Ion channel engineering for modulation and de novo generation of electrical excitability. *Curr Opin Biotechnol*. 2019;58: 100–107.
144. Zhang H, Cohen AE. Optogenetic Approaches to Drug Discovery in Neuroscience and Beyond. *Trends Biotechnol*. 2017;35: 625–639.
145. Kirkton RD, Bursac N. Genetic engineering of somatic cells to study and improve cardiac function. *Europace*. 2012;14 Suppl 5: v40–v49.
146. Kirkton RD, Badie N, Bursac N. Spatial profiles of electrical mismatch determine vulnerability to conduction failure across a host–donor cell interface. *Circ Arrhythm Electrophysiol*. 2013;6: 1200–1207.
147. Zhang H, Reichert E, Cohen AE. Optical electrophysiology for probing function and pharmacology of voltage-gated ion channels. *Elife*. 2016;5. doi:10.7554/eLife.15202
148. Mohanty SK, Reinscheid RK, Liu X, Okamura N, Krasieva TB, Berns MW. In-depth activation of channelrhodopsin 2-sensitized excitable cells with high spatial resolution using two-photon excitation with a near-infrared laser microbeam. *Biophys J*. 2008;95: 3916–3926.
149. Shah BS, Rush AM, Liu S, Tyrrell L, Black JA, Dib-Hajj SD, et al. Contactin associates with sodium channel Nav1.3 in native tissues and increases channel density at the cell surface. *J Neurosci*. 2004;24: 7387–7399.
150. Kralj JM, Hochbaum DR, Douglass AD, Cohen AE. Electrical Spiking in *Escherichia coli* Probed with a Fluorescent Voltage-Indicating Protein. *Science*. 2011;333. doi:10.1126/science.1204831

151. Chen K, Zuo D, Wang S-Y, Chen H. Kir2 inward rectification-controlled precise and dynamic balances between Kir2 and HCN currents initiate pacemaking activity. *FASEB J*. 2018;32: 3047–3057.
152. Khan KH. Gene expression in Mammalian cells and its applications. *Adv Pharm Bull*. 2013;3: 257–263.
153. Rodin SN, Riggs AD. Epigenetic silencing may aid evolution by gene duplication. *J Mol Evol*. 2003;56: 718–729.
154. Thomas M, Martinka S, Hughes T. Design for Fast Optogenetic Screen In Mammalian Cells For Next Gen Ca²⁺ Sensors. 2020. p. 2020.02.03.932426. doi:10.1101/2020.02.03.932426
155. Hille B. *Ionic Channels of Excitable Membranes*. Sinauer; 2001.
156. Fahrenbach JP, Mejia-Alvarez R, Banach K. The relevance of non-excitable cells for cardiac pacemaker function. *J Physiol*. 2007;585: 565–578.
157. Podgorski K, Ranganathan G. Brain heating induced by near-infrared lasers during multiphoton microscopy. *J Neurophysiol*. 2016;116: 1012–1023.
158. Zhao Y, Araki S, Wu J, Teramoto T, Chang Y-F, Nakano M, et al. An expanded palette of genetically encoded Ca²⁺ indicators. *Science*. 2011;333: 1888–1891.
159. Qian Y, Piatkevich KD, Mc Larney B, Abdelfattah AS, Mehta S, Murdock MH, et al. A genetically encoded near-infrared fluorescent calcium ion indicator. *Nat Methods*. 2019;16: 171–174.
160. Ibraheem A, Yap H, Ding Y, Campbell RE. A bacteria colony-based screen for optimal linker combinations in genetically encoded biosensors. *BMC Biotechnol*. 2011;11: 105.
161. Ding Y, Li J, Enterina JR, Shen Y, Zhang I, Tewson PH, et al. Ratiometric biosensors based on dimerization-dependent fluorescent protein exchange. *Nat Methods*. 2015;12: 195–198.
162. Alford SC, Abdelfattah AS, Ding Y, Campbell RE. A fluorogenic red fluorescent protein heterodimer. *Chem Biol*. 2012;19: 353–360.
163. Zhao Y, Abdelfattah AS, Zhao Y, Ruangkittisakul A, Ballanyi K, Campbell RE, et al. Microfluidic cell sorter-aided directed evolution of a protein-based calcium ion indicator with an inverted fluorescent response. *Integr Biol*. 2014;6: 714–725.
164. Shen Y, Dana H, Abdelfattah AS, Patel R, Shea J, Molina RS, et al. A genetically encoded Ca²⁺ indicator based on circularly permuted sea anemone red fluorescent protein eqFP578. *BMC Biol*. 2018;16: 9.

165. Storace D, Sepehri Rad M, Kang B, Cohen LB, Hughes T, Baker BJ. Toward Better Genetically Encoded Sensors of Membrane Potential. *Trends Neurosci.* 2016;39: 277–289.
166. Kost TA, Condreay JP, Ames RS, Rees S, Romanos MA. Implementation of BacMam virus gene delivery technology in a drug discovery setting. *Drug Discov Today.* 2007;12: 396–403.
167. Bernal Sierra YA, Rost BR, Pofahl M, Fernandes AM, Kopton RA, Moser S, et al. Potassium channel-based optogenetic silencing. *Nat Commun.* 2018;9: 4611.
168. Tewson PH, Martinka S, Shaner NC, Hughes TE, Quinn AM. New DAG and cAMP Sensors Optimized for Live-Cell Assays in Automated Laboratories. *J Biomol Screen.* 2016;21: 298–305.
169. Hirano M, Takebe M, Ishido T, Ide T, Matsunaga S. The C-terminal region affects the activity of photoactivated adenylyl cyclase from *Oscillatoria acuminata*. *Sci Rep.* 2019;9: 20262.
170. Fesenko EE, Kolesnikov SS, Lyubarsky AL. Induction by cyclic GMP of cationic conductance in plasma membrane of retinal rod outer segment. *Nature.* 1985;313: 310–313.
171. Rich TC, Fagan KA, Nakata H, Schaack J, Cooper DMF, Karpen JW. Cyclic Nucleotide-Gated Channels Colocalize with Adenylyl Cyclase in Regions of Restricted Camp Diffusion. *J Gen Physiol.* 2000;116: 147–162.
172. Yu CR, Power J, Barnea G, O'Donnell S, Brown HEV, Osborne J, et al. Spontaneous neural activity is required for the establishment and maintenance of the olfactory sensory map. *Neuron.* 2004;42: 553–566.
173. Butterweck A, Gergs U, Elfgang C, Willecke K, Traub O. Immunochemical characterization of the gap junction protein connexin45 in mouse kidney and transfected human HeLa cells. *J Membr Biol.* 1994;141: 247–256.
174. Cao G, Platasa J, Pieribone VA, Raccuglia D, Kunst M, Nitabach MN. Genetically Targeted Optical Electrophysiology in Intact Neural Circuits. *Cell.* 2013;154: 904–913.
175. Rich TC, Tse TE, Rohan JG, Schaack J, Karpen JW. In Vivo Assessment of Local Phosphodiesterase Activity Using Tailored Cyclic Nucleotide-Gated Channels as Camp Sensors. *J Gen Physiol.* 2001;118: 63–78.
176. Edelstein AD, Tsuchida MA, Amodaj N, Pinkard H, Vale RD, Stuurman N. Advanced methods of microscope control using µManager software. *J Biol Methods.* 2014;1. doi:10.14440/jbm.2014.36
177. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods.* 2012;9: 676–682.

178. Wu J, Liu L, Matsuda T, Zhao Y, Rebane A, Drobizhev M, et al. Improved orange and red Ca^{2+} indicators and photophysical considerations for optogenetic applications. *ACS Chem Neurosci*. 2013;4: 963–972.
179. Streit J, Kleinlogel S. Dynamic all-optical drug screening on cardiac voltage-gated ion channels. *Sci Rep*. 2018;8: 1153.
180. Hortigon-Vinagre MP, Zamora V, Burton FL, Green J, Gintant GA, Smith GL. The Use of Ratiometric Fluorescence Measurements of the Voltage Sensitive Dye Di-4-ANEPPS to Examine Action Potential Characteristics and Drug Effects on Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes. *Toxicol Sci*. 2016;154: 320–331.
181. Kirk MJ, Raliski BK, Miller EW. Monitoring neuronal activity with voltage-sensitive fluorophores. *Methods Enzymol*. 2020;640: 185–204.
182. Lou S, Adam Y, Weinstein EN, Williams E, Williams K, Parot V, et al. Genetically Targeted All-Optical Electrophysiology with a Transgenic Cre-Dependent Optopatch Mouse. *J Neurosci*. 2016;36: 11059–11073.