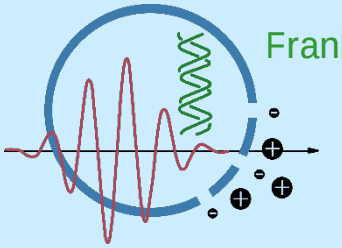


Frank Reidy Research Center for Bioelectrics

OLD DOMINION UNIVERSITY

AFOSR MURI

Topic 9: Exploiting biological electromechanics: Using electromagnetic energy to control biological systems

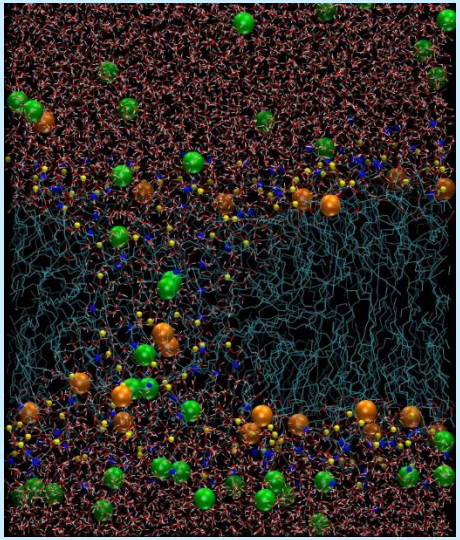
Project: Nanoelectropulse-induced electromechanical signaling and control of biological systems

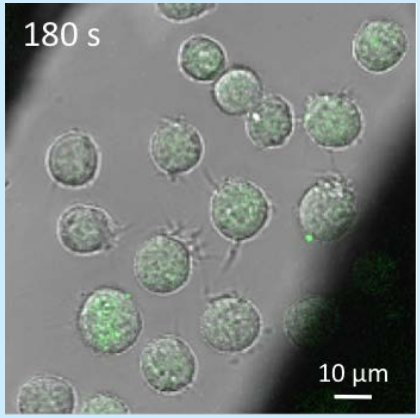
Task 2: **Molecular models of electrostimulated membrane transport**

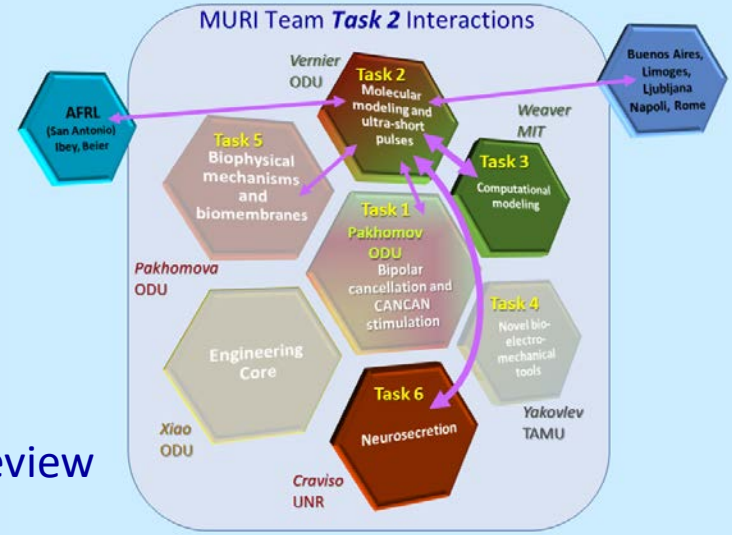
Membrane Biophysics of Biphasic Electrostimulated Molecular Transport

Esin B. Sözer[1], Federica Castellani[1,2], P. Thomas Vernier[1]

[1]Frank Reidy Research Center for Bioelectrics, Old Dominion University, Norfolk, VA USA; [2]Biomedical Engineering Institute, Frank Batten School of Engineering, Old Dominion University, Norfolk, VA, USA

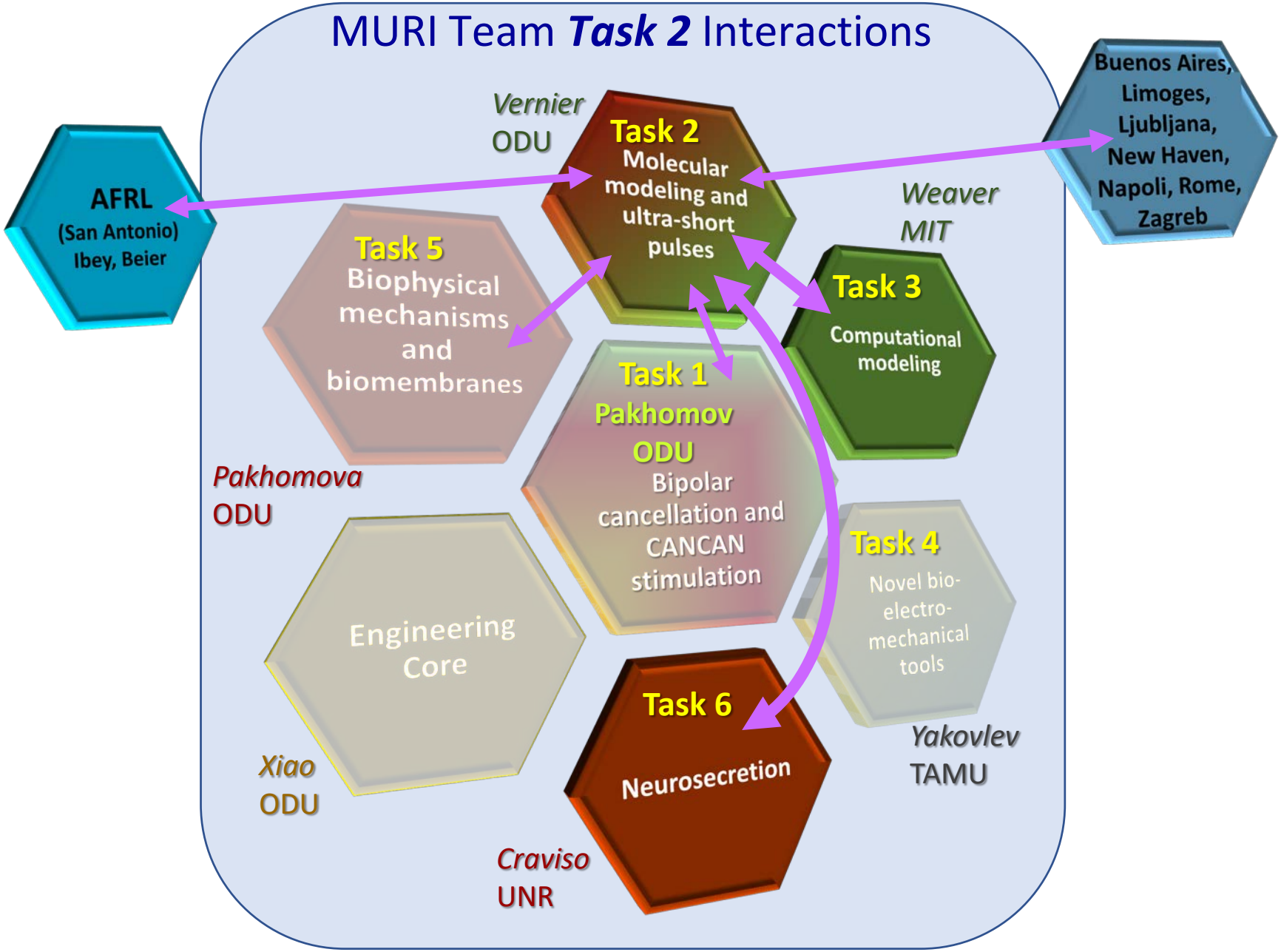






AFOSR MURI Program Review
April 18, 2018

MURI Team ***Task 2*** Interactions



MURI Progress Summary Report: Topic 9 Task 2

Topic 9: Exploiting biological electromechanics: Using electromagnetic energy to control biological systems

Task 2: Molecular models of electrostimulated membrane transport

Project: Nanoelectropulse-induced electromechanical signaling and control of biological systems

Federica Castellani, Esin B. Sözer, P. Thomas Vernier

Scientific objectives and technical approach:

General. ***Link molecular models of membranes in electric fields to continuum models and experimental observations*** in order to elucidate the processes associated with the electro-physical initiation of membrane responses to electric fields, including bipolar nanosecond electric pulse exposures.

Specific. Investigate the contribution of membrane permeabilization to bipolar pulse cancellation at the biomolecular level by conducting atomically detailed simulations of the transport of ions and small molecules through the electropores formed in phospholipid bilayers in permeabilizing electric fields.

Methodology — Model *and* Experiment

Models (molecular ⇆ continuum) ⇆ ***Experiments*** (in vitro ⇆ in vivo)

Tasks 2, 3 | Tasks 1, 2, 4, 5, 6

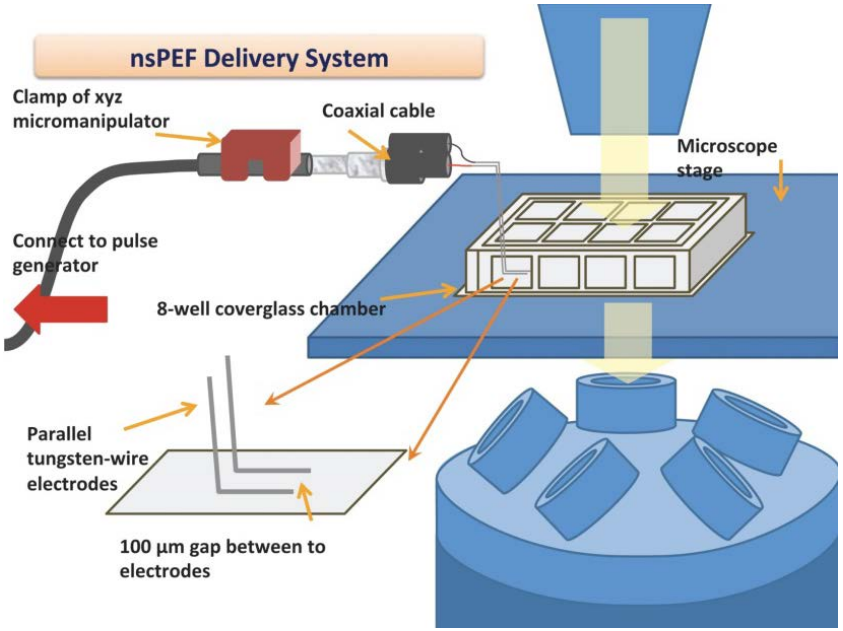
Observations give rise to models (simplified representations of reality), which drive experiments, which calibrate the models, which feed back again to empirical validation. This loop focuses *investigations of a large experimental space*.

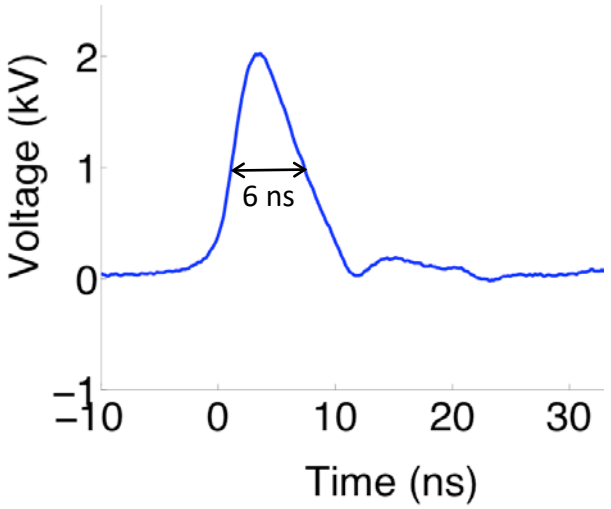
Outline

1. Bipolar pulse cancellation of multiple endpoints at the short end of “nanoseconds”.
2. Nanoscale ion transport through lipid electropores — Drift and diffusion currents, membrane charging and discharging (polarization and depolarization).
3. Nanoscale ion transport through lipid electropores — Competition with binding (Na^+, Ca^{2+}) to the phospholipid interface.
4. Cancellation and complexity.

YO-PRO-1 Influx Into Nanoelectropermeabilized U-937 Cells

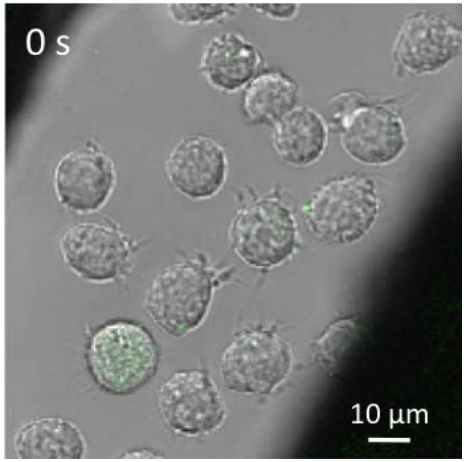
1 pulse, 6 ns, 20 MV/m

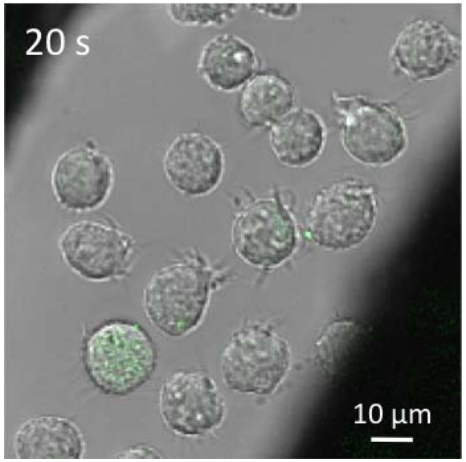


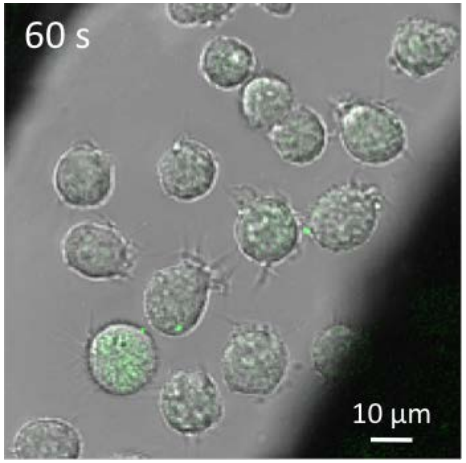


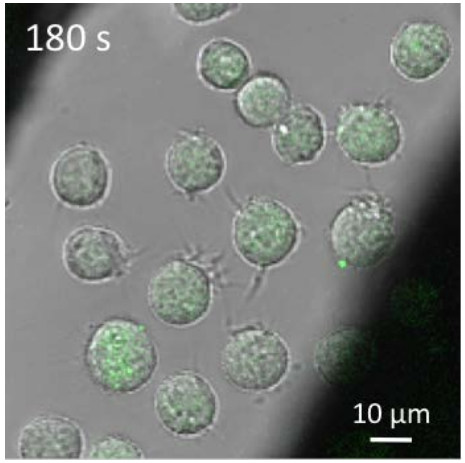
Winner, Pilla Award, BioEM 2016!

Esin Sözer





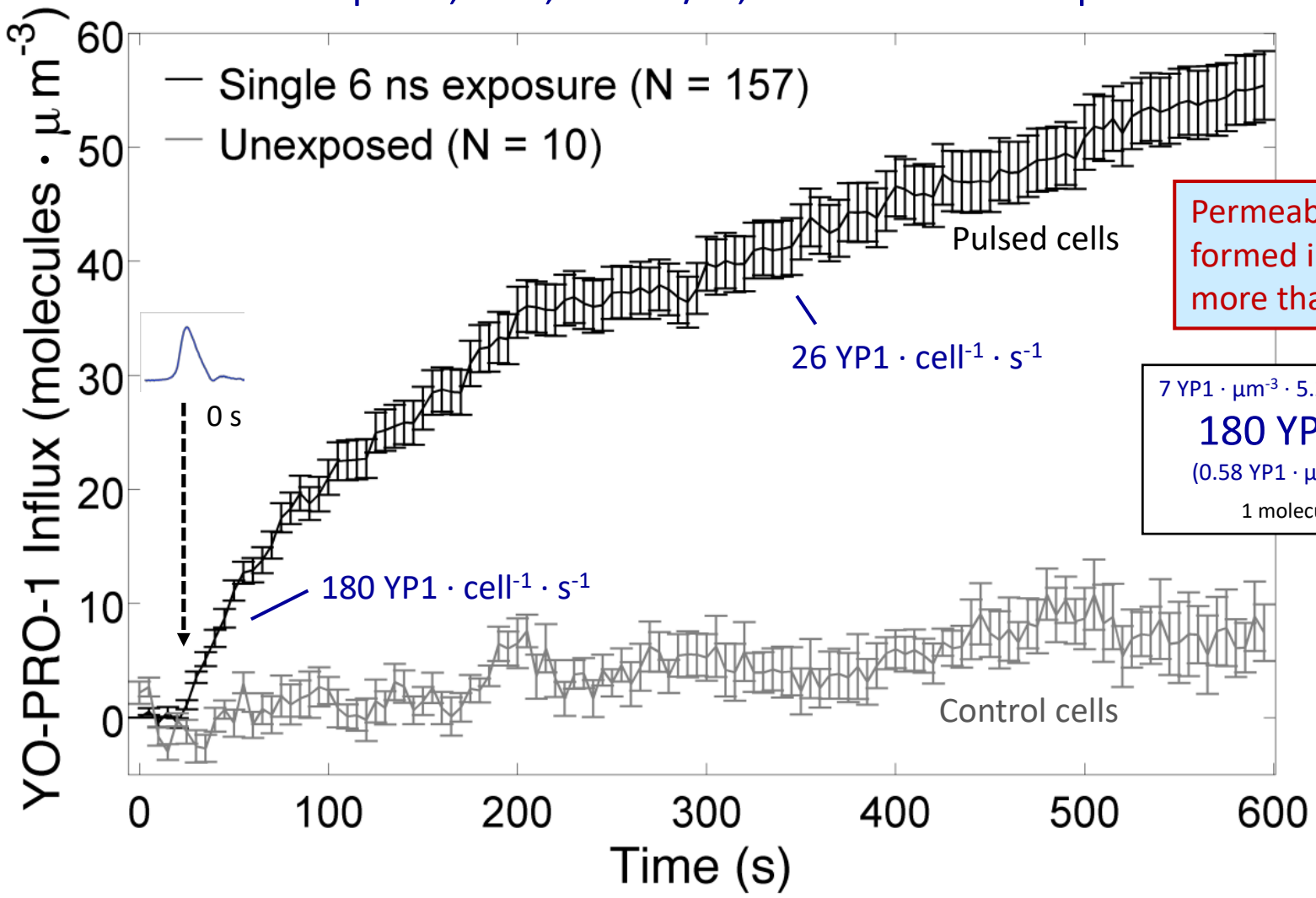




1. Wu, Y. H., D. Arnaud-Cormos, M. Casciola, J. M. Sanders, P. Leveque, and P. T. Vernier. 2013. Moveable wire electrode microchamber for nanosecond pulsed electric-field delivery. *IEEE Trans. Biomed. Eng.* 60:489-496.
2. Sözer, E. B., Z. A. Levine, and P. T. Vernier. 2017. Quantitative limits on small molecule transport via the electropermeome - measuring and modeling single nanosecond perturbations. *Sci Rep* 7:57.

Calibrated YO-PRO-1 Influx Into **Single-Pulse**-Permeabilized U-937 Cells

1 pulse, 6 ns, 20 MV/m; n = 157 from 9 experiments



Permeabilizing structures formed in ***6 ns*** persist for more than ***10 minutes***!

7 YP1 · µm^{-3} · 5.2 x 10^{2} µm^{3} · cell^{-1} / 20 s =

180 YP1 · cell^{-1} · s^{-1}

(0.58 YP1 · µm^{-2} (membrane) · s^{-1}).

1 molecule · µm^{-3} = 1.7 nM.

Influx of YP1 molecules after a single pulse, 6 ns, 20 MV/m. Images taken every 5 s; pulse delivered 22 s after the start of the recording. 157 cells monitored for 600 s in nine experiments. Error bars are standard error of the mean.

1. Sözer, E. B., Z. A. Levine, and P. T. Vernier. 2017. Quantitative limits on small molecule transport via the electropermeome - measuring and modeling single nanosecond perturbations. *Sci Rep* 7:57.

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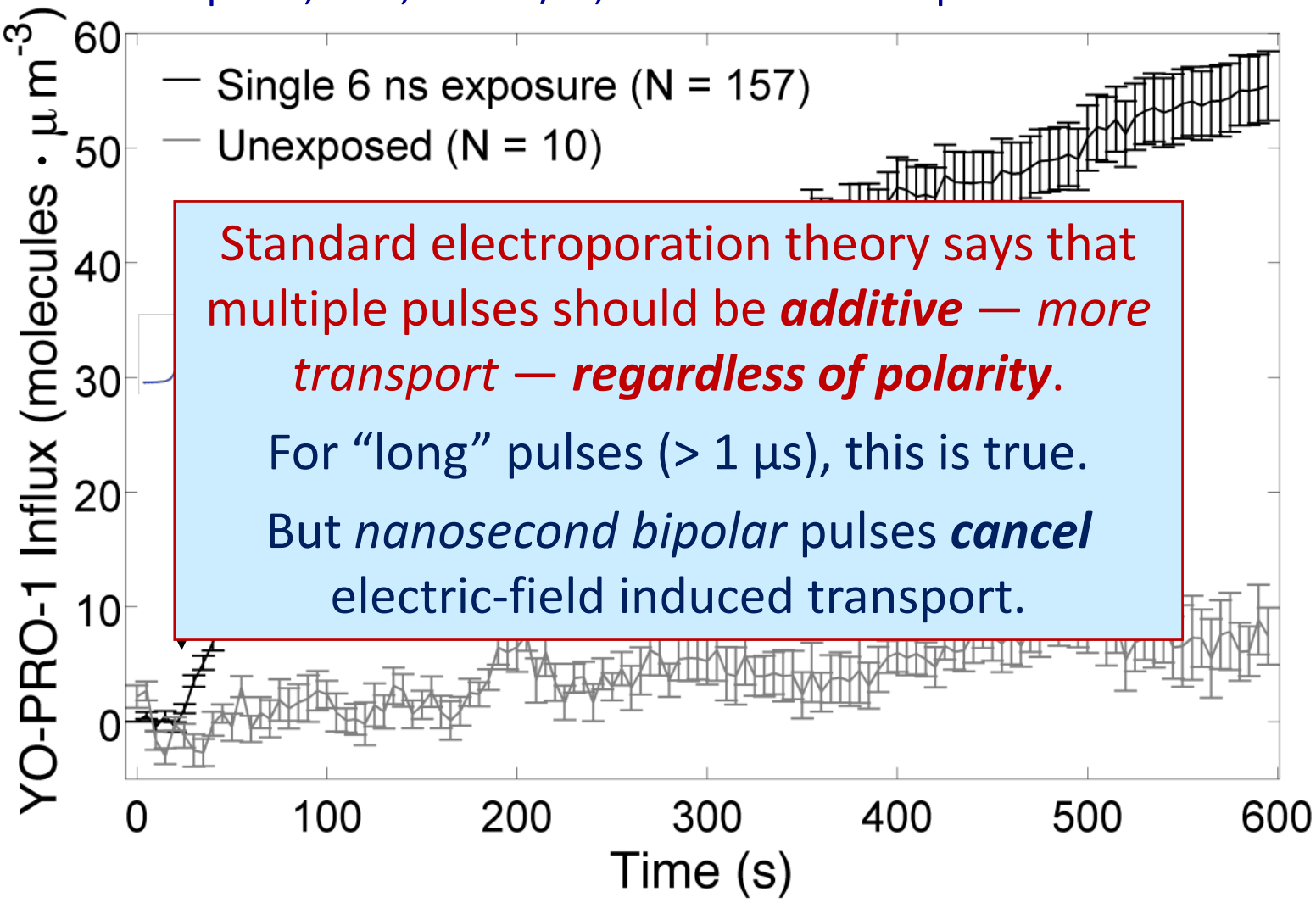


Standard electroporation theory says that multiple pulses should be ***additive*** — *more transport* — ***regardless of polarity***.

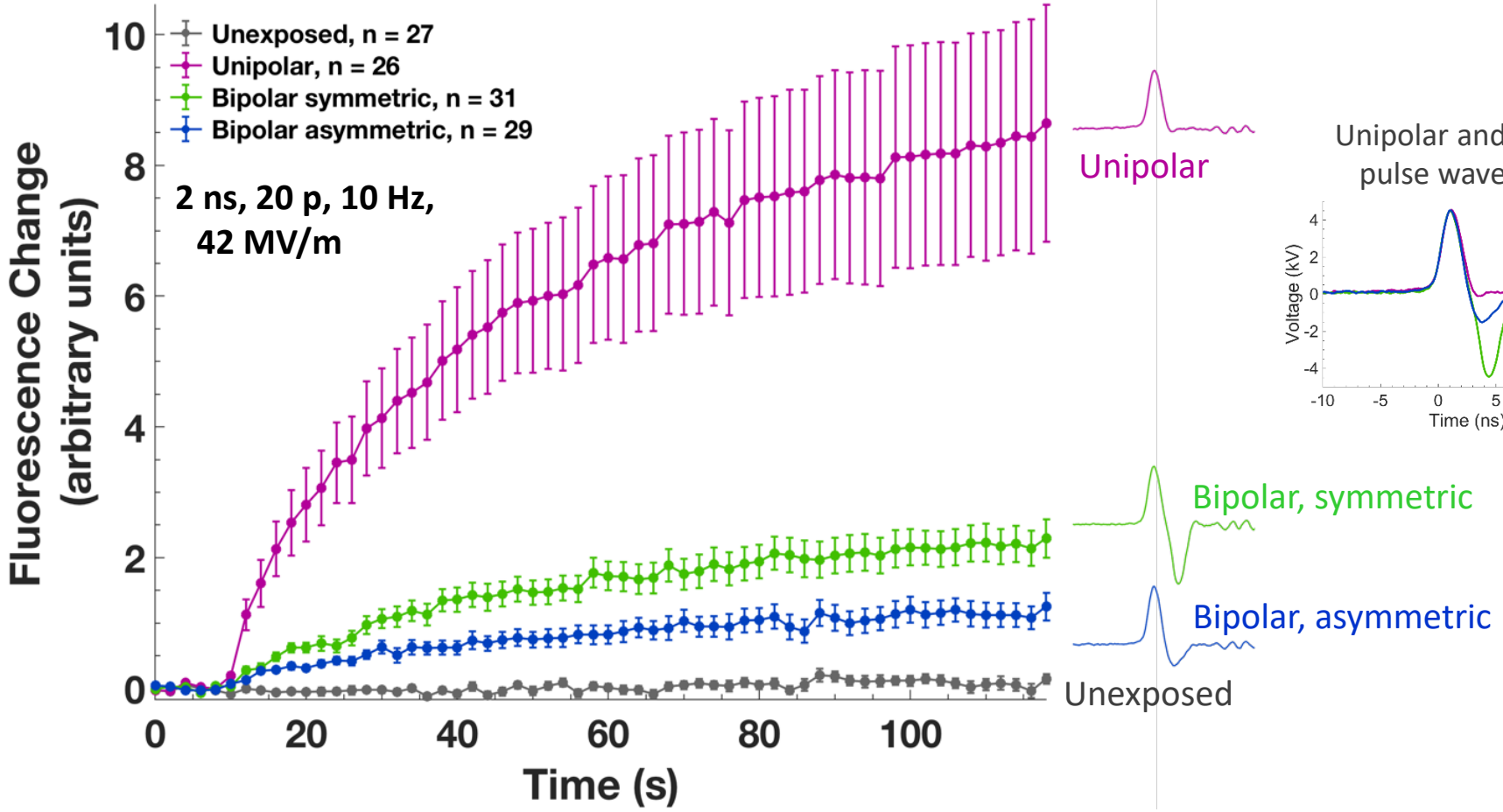
For "long" pulses (> 1 μs), this is true.

But *nanosecond bipolar* pulses ***cancel*** electric-field induced transport.

Influx of YP1 molecules after a single pulse, 6 ns, 20 MV/m. Images taken every 5 s; pulse delivered 22 s after the start of the recording. 157 cells monitored for 600 s in nine experiments. Error bars are standard error of the mean.

1. Sözer, E. B., Z. A. Levine, and P. T. Vernier. 2017. Quantitative limits on small molecule transport via the electropermeome - measuring and modeling single nanosecond perturbations. *Sci Rep* 7:57.

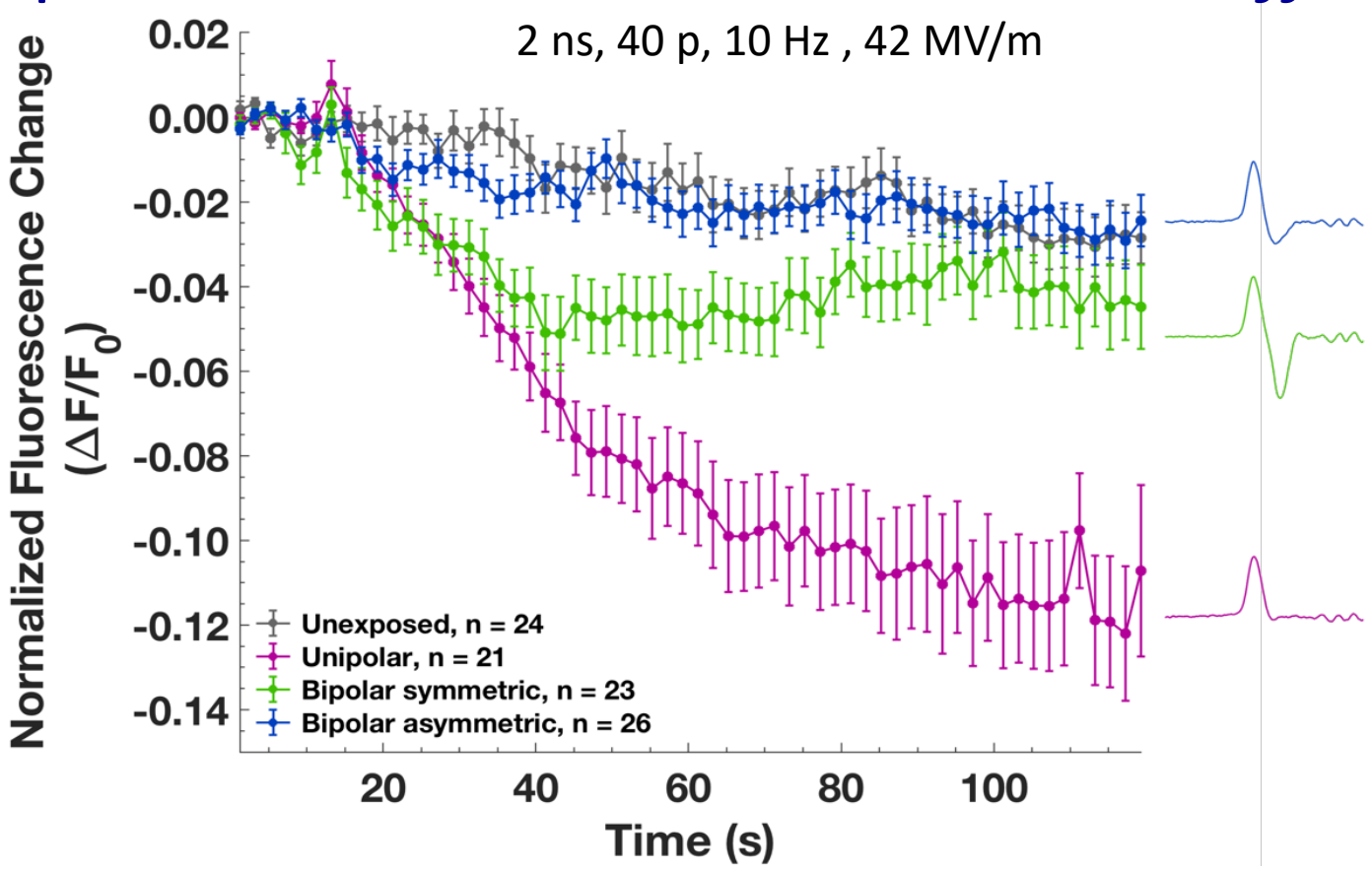
Attenuation of YO-PRO-1 Uptake By Multiple 2 ns Bipolar Pulses



2 ns bipolar pulse cancellation:

1. Observed for YO-PRO-1 (and Ca^{2+}, calcein, propidium) transport (and FM 1-43 fluorescence);
2. More effective with *asymmetric* (2^{nd} phase "smaller") pulses than with *symmetric* pulses.

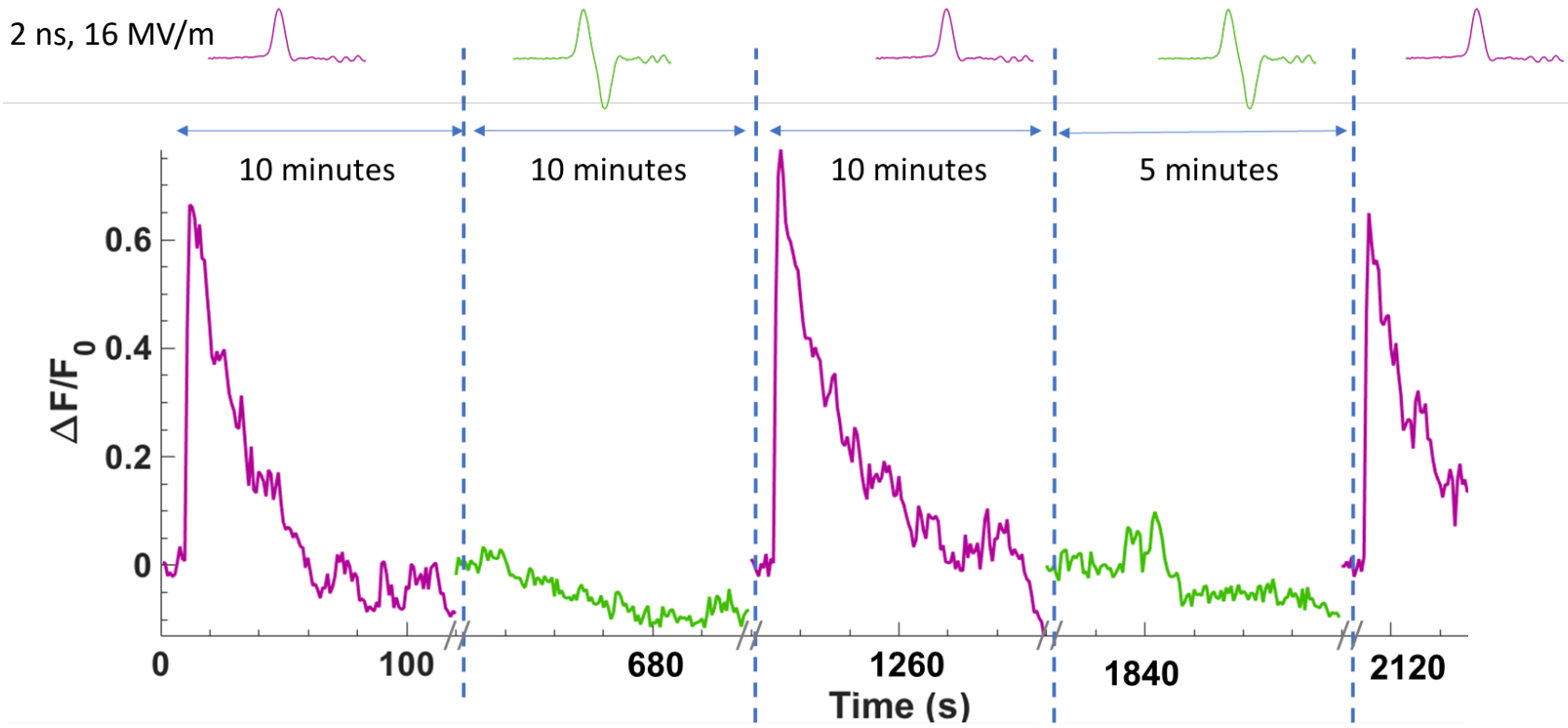
Bipolar Pulse Cancellation of Calcein *Efflux*



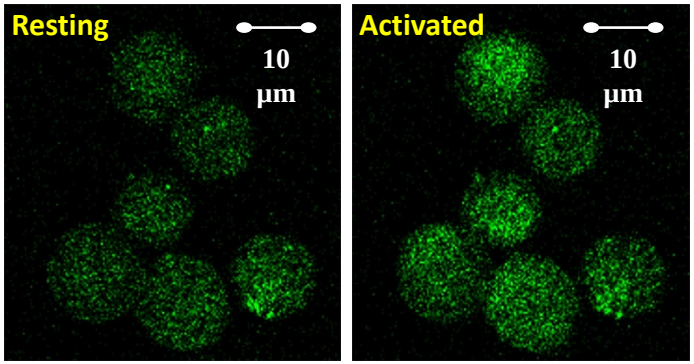
1. Calcein (anion) ***efflux*** is cancelled, similar to YO-PRO-1 (cation) ***influx***.
2. More effective cancellation with ***asymmetric*** (2nd phase "smaller") than with ***symmetric*** pulses — *similar to* results with longer pulses and different endpoints from **Task 1** (Pakhomov), **Task 5** (Pakhomova), **Task 6** (Craviso), and **AFRL San Antonio** (Ibey, Beier).

1. Sözer, E. B., C. F. Pocetti, and P. T. Vernier. 2018. Transport of charged small molecules after electropermeabilization — drift and diffusion. *BMC Biophys* 11:4.

Repetitive Bipolar Pulse (***2 ns***) Cancellation of Intracellular Ca^{2+} Increase in Adrenal Chromaffin Cells



Calcium Green-1 fluorescence.
Plots are mean of 10 cells.
Single pulse delivered 10 seconds into the recording in each interval.



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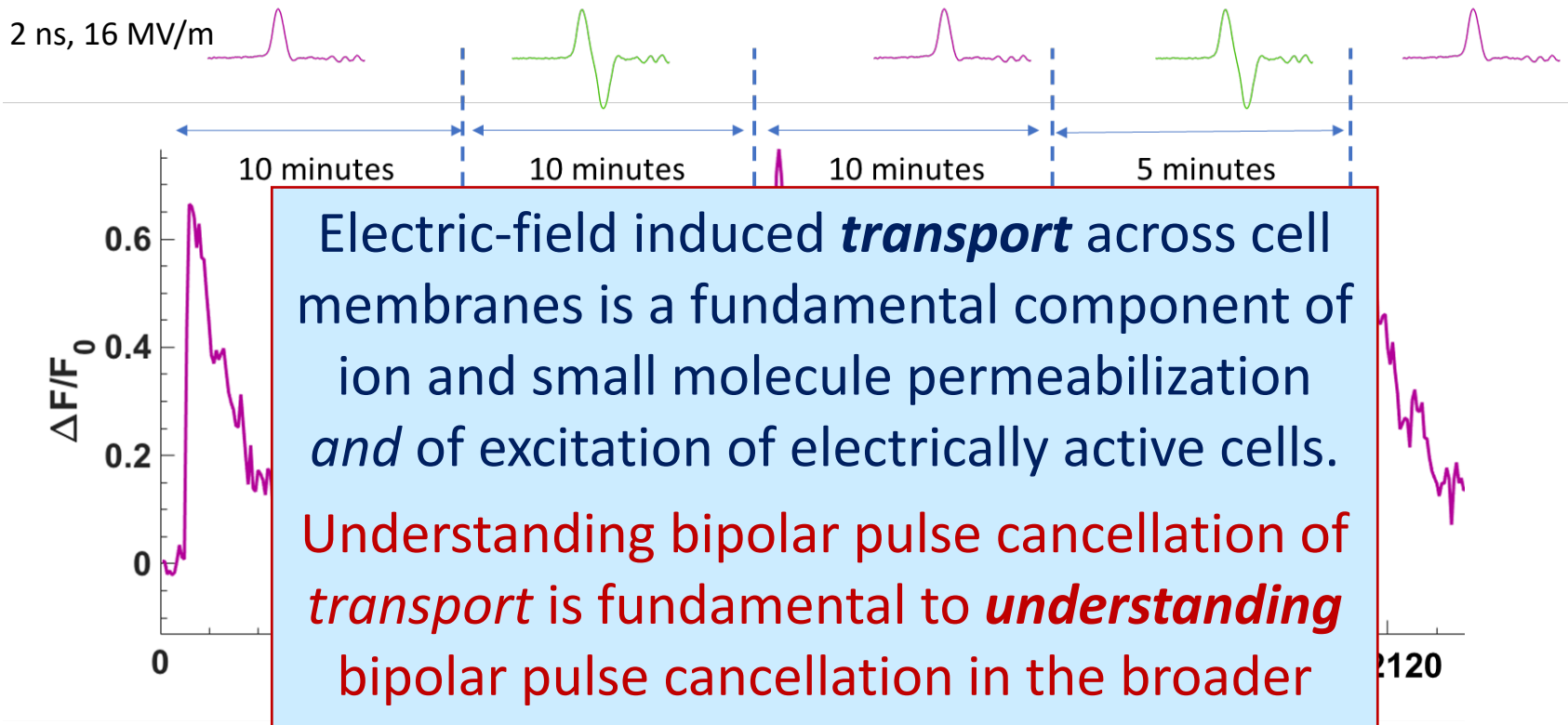
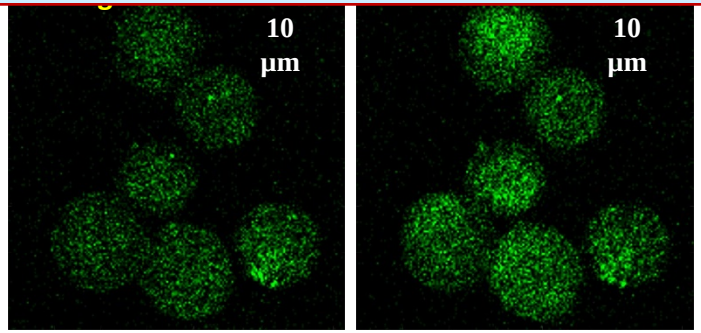


Electric-field induced ***transport*** across cell membranes is a fundamental component of ion and small molecule permeabilization *and* of excitation of electrically active cells.

Understanding bipolar pulse cancellation of *transport* is fundamental to ***understanding*** bipolar pulse cancellation in the broader sense, and to ***optimizing*** the applications.

Calcium Green-1 fluorescence.
Plots are mean of 10 cells.
Single pulse delivered 10 seconds into the recording in each interval.



Outline

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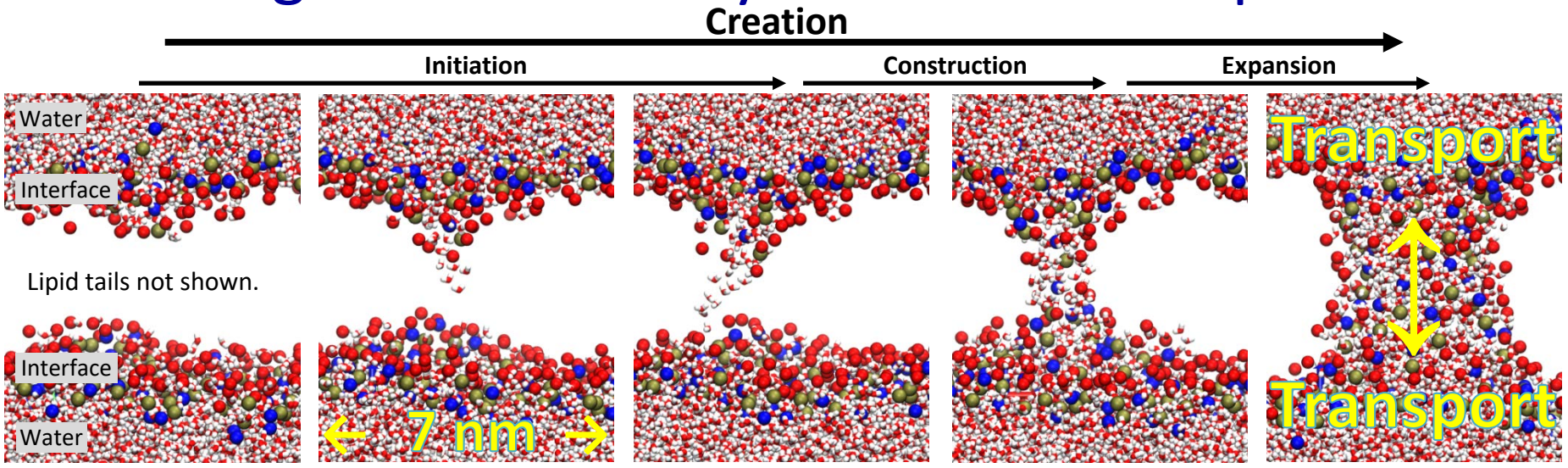
4. Cancellation and complexity.

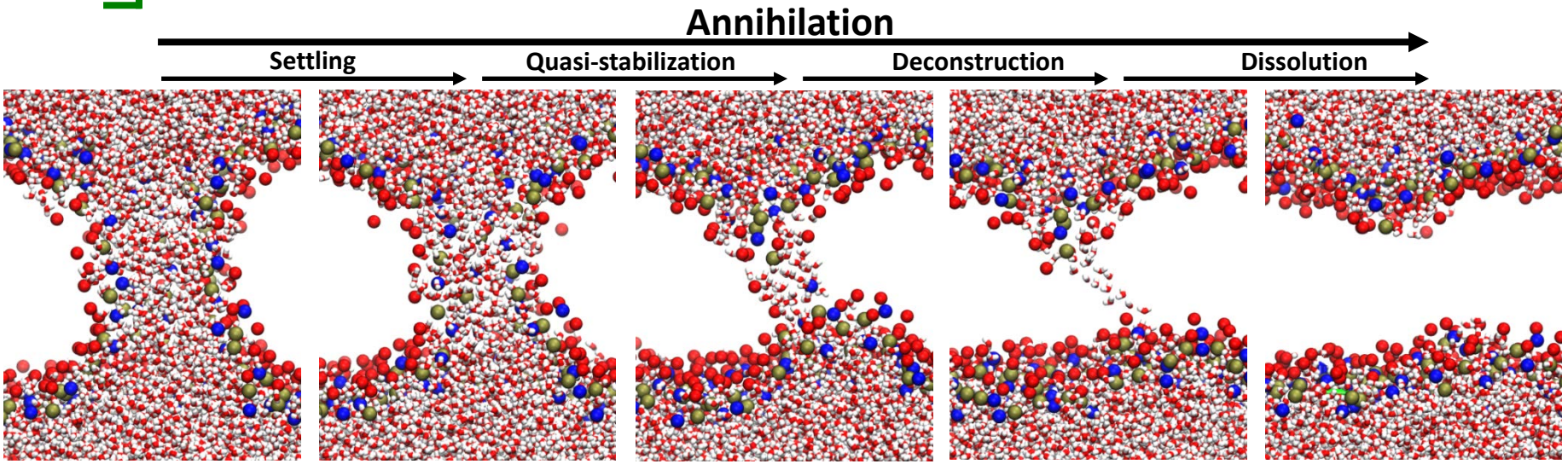
Stages in the Life Cycle of an Electropore



$E = E_{porating}$

Initiation is stochastic; Construction time is 1–2 ns.

Gold – P, Blue – N, Red (large) – acyl and ester O, Red (small) – water O, White – water H

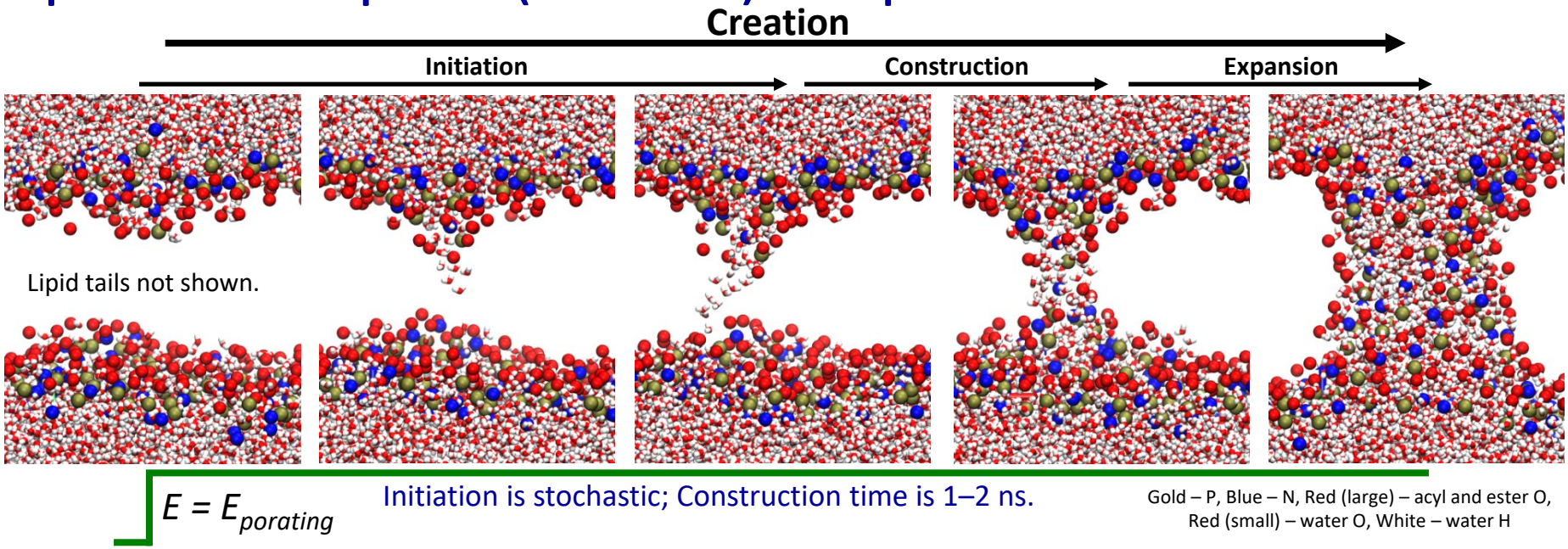


$E = 0$

Quasi-stabilization is a few nanoseconds; deconstruction is stochastic, but < 100 ns.

Levine, Z. A. and P. T. Vernier. 2010. Life cycle of an electropore: field-dependent and field-independent steps in pore creation and annihilation. *J. Membrane Biol.* 236:27-36.

Lipid Electropore (Lack of) Response to Field Reversal



Reversing the field does not reverse pore formation.

After water and head group dipole reorientation (≈1 ns), pore expansion continues.

Levine, Z. A. and P. T. Vernier. 2010. Life cycle of an electropore: field-dependent and field-independent steps in pore creation and annihilation. *J. Membrane Biol.* 236:27-36.

Mechanisms for Bipolar Pulse Cancellation

Assisted Membrane Discharge Hypothesis [1]

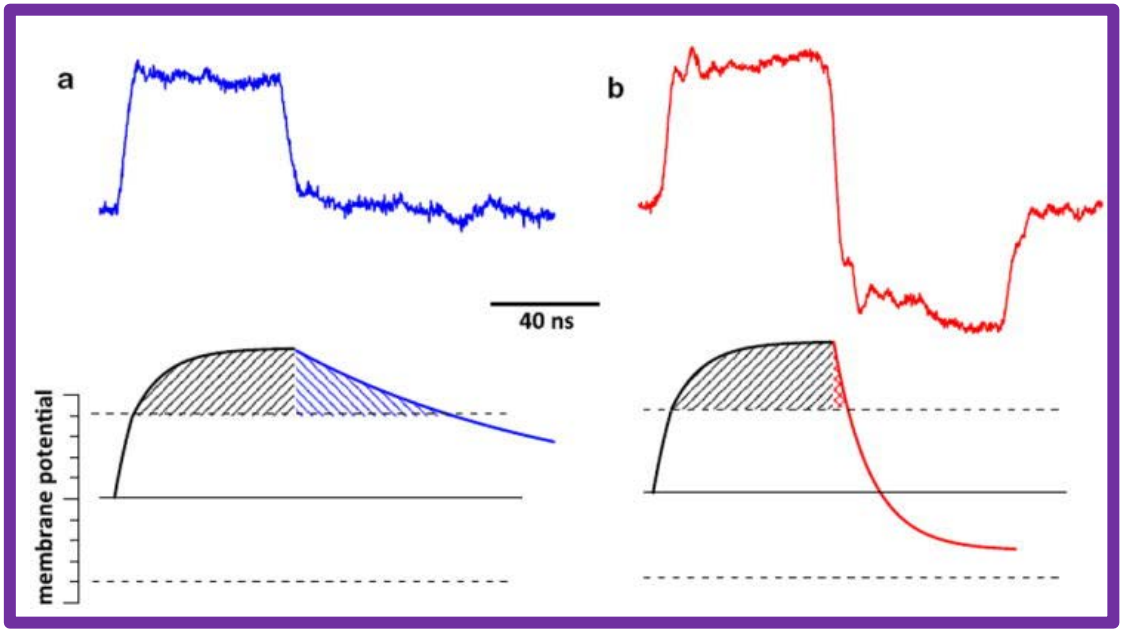


Fig. 6 [1]. The membrane voltage (arbitrary units) goes from the baseline (*solid line*) to the critical electroporation voltage (*dashed line*) and above it. The time when the membrane voltage exceeds the critical level is shown by *shading*. The bipolar pulse reduces this time but does not bring the voltage below the negative critical value.''

After unipolar pulse-induced charging, a cell membrane discharges over a period of hundreds of nanoseconds, a prolonged period of electrical stress.

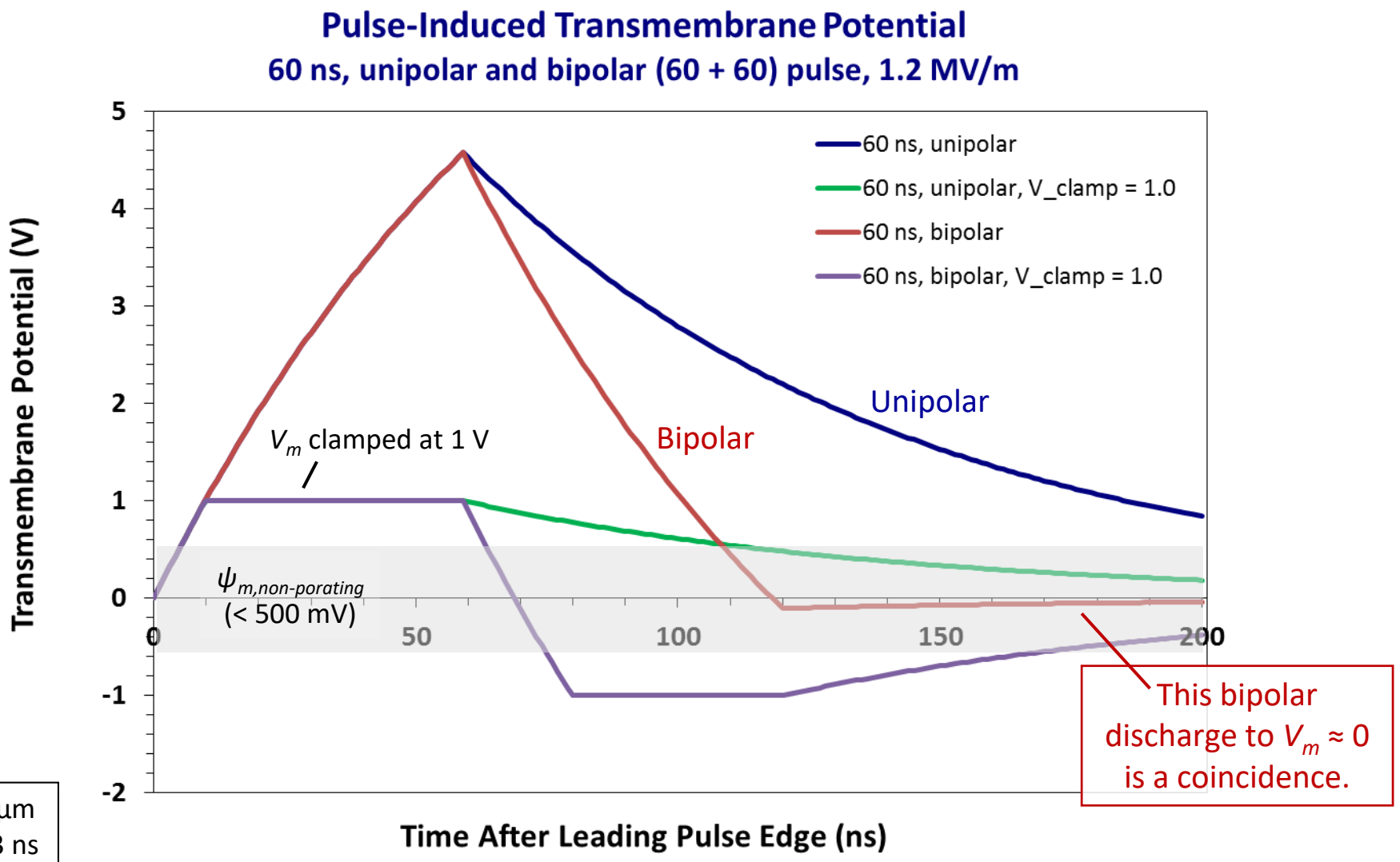
The second phase of a bipolar pulse could reduce the total time under stress.

This mechanism is based on the properties of the intact membrane, but it involves the transport of charged species ***to*** and ***through*** the permeabilized membrane!

1. Pakhomov, A. G., I. Semenov, S. Xiao, O. N. Pakhomova, B. Gregory, K. H. Schoenbach, J. C. Ullery, H. T. Beier, S. R. Rajulapati, and B. L. Ibey. 2014. Cancellation of cellular responses to nanoelectroporation by reversing the stimulus polarity. *Cell. Mol. Life Sci.* 71:4431-4441.
2. Vernier, P. T., Y. Sun, L. Marcu, C. M. Craft, and M. A. Gundersen. 2004. Nanoelectropulse-induced phosphatidylserine translocation. *Biophys. J.* 86:4040-4048.

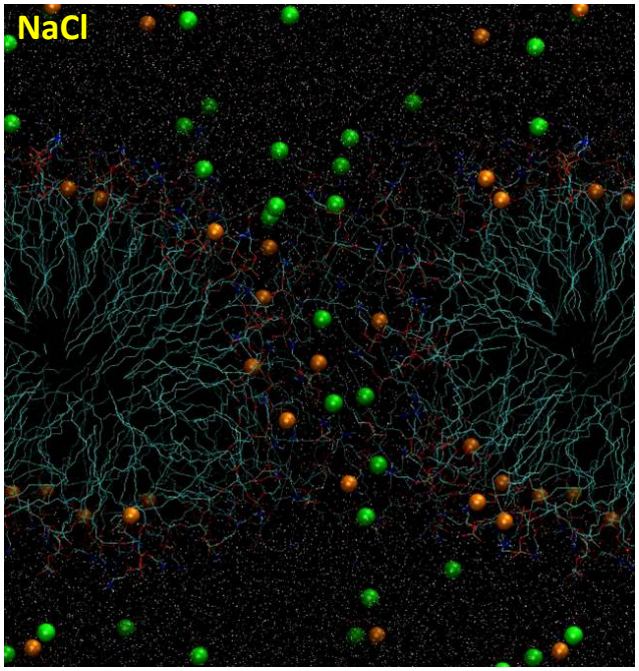
Charge-Discharge With *Unipolar* and *Bipolar* Pulses

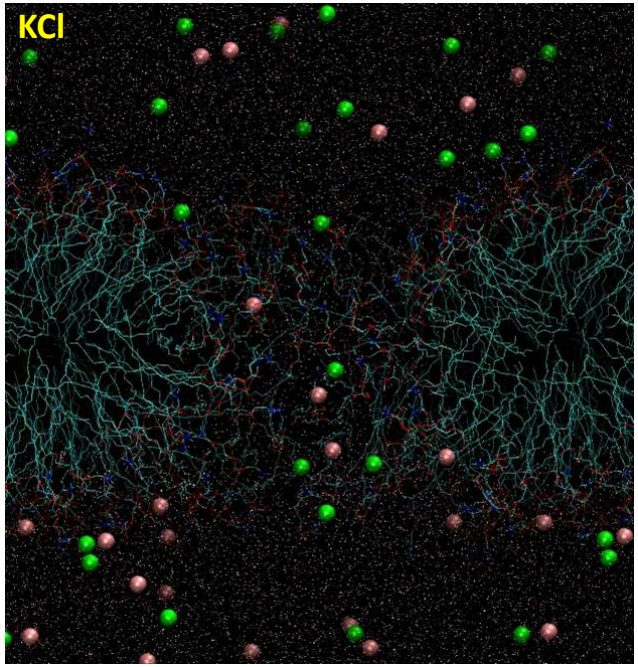
When $V_{membrane}$ cannot exceed some maximum value



Note: Post-***permeabilization*** charge equilibration kinetics are ***very different***.

Molecular Simulations of Electrophoretic Ion Transport Through Lipid Nanopores





Electric field-stabilized pores [1] in POPC bilayers with NaCl and KCl [2]

128 POPC

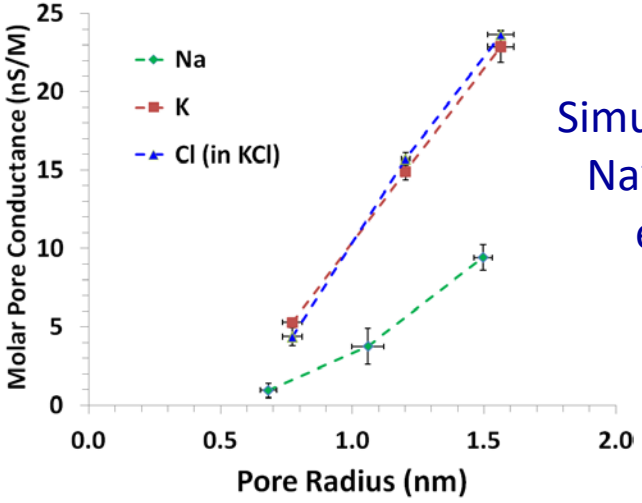
NaCl: 40 Na^+, 40 Cl^-, 8926 SPC water (0.11 M)

KCl: 22 K^+, 22 Cl^-, 8962 SPC water (0.12 M)

Lipid electropores: membrane discharge pathway?

Na ●
K ●
Cl ●

For $r_{pore,\ POPC}$ = 1 nm,
$G_{KCl,\ molar}$ = 10 nS/M [2] →
G_{pore} = 1 nS for 100 mM KCl.

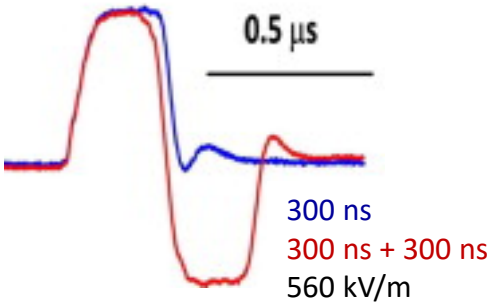


Simulated molar conductance of Na^+, K^+, and Cl^- for single lipid electropores in POPC [2]

1. Fernández, M. L., M. Risk, R. Reigada, and P. T. Vernier. 2012. Size-controlled nanopores in lipid membranes with stabilizing electric fields. *Biochem. Biophys. Res. Commun.* 423:325-330.
2. Ho, M. C., M. Casciola, Z. A. Levine, and P. T. Vernier. 2013. Molecular dynamics simulations of ion conductance in field-stabilized nanoscale lipid electropores. *J. Phys. Chem. B* 117:11633-11640.

Electrophoretic Sodium Transport Through Lipid Nanopores

Does ***Immediate (Bipolar Pulse) Depolarization*** "Heal" Permeabilization Damage?



Bipolar Cancellation by Assisted Membrane Discharge?

"E-field reversal can speed up membrane discharge and thereby reduce nsEP effects." [1]

How many Na^+ ions must be transported to ***discharge*** a membrane from V_m = 1 V?

$$\frac{C_{membrane}}{m^2} = \frac{1 \times 10^{-2}\ F}{m^2} = \frac{1 \times 10^{-2}\ C}{V \cdot m^2} =$$

$$\frac{1 \times 10^{-15}\ C}{1000\ mV \cdot \mu m^2} \cdot \frac{1\ Na^+}{1.6 \times 10^{-19}\ C} = \frac{6.2 \times 10^{4}\ Na^+}{1000\ mV \cdot \mu m^2} \longrightarrow 8 \times 10^7\ Na^+/cell$$

r_{cell} = 10 µm radius (A = 1.3 x 10^{-9} m^2, V = 4.2 x 10^{-15} m^3)

MD: For r_{pore} = 1 nm, G_{pore} = 450 pS ($[Na^+]_e$ = 150 mM), $V_{pore,\ charged}$ ≈ 200 mV, φ_{pore} = 0.56 Na^+/ns, r_{cell} = 10 µm, A_{pores} = 8.1 x 10^{-12} m^2, Na^+ transport = 1.4 x 10^6 Na^+/ns,…

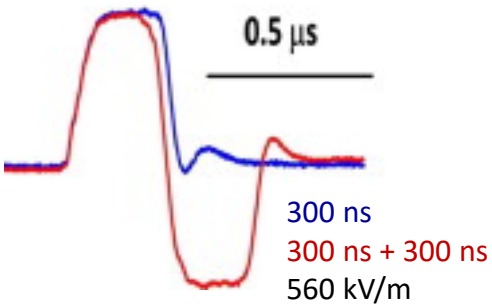
… a single 6 ns, 10 MV/m pulse (3 x 10^6 pores) will electrophoretically transport (*during* the pulse) 8 x 10^6 Na^+, 10% of the amount needed (8 x 10^7) to discharge the membrane.

(1 pulse ***could*** "depolarize" a membrane with $\psi_{m,resting}$ < 100 mV.)

1. Pakhomov, A. G., I. Semenov, S. Xiao, O. N. Pakhomova, B. Gregory, K. H. Schoenbach, J. C. Ullery, H. T. Beier, S. R. Rajulapati, and B. L. Ibey. 2014. Cancellation of cellular responses to nanoelectroporation by reversing the stimulus polarity. *Cell. Mol. Life Sci.* 71:4431-4441.

Diffusive Sodium Transport Through Lipid Nanopores

Molecular Models and Experimental Data — Membrane Discharge



How many Na^+ ions must be transported to ***discharge*** a membrane from V_m = 1 V?

r_{cell} = 10 µm radius (A = 1.3 x 10^{-9} m^2, V = 4.2 x 10^{-15} m^3)

$$\frac{C_{membrane}}{m^2} = \frac{1 \times 10^{-2}\ F}{m^2} = \frac{1 \times 10^{-2}\ C}{V \cdot m^2} =$$

$$\frac{1 \times 10^{-15}\ C}{1000\ mV \cdot \mu m^2} \cdot \frac{1\ Na^+}{1.6 \times 10^{-19}\ C} = \frac{6.2 \times 10^{4}\ Na^+}{1000\ mV \cdot \mu m^2} \longrightarrow 8 \times 10^7\ Na^+/cell$$

For r_{pore} = 1 nm, D_{Na+} = 1.33 x 10^{-9} $m^2 \cdot s^{-1}$, [Na^+] = 150 mM, l_{pore} = 5 nm, φ_{pore} = 5.7 x 10^7 $Na^+ \cdot s^{-1} \cdot pore^{-1}$, (0.057 Na^+/ns), N_{pore} = 2.6 x 10^6 pores,…

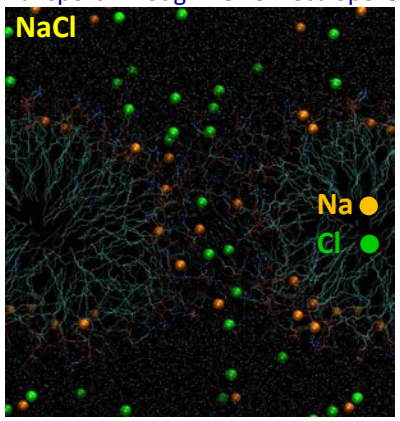
Na^+ transport = 5.7 x 10^7 $Na^+ \cdot s^{-1} \cdot pore^{-1}$ · 2.6 x 10^6 pore = 1.5 x 10^{14} Na^+/s.

… *diffusive transport* after a single 6 ns, 10 MV/m pulse (3 x 10^6 pores) ***will discharge the membrane in 500 ns*** at 2 x 10^{14} Na^+/s.

(But phase 2 of a bipolar pulse is effective 5 µs later.)

Cancellation mechanism(s) must be more than assisted membrane discharge.

Molecular Simulation of Sodium Transport Through POPC Electropore



Electric field-stabilized pore in POPC bilayer with NaCl [1]

128 POPC

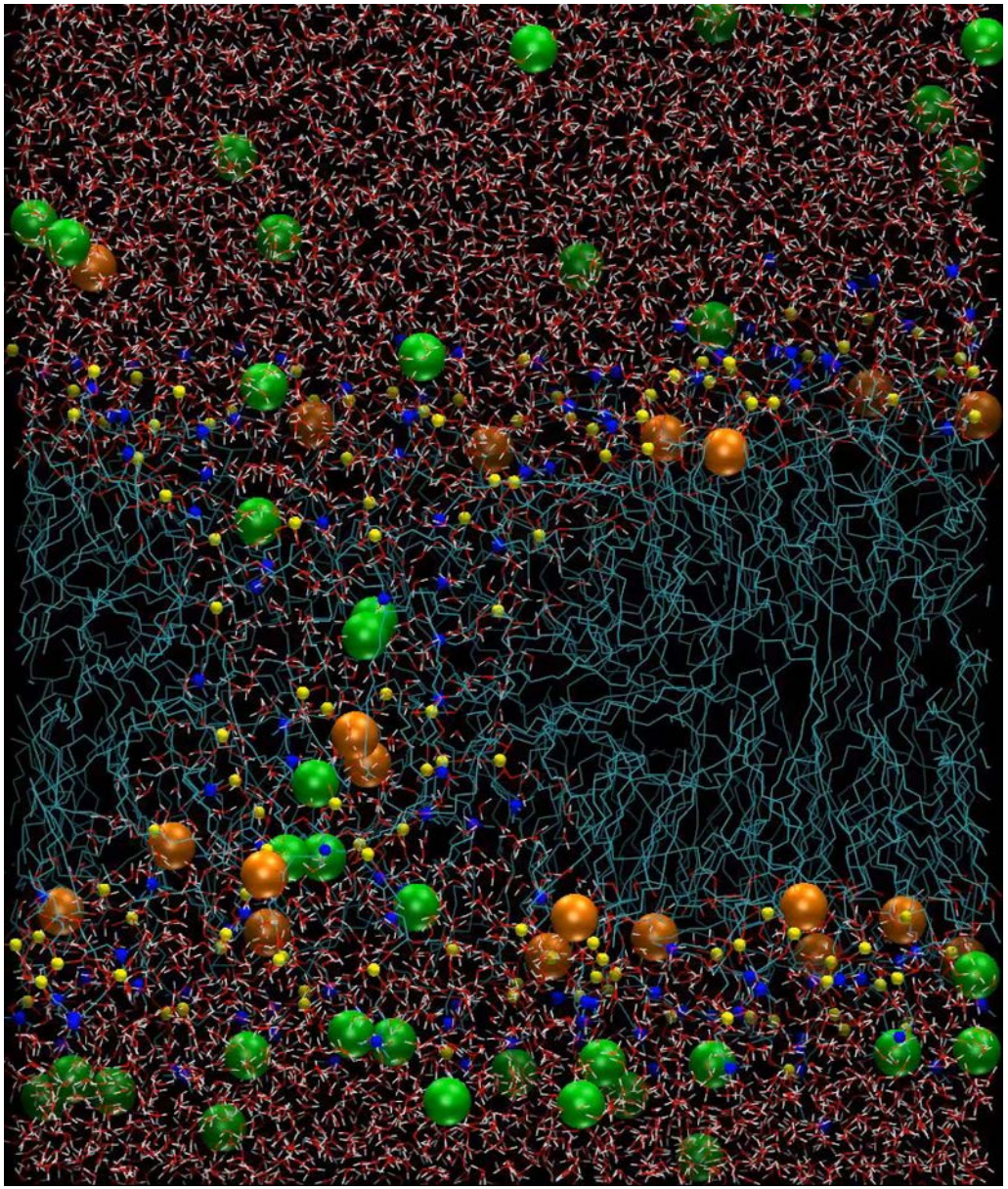
NaCl: 40 Na^+, 22 Cl^-, 8926 SPC water (0.11 M)

1. Ho, M. C., M. Casciola, Z. A. Levine, and P. T. Vernier. 2013. Molecular dynamics simulations of ion conductance in field-stabilized nanoscale lipid electropores. *J. Phys. Chem. B* 117:11633-11640.
2. Romeo, S., Y. H. Wu, Z. A. Levine, M. A. Gundersen, and P. T. Vernier. 2013. Water influx and cell swelling after nanosecond electropermeabilization. *Biochim. Biophys. Acta* 1828:1715-1722.

Outline

1. Bipolar pulse cancellation of multiple endpoints at the short end of “nanoseconds”.
2. Nanoscale ion transport through lipid electropores — Drift and diffusion currents, membrane charging and discharging (polarization and depolarization).
3. Nanoscale ion transport through lipid electropores — Competition with binding (Na^+, Ca^{2+}) to the phospholipid interface.
4. Cancellation and complexity.

Ca^{2+} Transport Through a Field-Stabilized Lipid Electropore

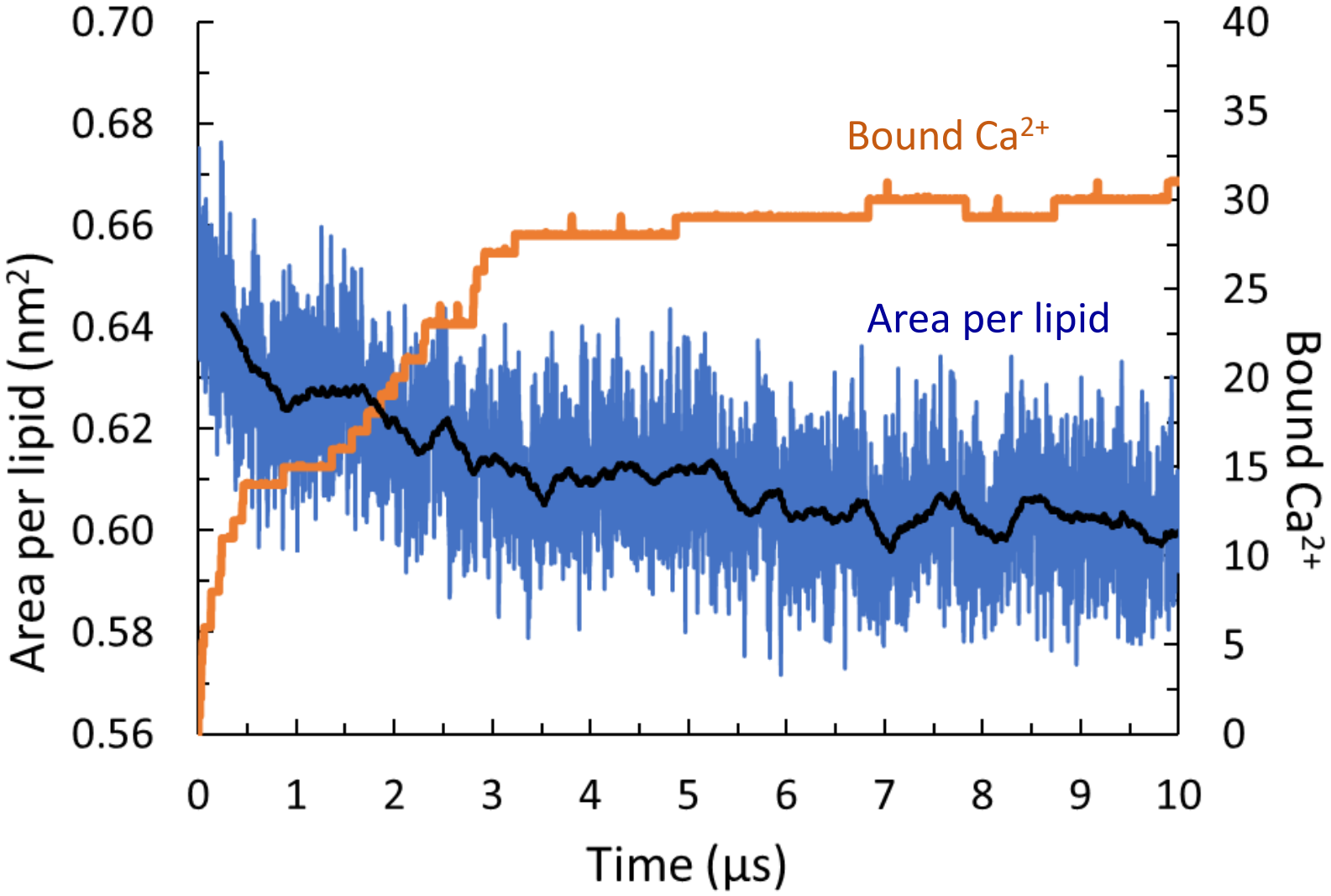


Federica Castellani

Simulated Ca^{2+}, Na^{+}, and K^{+} currents through lipid electropores provide boundaries on transport mechanisms for nanosecond pulse-induced membrane depolarization and ion flux.

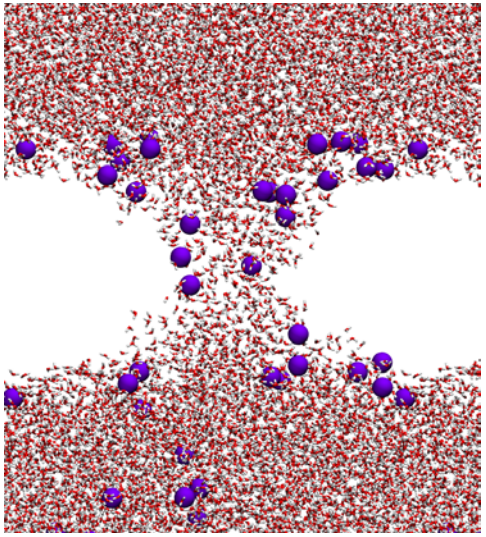
- Ca^{2+}
- Cl^{-}

Area Per Lipid and Ca^{2+} Binding To POPC



Area per lipid sampled every 10 ps, averaged every 1000 ps.
Ca^{2+} binding sampled every 100 ps, averaged every 1000 ps.

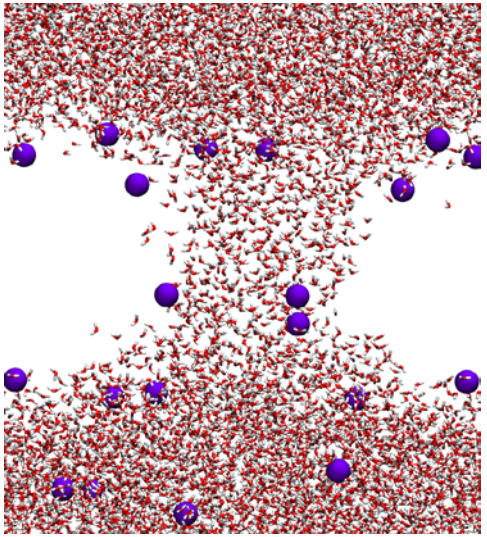
Ca^{2+}-Phospholipid Interface Binding Kinetics and Pore Annihilation Time



$\tau_{binding,Ca2+\text{-}POPC} \approx 10\ \mu s$; $\tau_{binding,K+\text{-}POPC} < 10$ ns.

$\tau_{annihilation,10ns\text{-}pore,K+} = \tau_{annihilation,100\mu s\text{-}pore,K+}$.

$\tau_{annihilation,10ns\text{-}pore,\ Ca2+} \neq \tau_{annihilation,100\mu s\text{-}pore,Ca2+}$.



Lipid electropores formed in POPC in the presence of Ca^{2+} will not reach Ca^{2+}-POPC equilibrium for pulse widths less than 10 µs.

Pores formed with ***10 ns*** pulses (pore walls ***not*** Ca^{2+}-saturated) will have a structure that is different from that of pores formed with ***100 µs*** pulses (Ca^{2+}-saturated pore walls) [1,2].

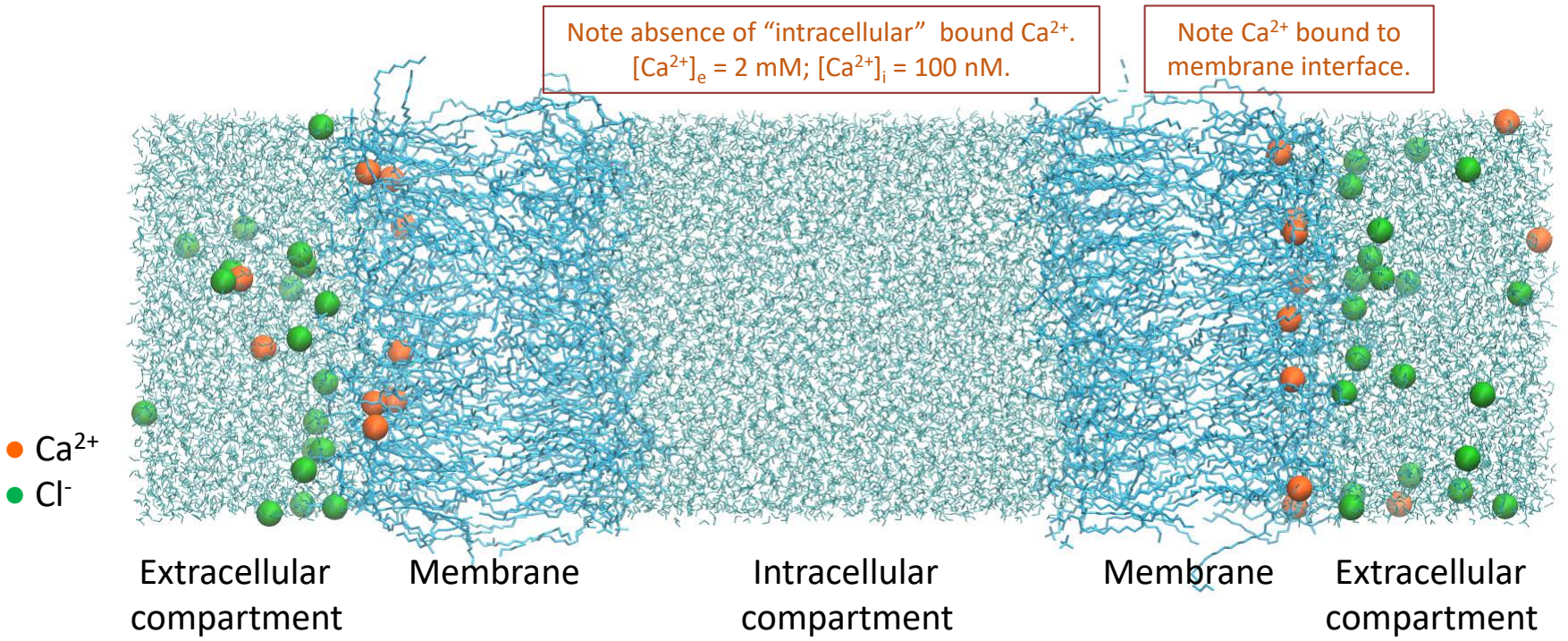
Is this why bipolar pulse cancellation works only with nanosecond pulses?

Multiple 10 ns pulse exposures may produce Ca^{2+}-saturated pores.

1. Böckmann, R. A., and H. Grubmüller. 2004. Multistep binding of divalent cations to phospholipid bilayers: A molecular dynamics study. *Angew. Chem. Int. Ed. Engl.* 43:1021-1024.
2. Levine, Z. A., and P. T. Vernier. 2012. Calcium and phosphatidylserine inhibit lipid electropore formation and reduce pore lifetime. *J. Membr. Biol.* 245:599-610.

Kinetics of Ca^{2+} Transport and Binding to Membrane Interface

Double lipid bilayer system — transport from high (extracellular) to low (intracellular) $[Ca^{2+}]$



Ca^{2+} binds to a phospholipid bilayer interface. ***Equilibration occurs in two stages over about 10 μs*** (new!!) in molecular simulations.

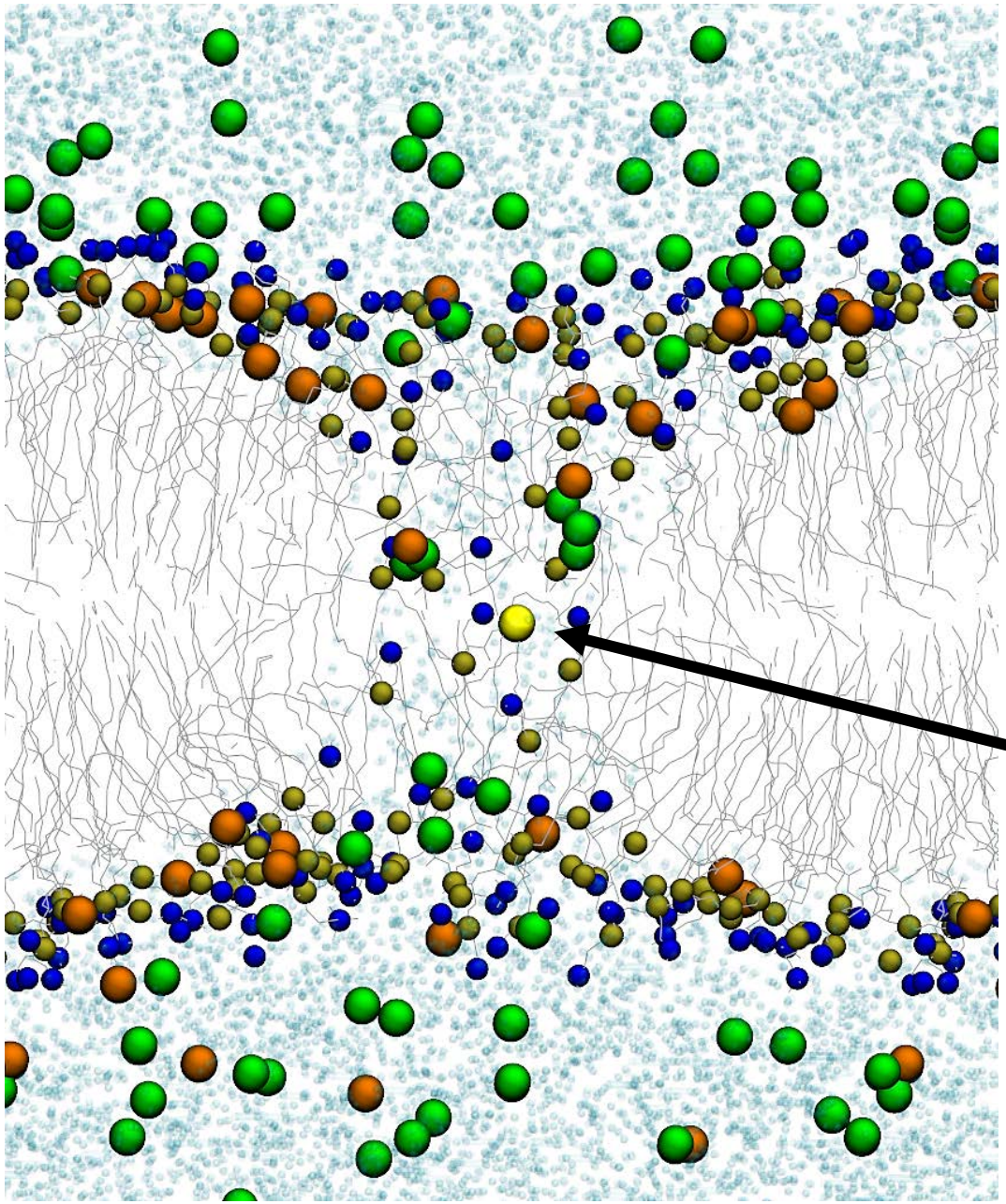
Cells maintain a 10000:1 extracellular:intracellular Ca^{2+} concentration gradient.

How is Ca^{2+} redistributed during and after lipid electropore formation?

Does Ca^{2+} diffuse freely into the cytoplasm (available for signaling) or is it partitioned quickly into the intracellular leaflet of the lipid bilayer?

What happens when the electric field polarity reverses (bipolar pulse)?

Nanoscale Material Transport Through Membrane Defects and Discontinuities

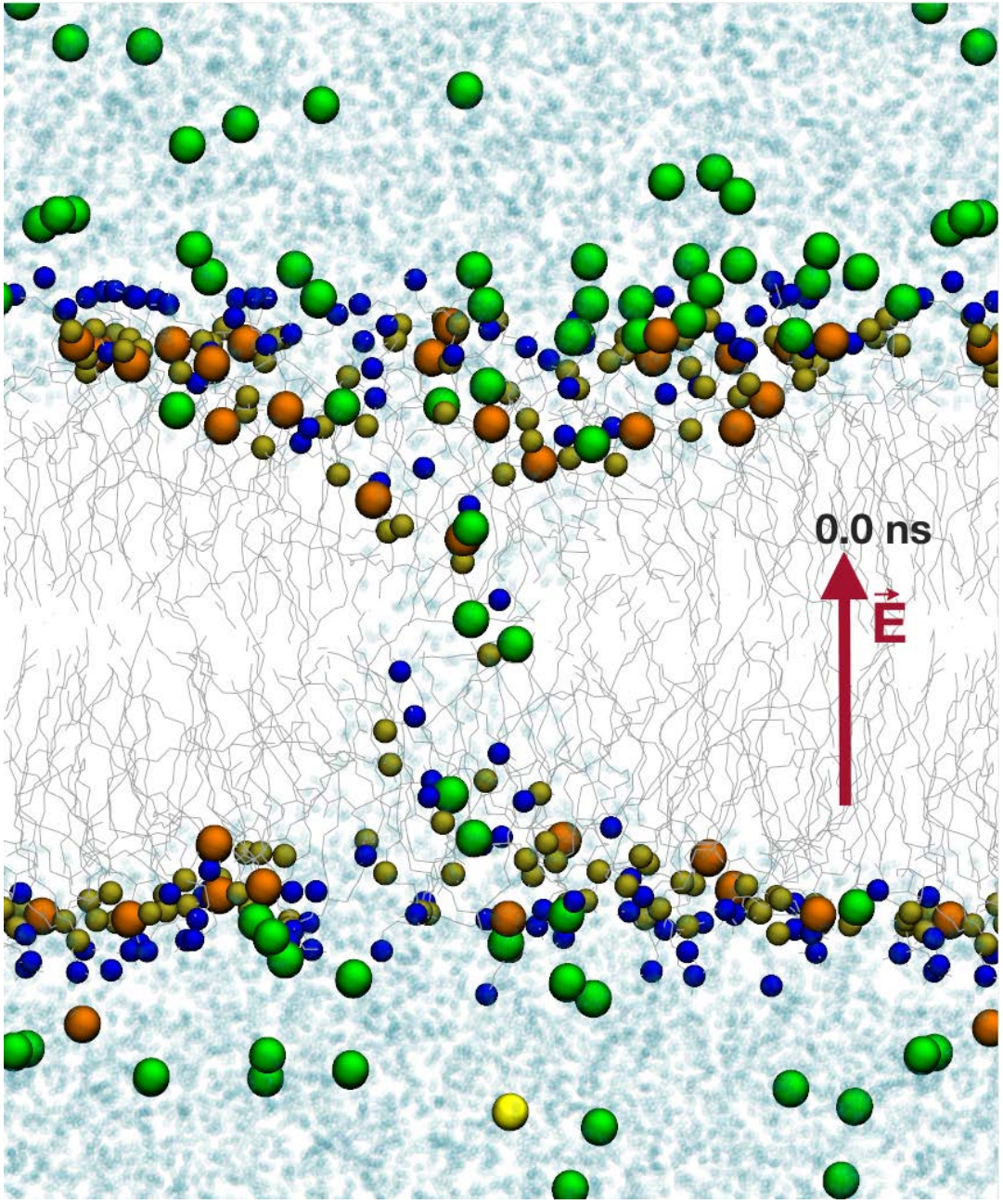


Ca^{2+} and Cl^{-} in a porated POPC bilayer

Highlighted Ca^{2+}

Light blue – water molecules
Gray – hydrocarbon tails
Orange – calcium ions
Green – chloride ions
Blue – nitrogen atoms
Gold – phosphorus atoms

Nanoscale Material Transport Through Membrane Defects and Discontinuities



Ca^{2+} and Cl^{-} bipolar pulse-driven (66 MV/m) electro-transport in a porated POPC bilayer

Field reverses at 2.1 ns.

Light blue – water molecules
Gray – hydrocarbon tails
Orange – calcium ions
Green – chloride ions
Blue – nitrogen atoms
Gold – phosphorus atoms

Graphics and video by Federica Castellani and Esin Sözer with VMD and Matlab.

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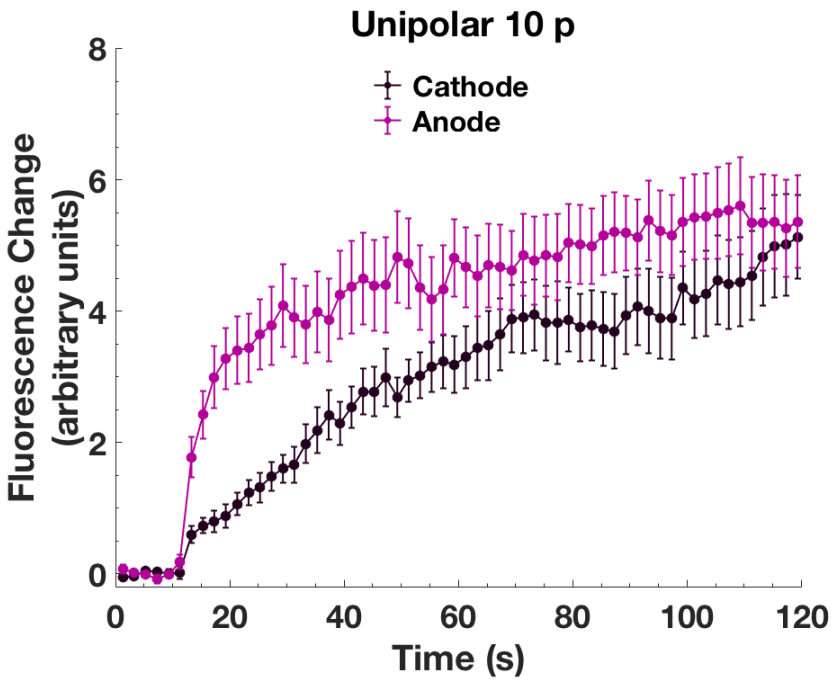
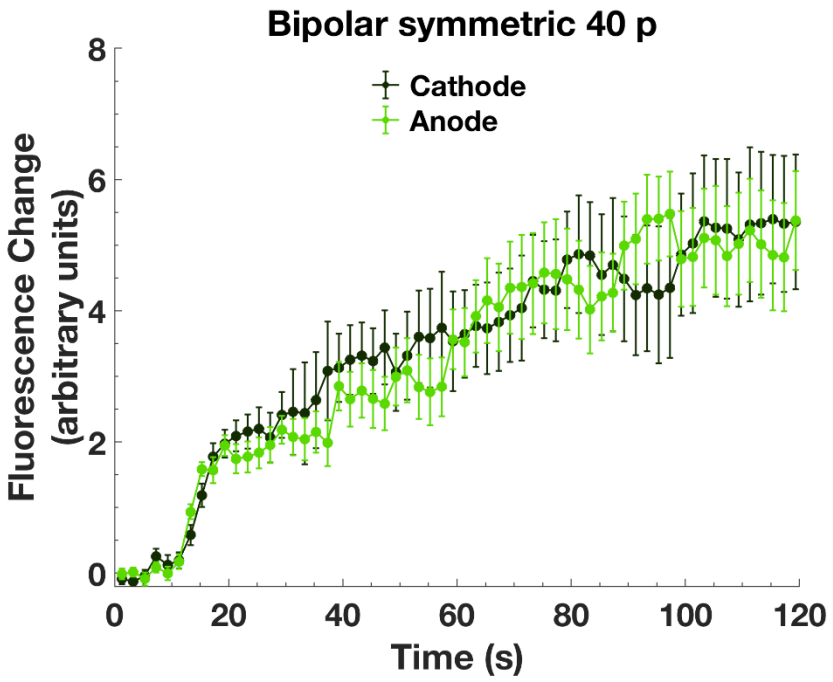
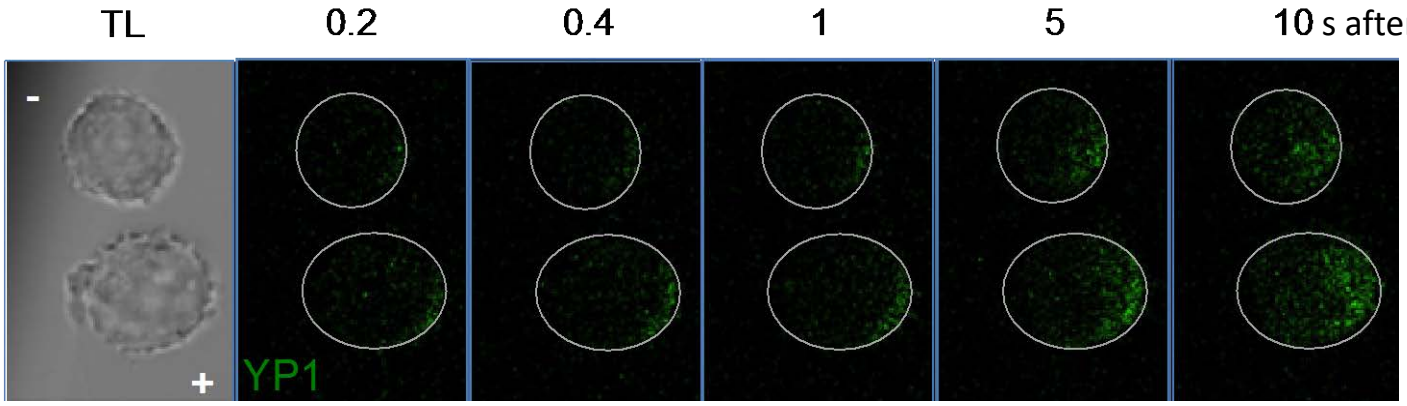
Bipolar Cancellation of Molecular Transport Pattern of YO-PRO-1

Equally permeabilizing number of pulses



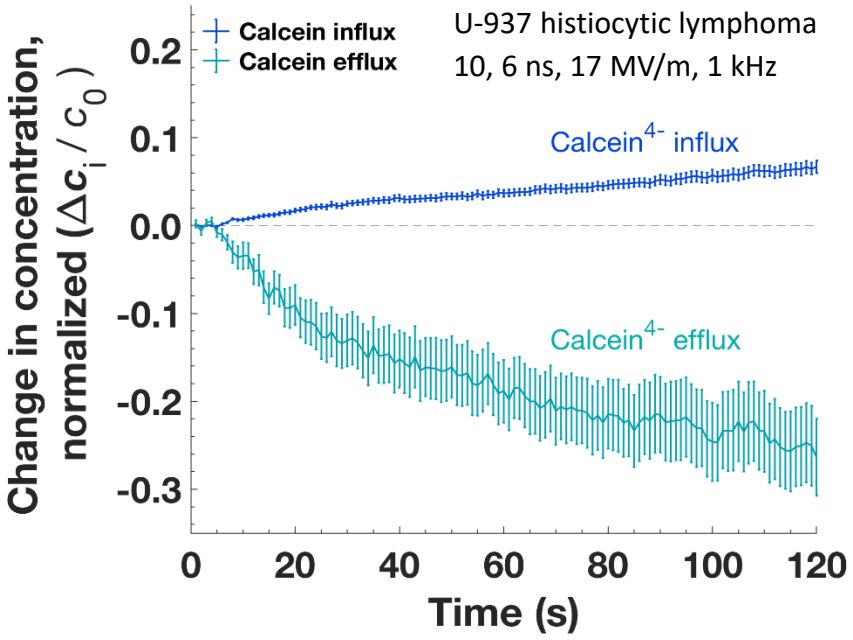




Images from: Sözer, E. B., C. F. Pocetti, and P. T. Vernier. 2017. Asymmetric patterns of small molecule transport after nanosecond and microsecond electropermeabilization. *J Membr Biol.*, in press.

Collaborative Initiative With Joshua Zimmerberg at NICHD, NIH

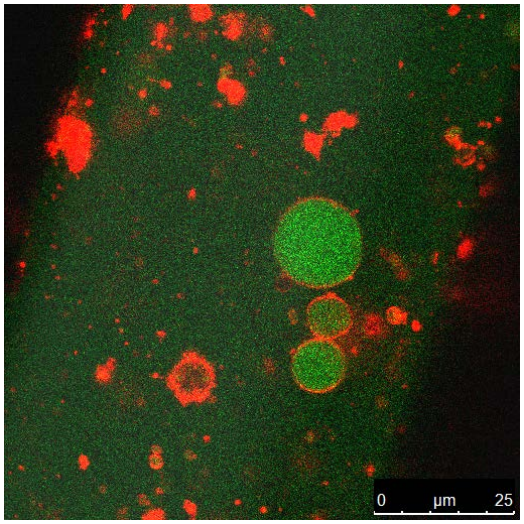
"A molecular understanding of complex events at the cell membrane requires the synergy of approaches and perspectives."
https://irp.nih.gov/pi/joshua-zimmerberg



Joshua Jay Zimmerberg, M.D.,Ph.D., Senior Investigator, Section on Membrane and Cellular Biophysics, NICHD/DIR

Objectives at NIH (First visit, Sözer, 2018 Apr 30)

1. Determine whether molecular transport of calcein after 6 ns electropermeabilization is similar for GUVs and cells.
2. Compare membrane permeabilization by laser and by 6 ns electric pulse exposure by imaging calcein-loaded GUVs after laser pulses of varying intensity to find transport equivalent to that observed in objective 1.
3. Correlate with anticipated GUV results from Task 4 (Yakovlev).

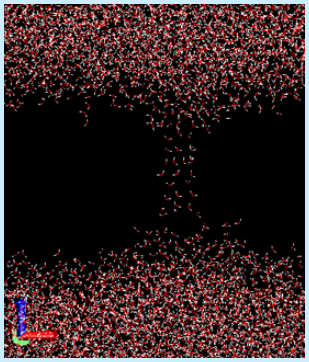


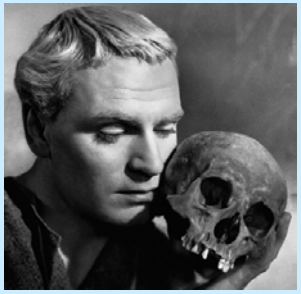
Calcein-loaded, DiI-labeled GUVs.

Summary

1. Bipolar pulse cancellation of small molecule transport is observed with pulses as short as 2 ns. This extends the stimulus time-frequency boundary for applications like CAN-CAN nearly to picoseconds-gigahertz.
2. “Undo” (cancellation by the second phase of a bipolar pulse) occurs in 2 ns, and must almost certainly be a one-step, physical, process. Residence time ***in*** the phospholipid interface for Ca^{2+}, propidium, and YO-PRO-1 is significantly longer than 2 ns, so “undo” is not removal from the interface.
3. Molecular simulations indicate that lipid pores ***could*** provide a pathway for the hypothetical facilitated discharge, based on ion transport, but with arbitrary parametric constraints.
4. The time scale of Ca^{2+}-phospholipid binding kinetics is similar to the bipolar phase 2 delay window for cancellation.
5. Cancellation complexity spurs collaborative synergy: University of Nevada, Reno; MIT; AFRL San Antonio; NICHD, NIH.

Biomolecular Manipulations with Uni- and Bipolar Pulsed Electric Fields





… and now remains
That we find out the cause of this effect,
Or rather say, the cause of this defect,
For this effect defective comes by cause.

Polonius, *Hamlet, Act ii. Sc. 2*.

Bipolar Pulse Attenuators — 2018


Delia Arnaud-Cormos


Maura Casciola


Federica Castellani


Gale Craviso


M. Laura Fernández


Nadica Ivošević DeNardis


Peter Kramar


Norm Leblanc


Philippe Leveque


Zach Levine


Caterina Merla


Andrei Pakhomov


Olga Pakhomova


Marcelo Risk


Iurii Semenov


Esin Sözer


Jim Weaver

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