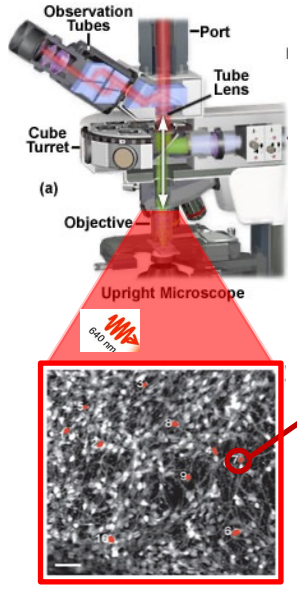
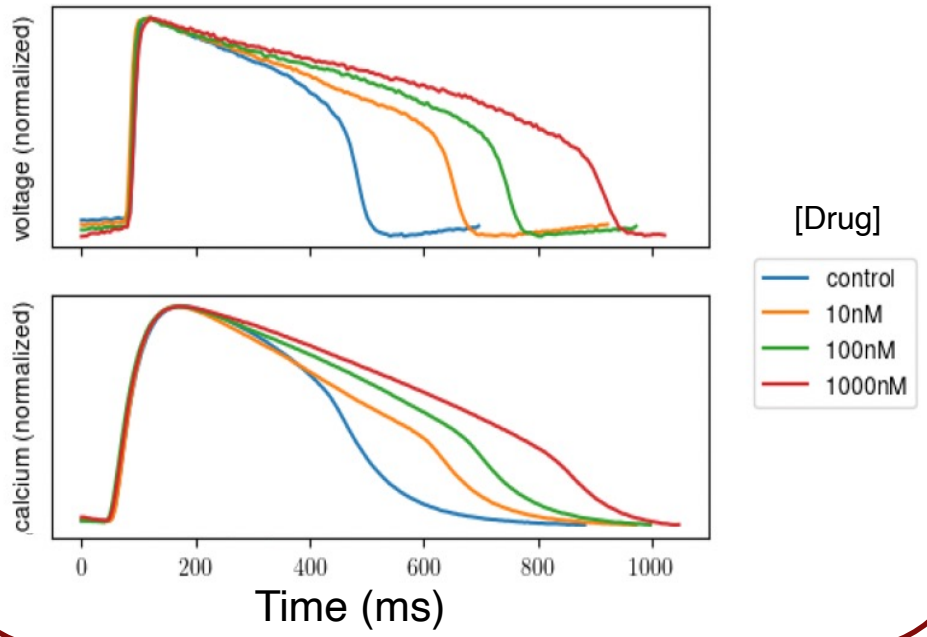
Cardiac MPS assay measurements

FLUORESCENCE MICROSCOPY

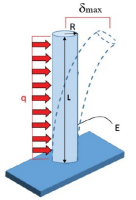


Optically recorded voltage and calcium



MECHANICS

Engineered post deformation

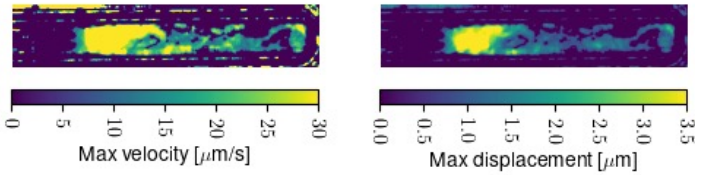


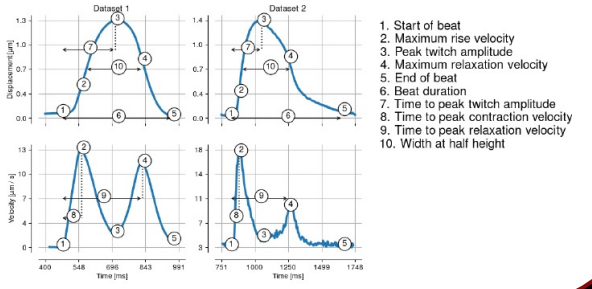
$$F = qL = \frac{8EI\delta_{max}}{L^3}$$

with $\delta_{max} = \frac{qL^4}{8EI}$

And $I = \frac{1}{4}\pi R^4$

Pixel tracking

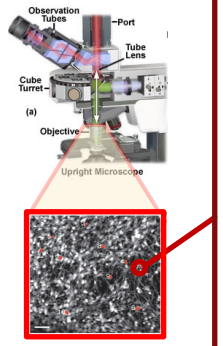




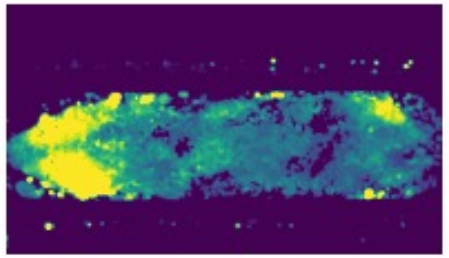
1. Start of beat
2. Maximum rise velocity
3. Peak twitch amplitude
4. Maximum relaxation velocity
5. End of beat
6. Beat duration
7. Time to peak twitch amplitude
8. Time to peak contraction velocity
9. Time to peak relaxation velocity
10. Width at half height

Measurement constraints in MPS data streams

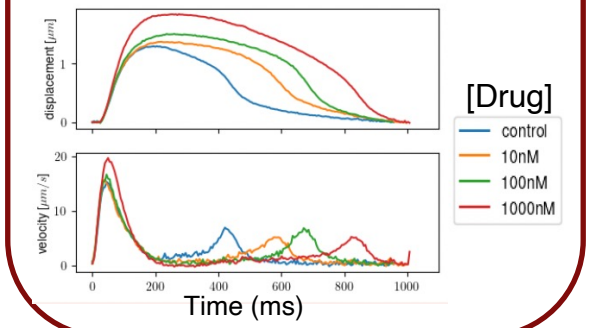
ELECTRO-MECHANICAL INVERSE PROBLEM



Optical tissue displacement

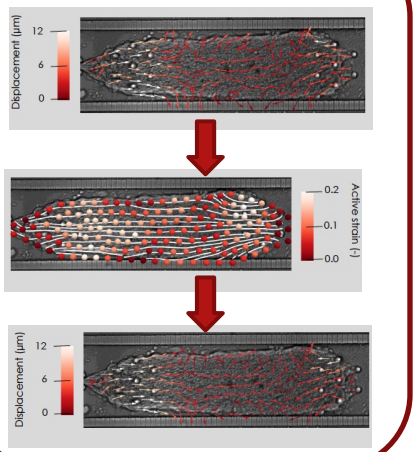


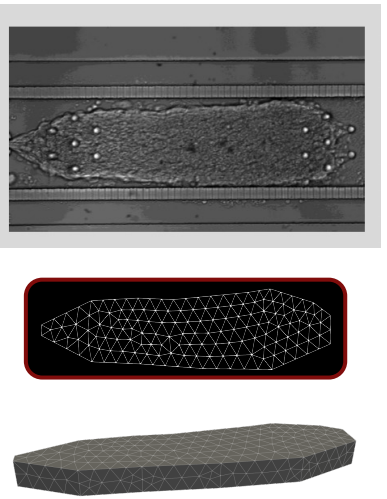
Optical displacement and velocity



$\min(C_{(d,m)})$

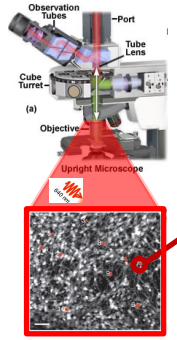
Simulated tissue displacement

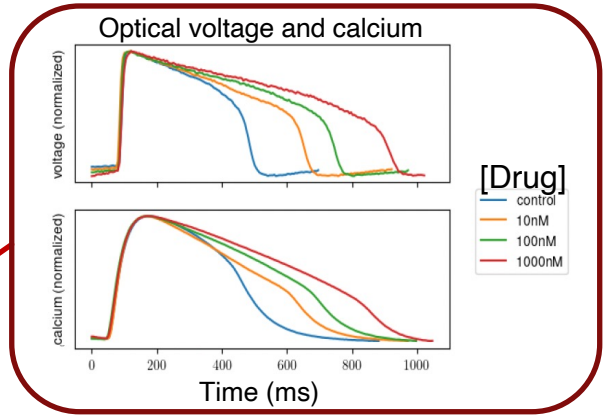




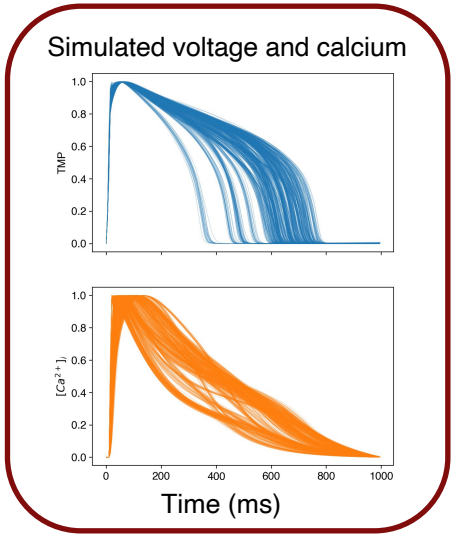
Measurement constraints in MPS data streams

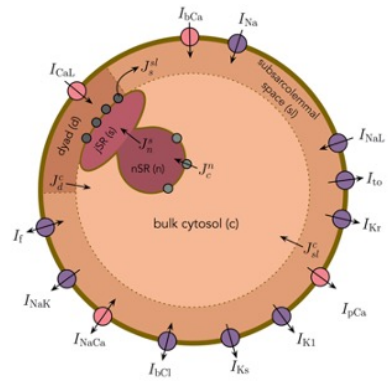
ELECTRO-IONIC INVERSE PROBLEM





$\min(C_{(d,m)})$

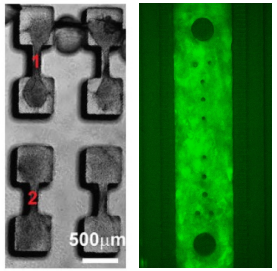




Measurement constraints in MPS data streams

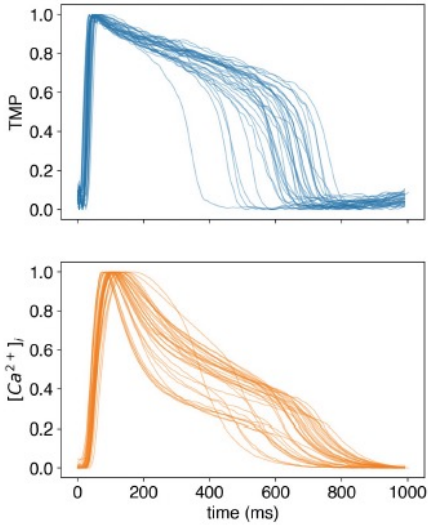
SOURCES OF VARIABILITY IN THE MICROTISSUE ASSAYS

Observational unit



HTS

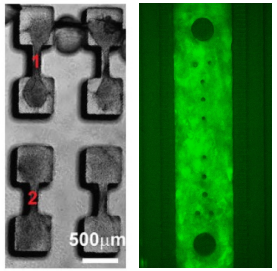
MPS



Measurement constraints in MPS data streams

SOURCES OF VARIABILITY IN THE MICROTISSUE ASSAYS

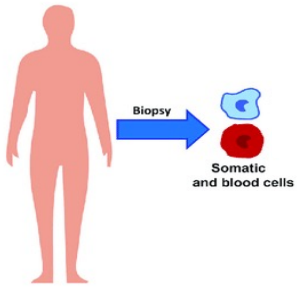
Observational unit



HTS MPS

Genetic variation

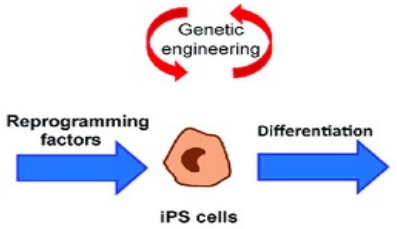
Patient
(10-40% of genetic variation[1])



Genotype

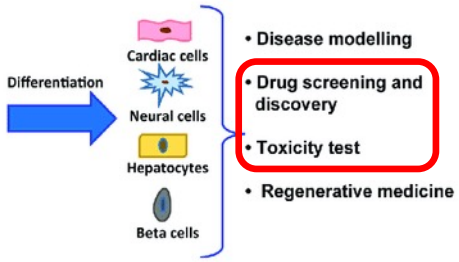
Disease

Reprogramming
(10-30% of genetic variation[2])

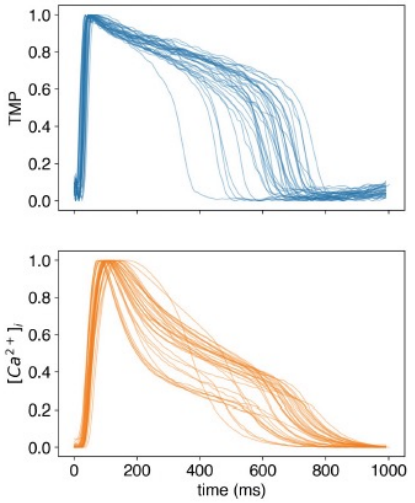


Phenotypic variation

Differentiation
(~ 60% of transcriptome variation[3])



Assay
(?)

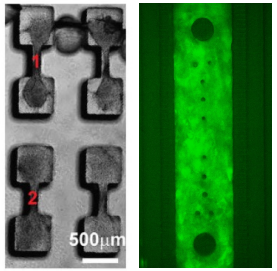


1. Kilpinen *et al.* 2017
2. D'Antonio *et al.* 2018
3. Volpato *et al.* 2018

Measurement constraints in MPS data streams

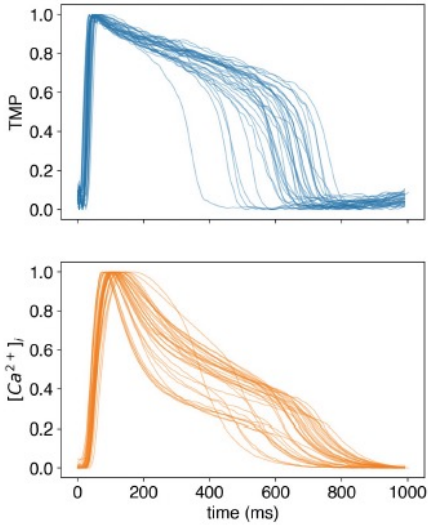
SOURCES OF VARIABILITY IN THE MICROTISSUE ASSAYS

Observational unit



HTS MPS

Question: How are we addressing most of this variability through experimental design?

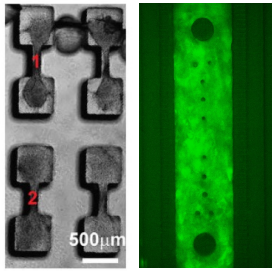


Measurement constraints in MPS data streams

SOURCES OF VARIABILITY IN THE MICROTISSUE ASSAYS

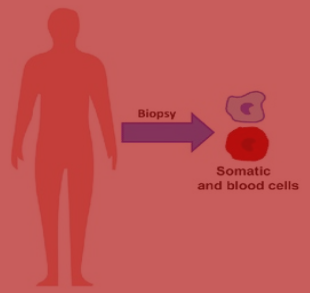
Genetic variation | **Phenotypic variation**

Observational unit



HTS MPS

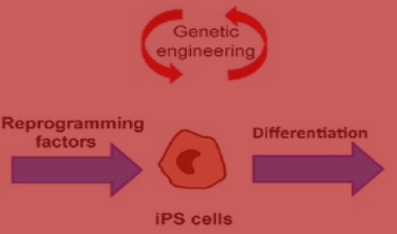
Patient
(10-40% of genetic variation[1])



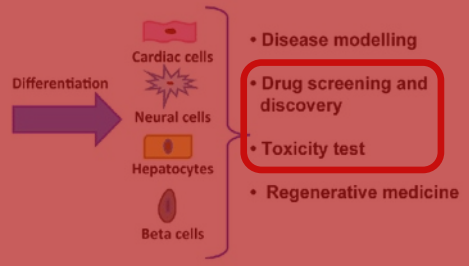
Genotype

Disease

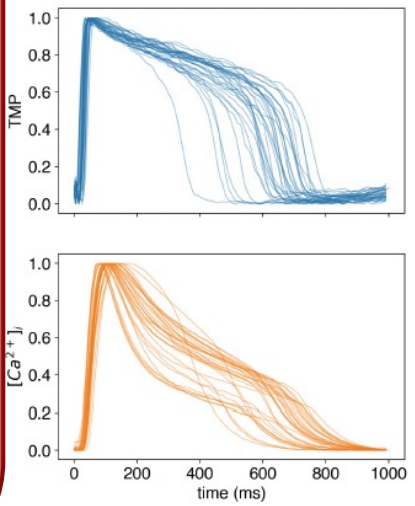
Reprogramming
(10-30% of genetic variation[2])



Differentiation
(~ 60% of transcriptome variation[3])



Assay
(?)



1. Kilpinen *et al.* 2017
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3. Volpato *et al.* 2018

Measurement constraints in MPS data streams

THE PYRAMID OF FRUSTRATION IN LIVE CELL MICROSCOPY

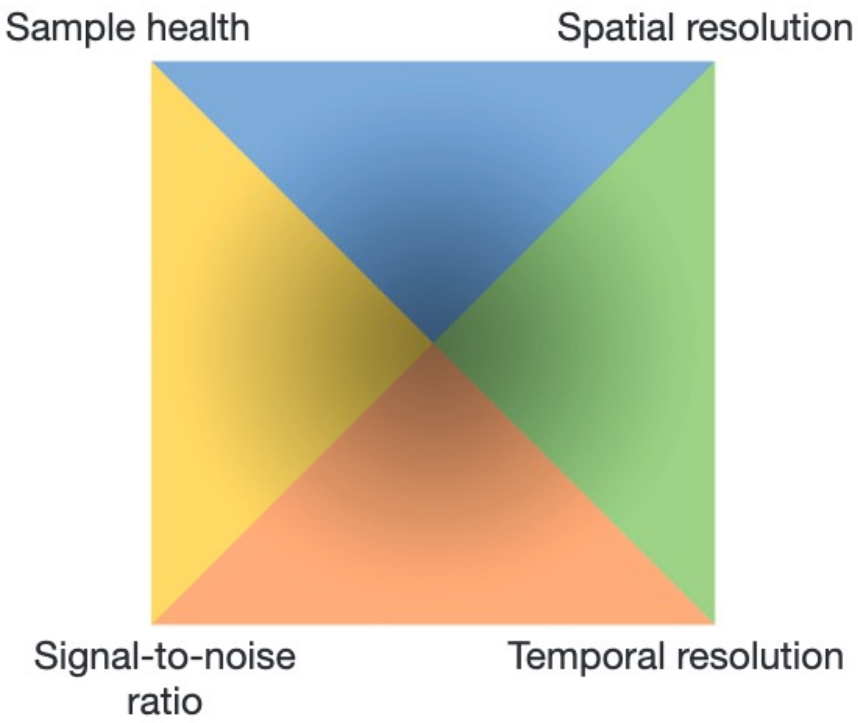


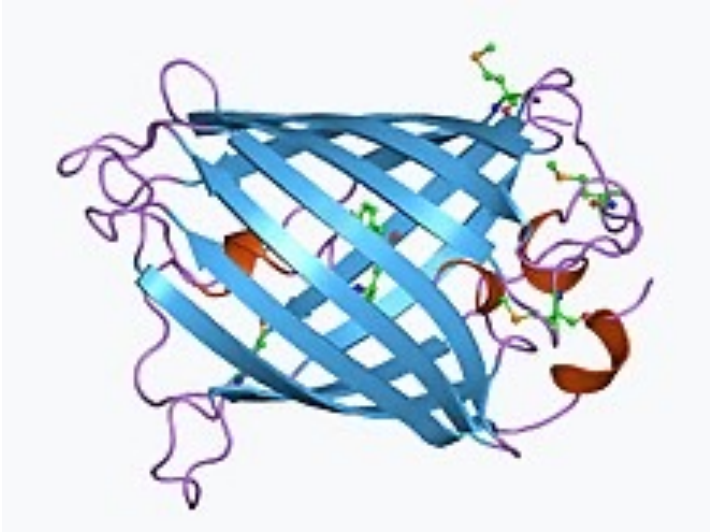
Figure 2 | The four main considerations for live imaging. This is also known as the 'pyramid of frustration', as no single parameter can be optimized without compromising the others.

Laissue *et al*. *Nature Methods*. 2018

Basis of Fluorescence

WHAT IS FLUORESCENCE?

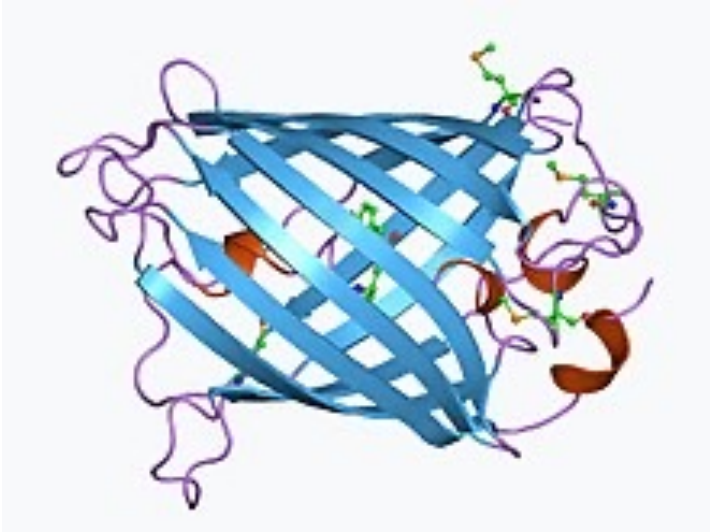
Green Fluorescent Protein - GFP

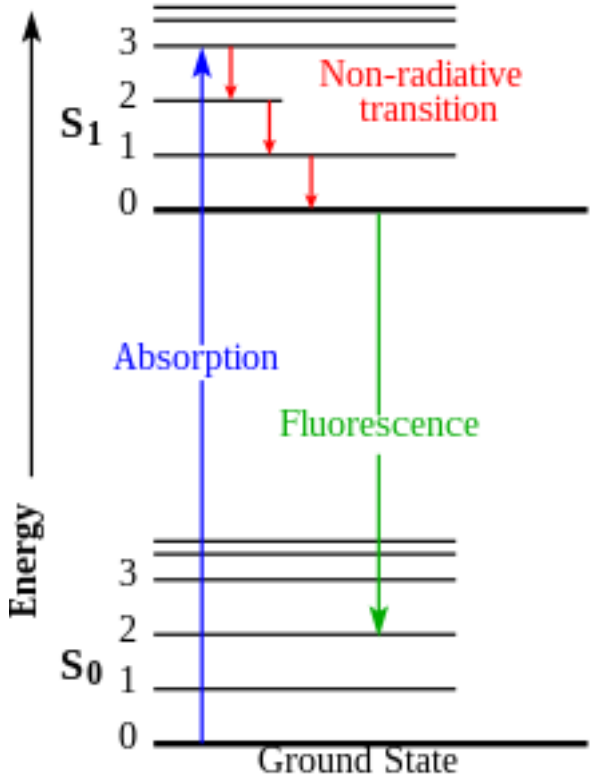


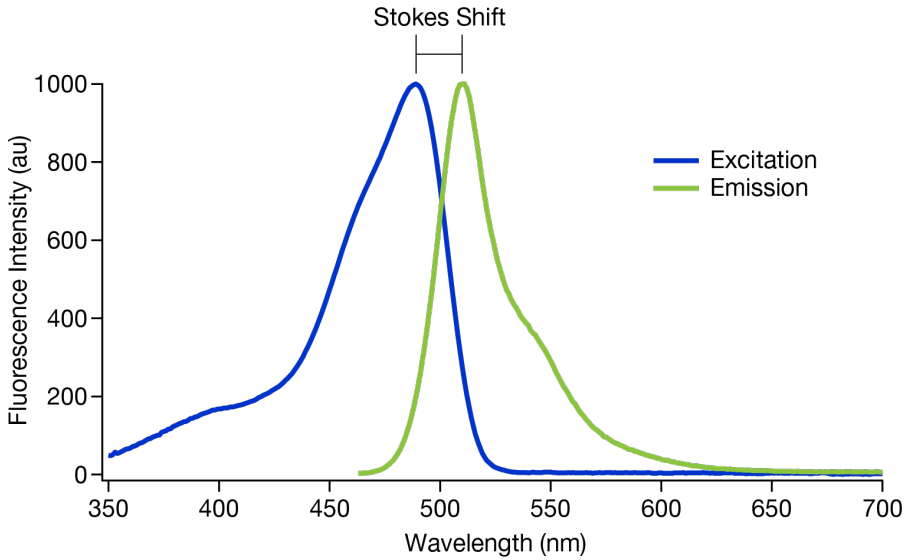
Basis of Fluorescence

ENERGY ABSORPTION AND EMISSION

Green Fluorescent Protein - GFP

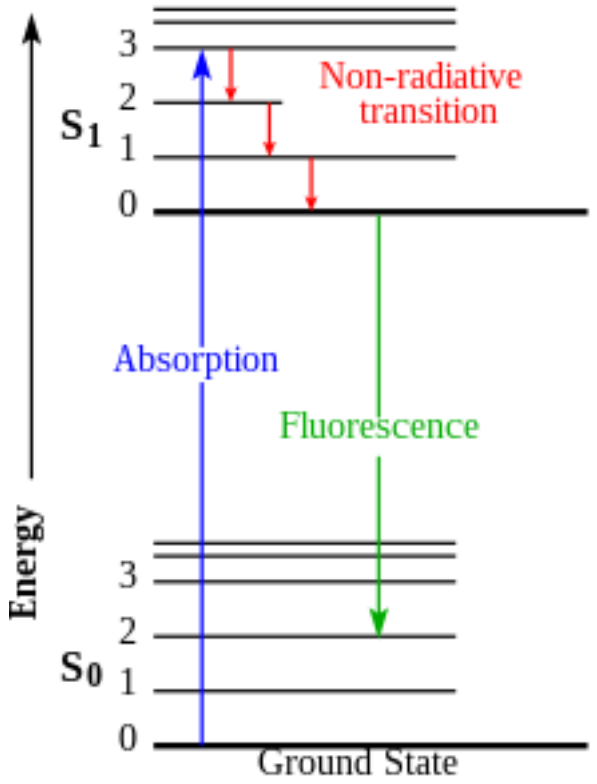


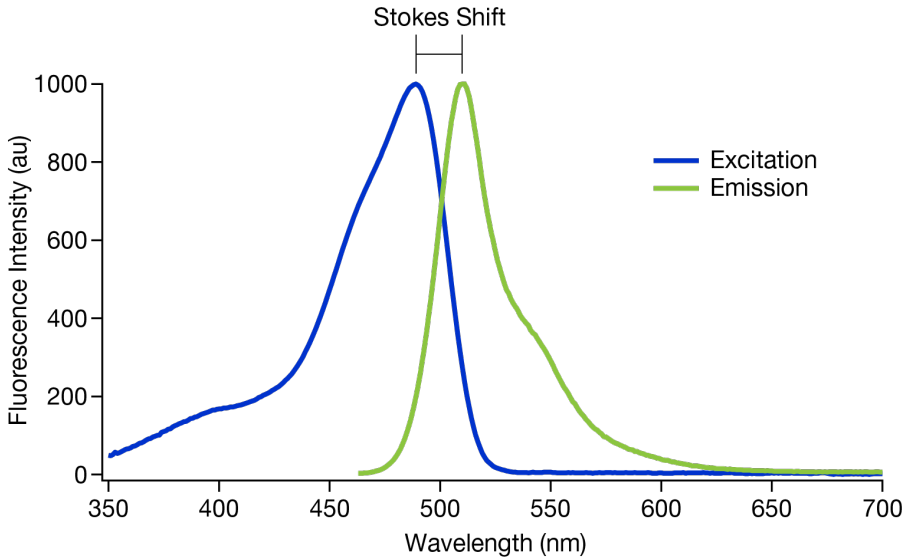




Basis of Fluorescence

WHAT IS FLUORESCENT QUANTUM YIELD?





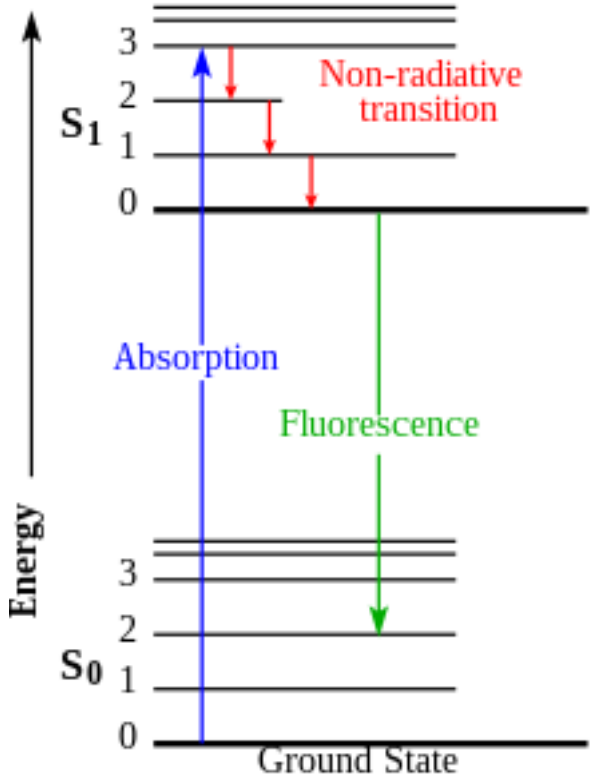
Basis of Fluorescence

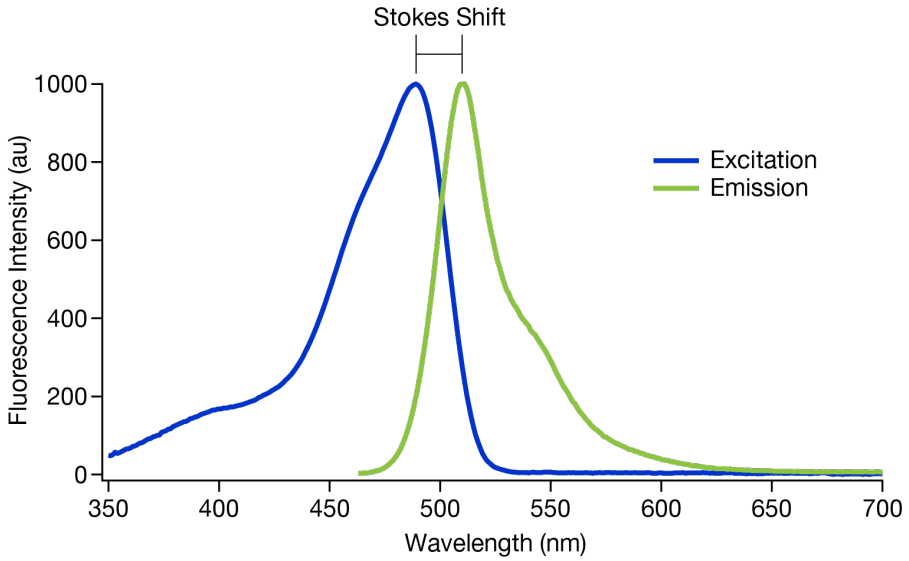
WHAT IS FLUORESCENT QUANTUM YIELD?

Quantum yield

$$\Phi(\lambda) = \frac{\text{number of events}}{\text{number of photons absorbed}}$$

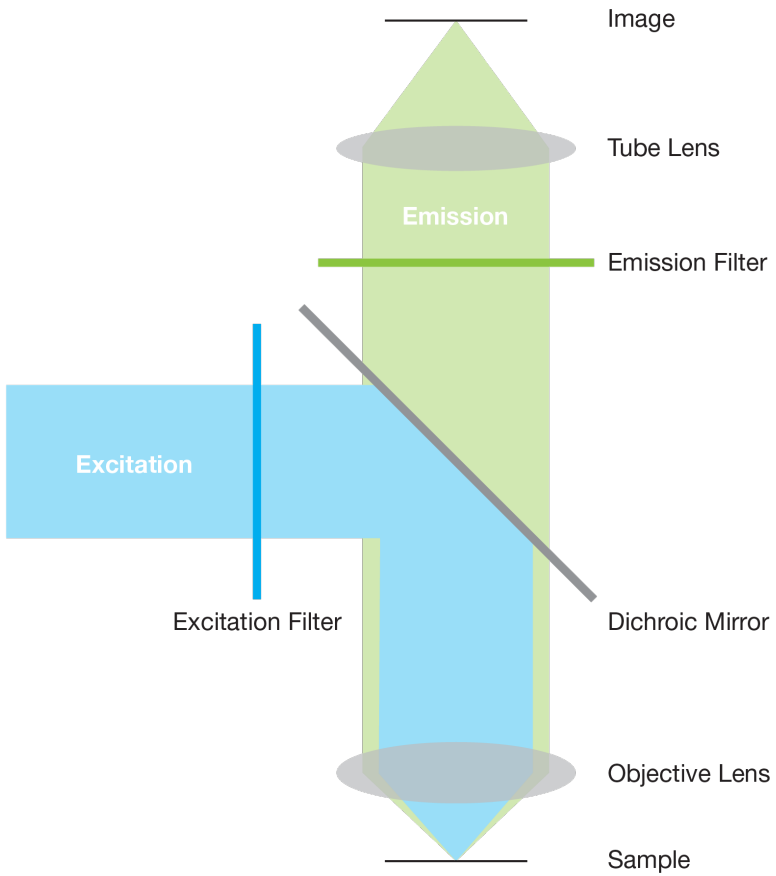
Compound	Solvent	Literature Quantum yield	Emission range / nm
Cresyl violet	Methanol	0.54	600-650
Rhodamine 101	Ethanol + 0.01% HCl	1.00	600-650
Quinine sulfate	0.1M H_2SO_4	0.54	400-600
Fluorescein	0.1M NaOH	0.79	500-600
Norharmane	0.1M H_2SO_4	0.58	400-550
Harmane	0.1M H_2SO_4	0.83	400-550
Harmine	0.1M H_2SO_4	0.45	400-550
2-methylharmane	0.1M H_2SO_4	0.45	400-550
Chlorophyll A	Ether	0.32	600-750
Zinc phthalocyanine	1% pyridine in toluene	0.30	660-750
Benzene	Cyclohexane	0.05	270-300
Tryptophan	Water, pH 7.2, 25C	0.14	300-380
2-Aminopyridine	0.1M H_2SO_4	0.60	315-480
Anthracene	Ethanol	0.27	360-480
9,10-diphenyl anthracene	Cyclohexane	0.90	400-500





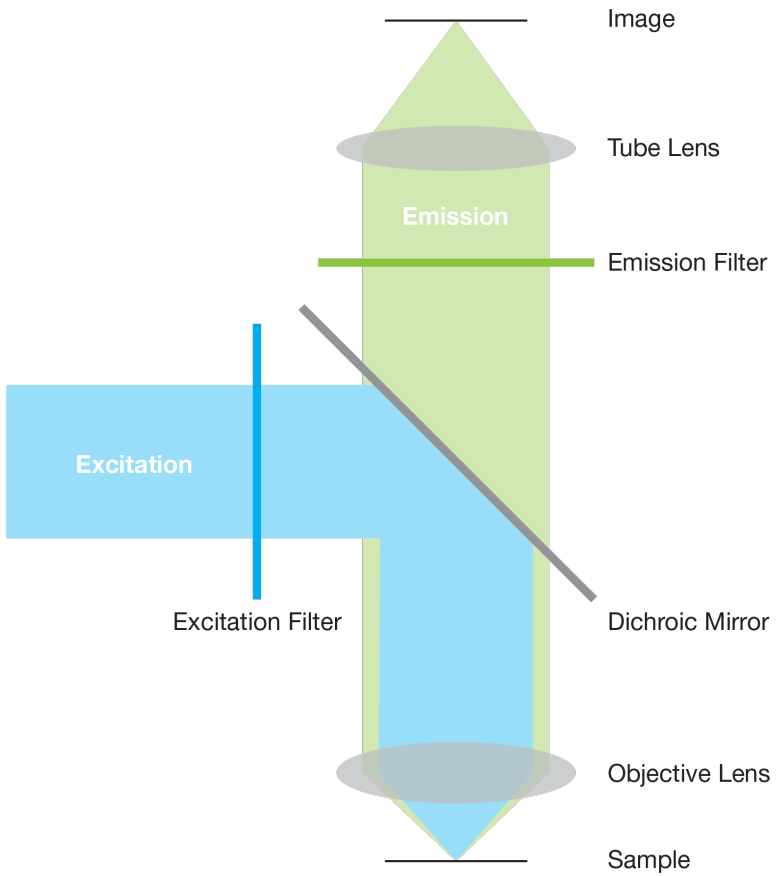
Fluorescence microscopy

WHAT ARE THE DETERMINANTS OF FLUORESCENCE INTENSITY?



Fluorescence microscopy

WHAT ARE THE DETERMINANTS OF FLUORESCENCE INTENSITY?



$$I_f = kI_o\Phi[\varepsilon bc]$$

k optical path loss coefficient

I_0 incident light intensity

Φ quantum yield

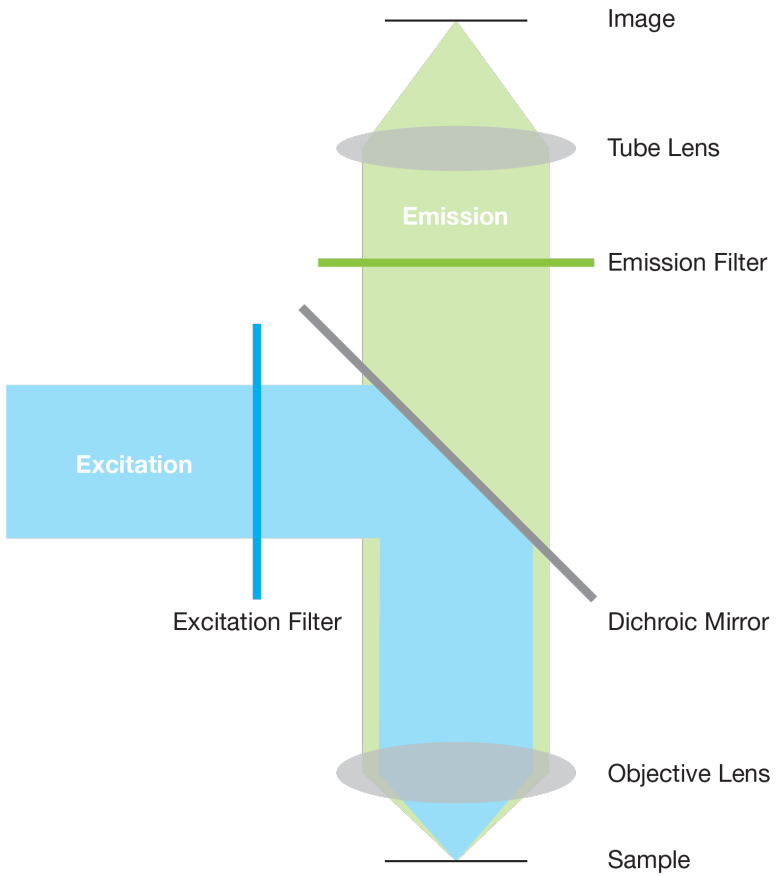
ϵ molar absorptivity

b light path length

c concentration of the fluorophore

Fluorescence microscopy

WHAT ARE THE DETERMINANTS OF FLUORESCENCE INTENSITY?



$$I_f = kI_o\Phi[\varepsilon bc]$$

k optical path loss coefficient

I_0 incident light intensity

Φ quantum yield

ϵ molar absorptivity

b light path length

c concentration of the fluorophore

$$I_f = N_f P_{abs} P_{em}$$

N_f number of fluorophores in the light path

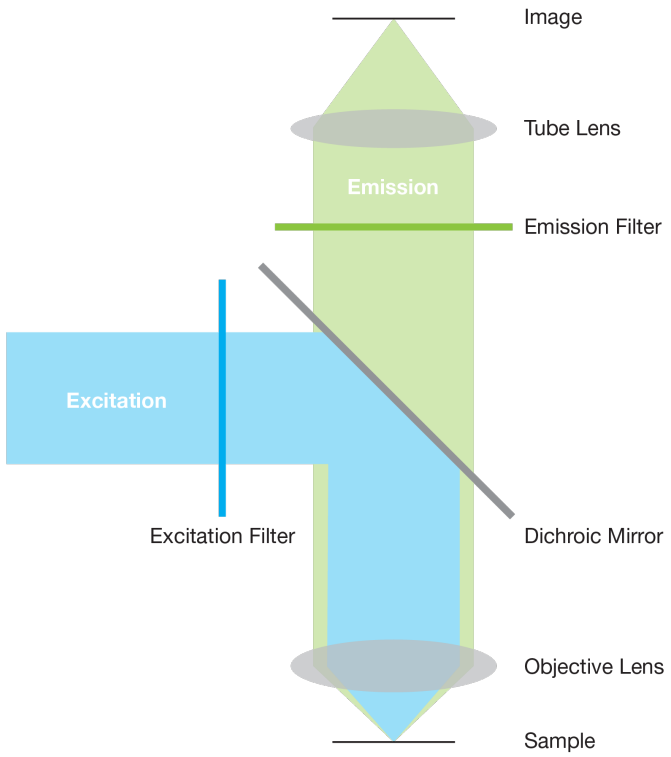
$P_{abs} = f(\lambda_{ex}, I_0, k, b_{ex})$

$P_{em} = f(\lambda_{em}, k, b_{em})$

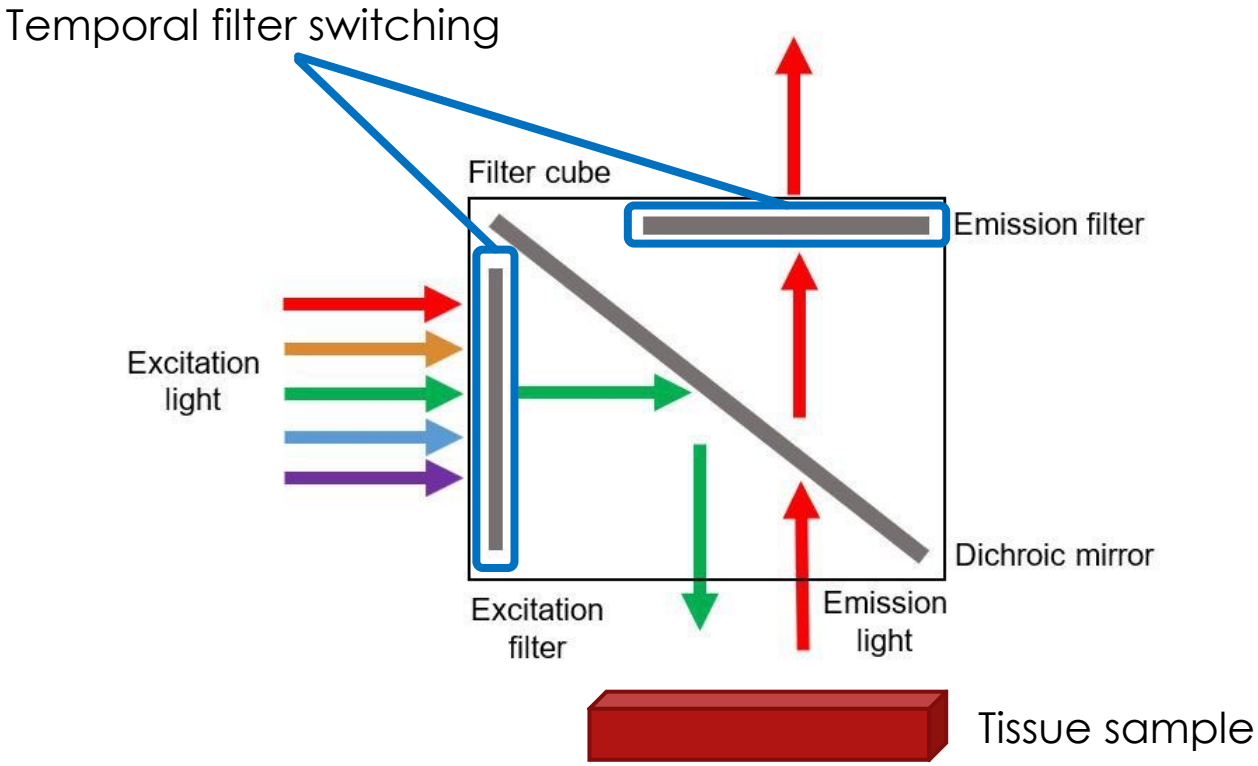
Fluorescence microscopy

THE LIGHT PATH

Single-colour imaging



Multi-colour(fluorophore) imaging

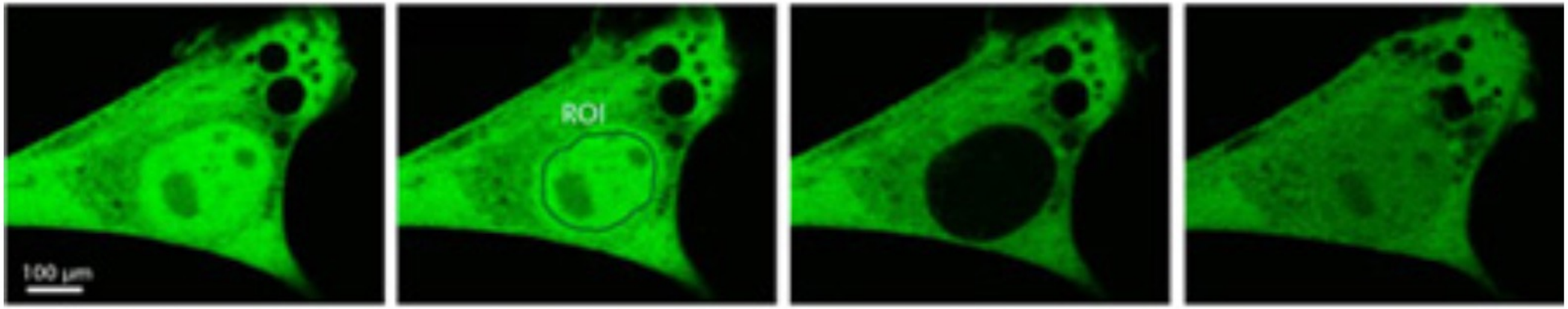


Fluorescence microscopy

WHAT IS PHOTOBLEACHING?

Fluorescence microscopy

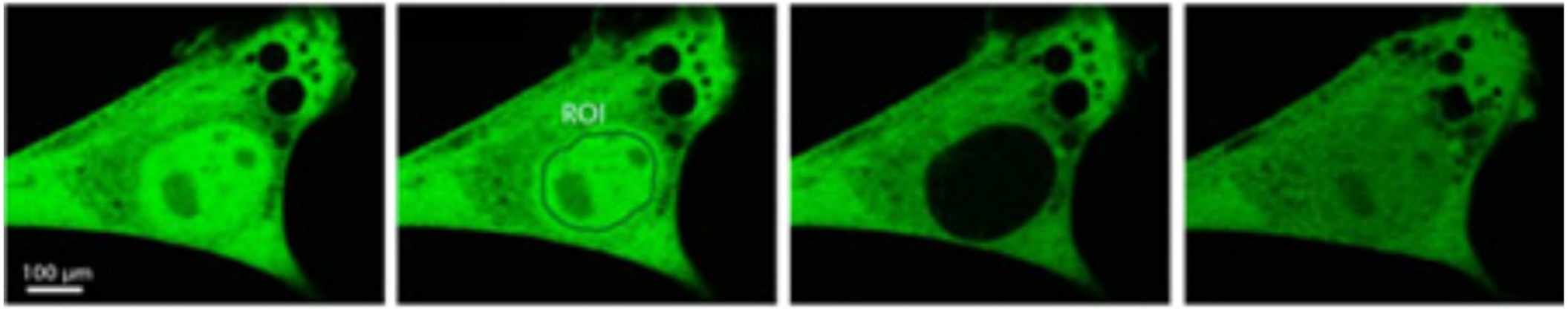
WHAT IS PHOTOBLEACHING?



Pre-bleach | Bleach ROI | Post-bleach | Recovered

Fluorescence microscopy

WHAT IS PHOTOBLEACHING?



Pre-bleach **Bleach ROI** **Post-bleach** **Recovered**

Proportional to illumination time and I_0

$$I_f = N_f P_{abs} P_{em}$$

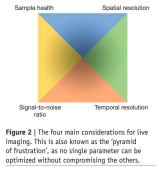


Figure 2 | The four main considerations for live imaging. This is also known as the 'pyramid of frustration', as no single parameter can be optimized without compromising the others.

Fluorescence microscopy

WHAT IS PHOTOTOXICITY?

Published: 29 June 2017

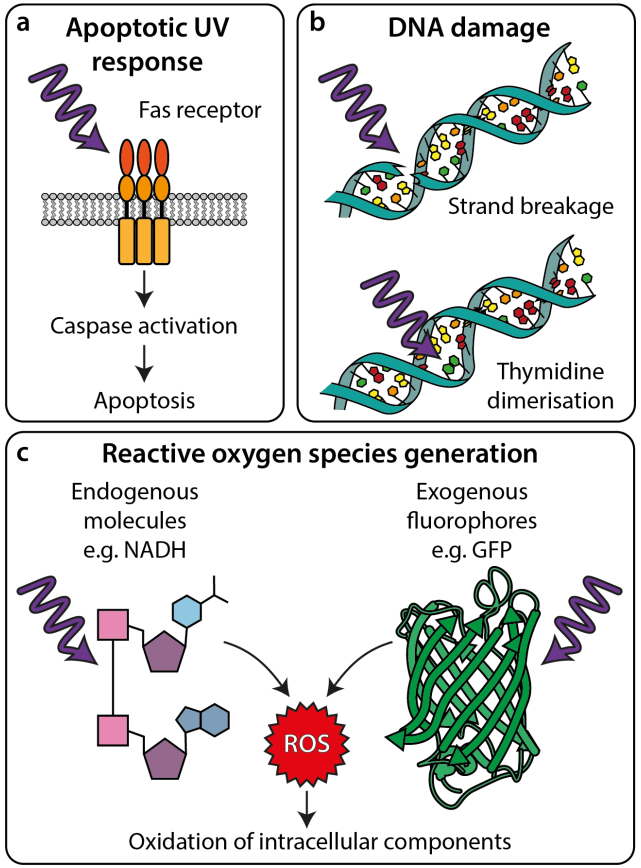
Assessing phototoxicity in live fluorescence imaging

P Philippe Laissue ✉, Rana A Alghamdi, Pavel Tomancak, Emmanuel G Reynaud & Hari Shroff

Nature Methods **14**, 657–661 (2017) | Cite this article

Fluorescence microscopy

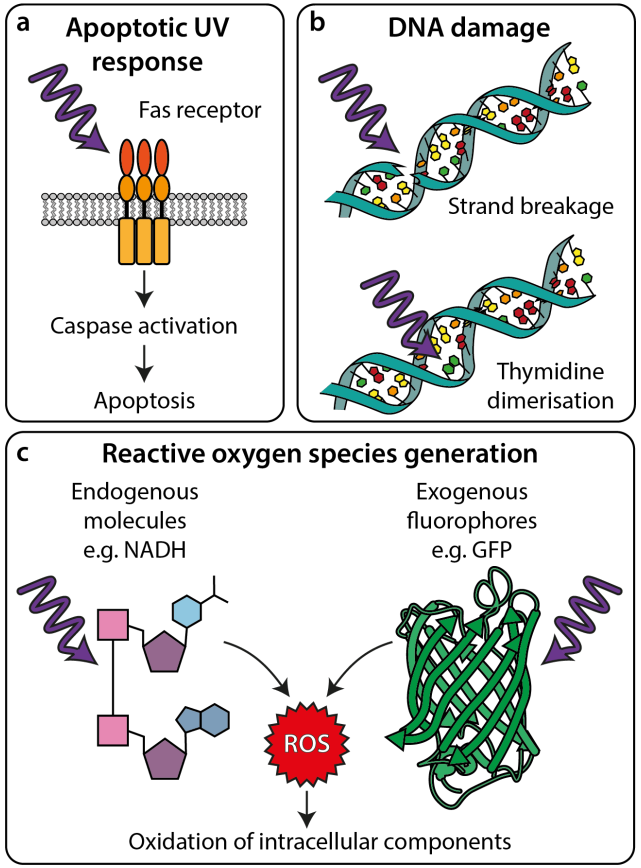
WHAT IS PHOTOTOXICITY?



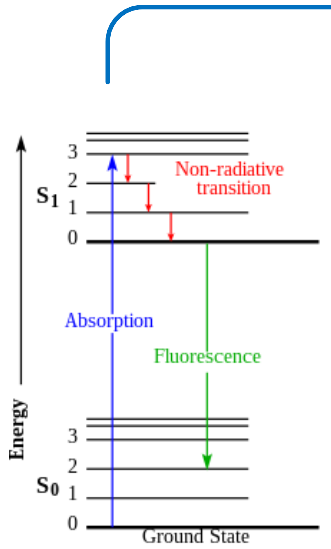
Zhou *et al.*, *Biochemistry*, 2021

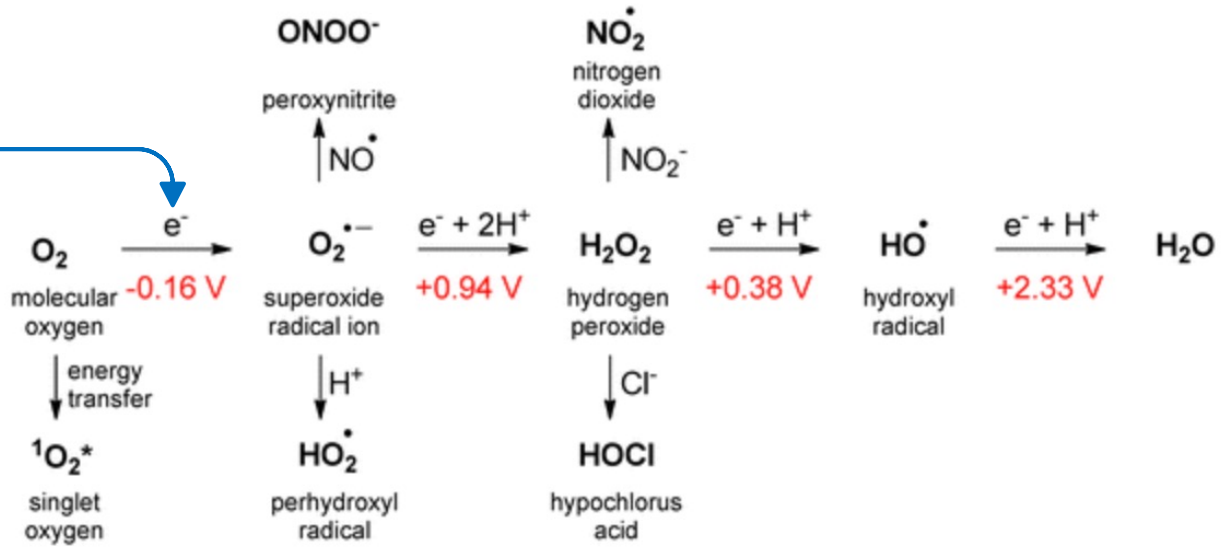
Fluorescence microscopy

WHAT IS PHOTOTOXICITY?



Zhou *et al.*, *Biochemistry*, 2021





Synthetic calcium fluorophores

CALCIUM-BINDING IS STEP 1: WHAT ARE DESIRABLE CHARACTERISTICS?

Resting intracellular [Ca^{2+}] is ~ 100 nM

Question: You have 1 teaspoon (5 g) of $CaCl_2$, how much water to you need to dissolve this in to achieve resting [Ca^{2+}]?

Synthetic calcium fluorophores

CALCIUM-BINDING IS STEP 1: WHAT ARE DESIRABLE CHARACTERISTICS?

Resting intracellular [Ca^{2+}] is ~ 100 nM

Question: You have 1 teaspoon (5 g) of $CaCl_2$, how much water to you need to dissolve this in to achieve resting [Ca^{2+}]?

Answer: ~ 450 tonnes

Synthetic calcium fluorophores

CALCIUM-BINDING IS STEP 1: WHAT ARE DESIRABLE CHARACTERISTICS?

Question: What fraction of that resting 100 nM Ca^{2+} is free compared to bound to intracellular molecules?

Synthetic calcium fluorophores

CALCIUM-BINDING IS STEP 1: WHAT ARE DESIRABLE CHARACTERISTICS?

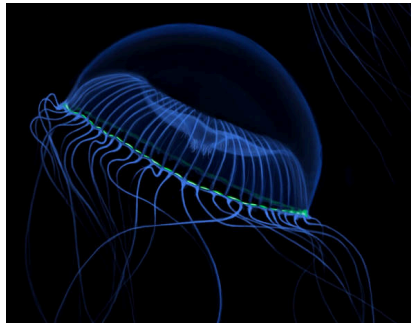
Question: What fraction of that resting 100 nM Ca^{2+} is free compared to bound to intracellular molecules?

Answer: ~ 1%

What is the implication for creating Ca^{2+}-sensitive fluorophores?

Calcium-sensitive fluorophores

ENDOGENOUS CALCIUM FLUOROPHORES



Aequorea Victoria

Extraction, Purification and Properties of Aequorin, a Bioluminescent Protein from the Luminous Hydromedusan, *Aequorea*[1]

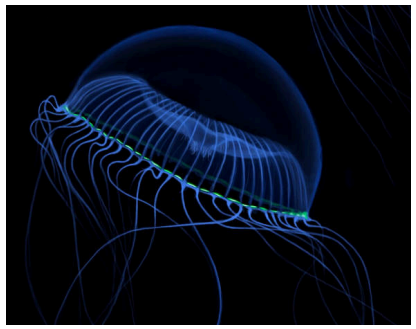
OSAMU SHIMOMURA,* FRANK H. JOHNSON AND YO SAIGA
Department of Biology, Princeton University, Princeton, New Jersey, and the Friday Harbor Laboratories, University of Washington, Friday Harbor, Washington

Shimomura *et al*. 1962

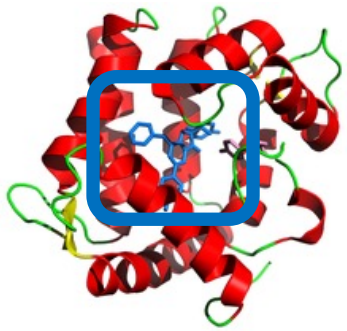
Calcium-sensitive fluorophores

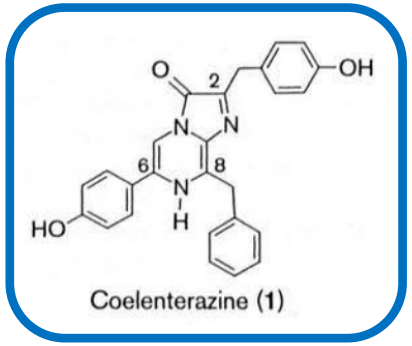
ENDOGENOUS CALCIUM FLUOROPHORES

Aequorin



Aequorea Victoria





Coelenterazine (1)

Blue emitted light

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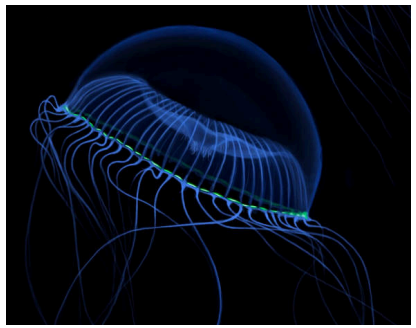
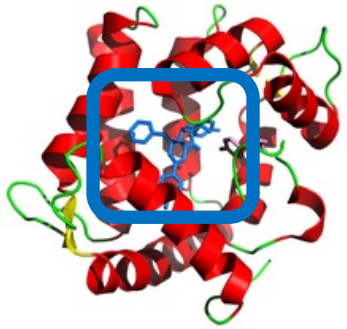
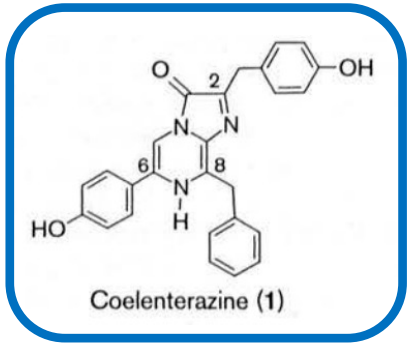


Aequorea Victoria





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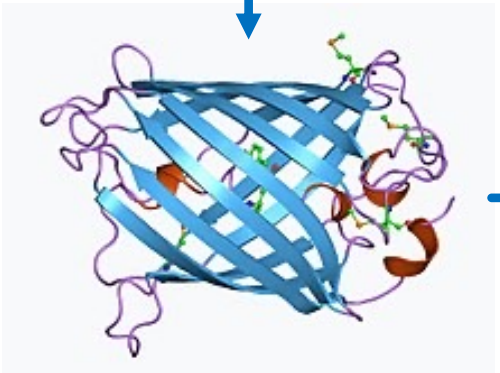
Blue emitted light

GFP



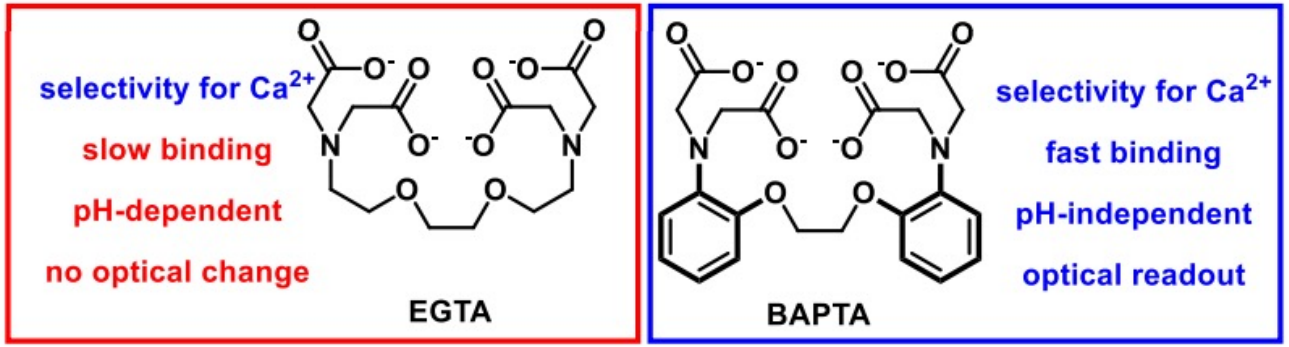
Green emitted light

Förster
Resonance
Energy
Transfer

Synthetic calcium fluorophores

CALCIUM-BINDING IS STEP 1

BAPTA is the foundation of
most synthetic Ca^{2+} indicators



Zhou *et al.*, *Biochemistry*, 2021

Pros

1. K_d = 100 nM (perfect for biology)
2. pH-independent
3. Fast

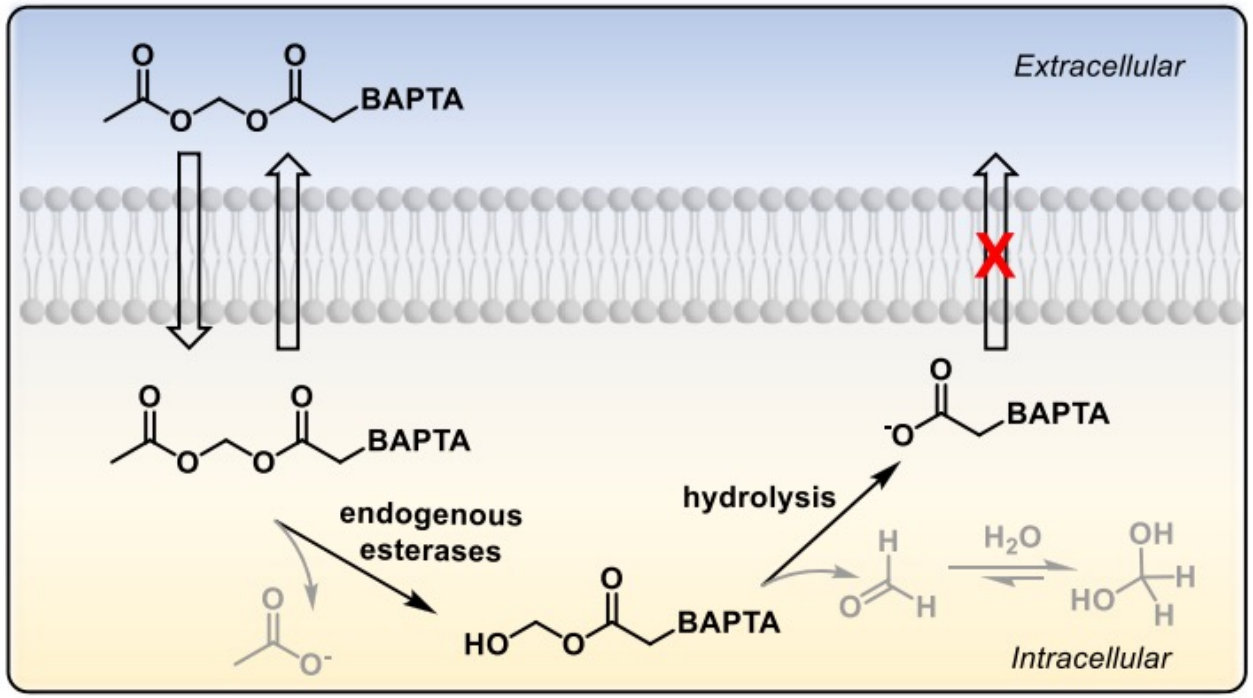
Cons

1. Cell impermeant
2. Absorption peak $\lambda \sim 200$ nm
3. Emission peak $\lambda < 250$ nm

Delivering calcium fluorophores

HOW DO CALCIUM FLUOROPHORES GET INTO CELLS?

AM esters create cell-reversible lipid solubility



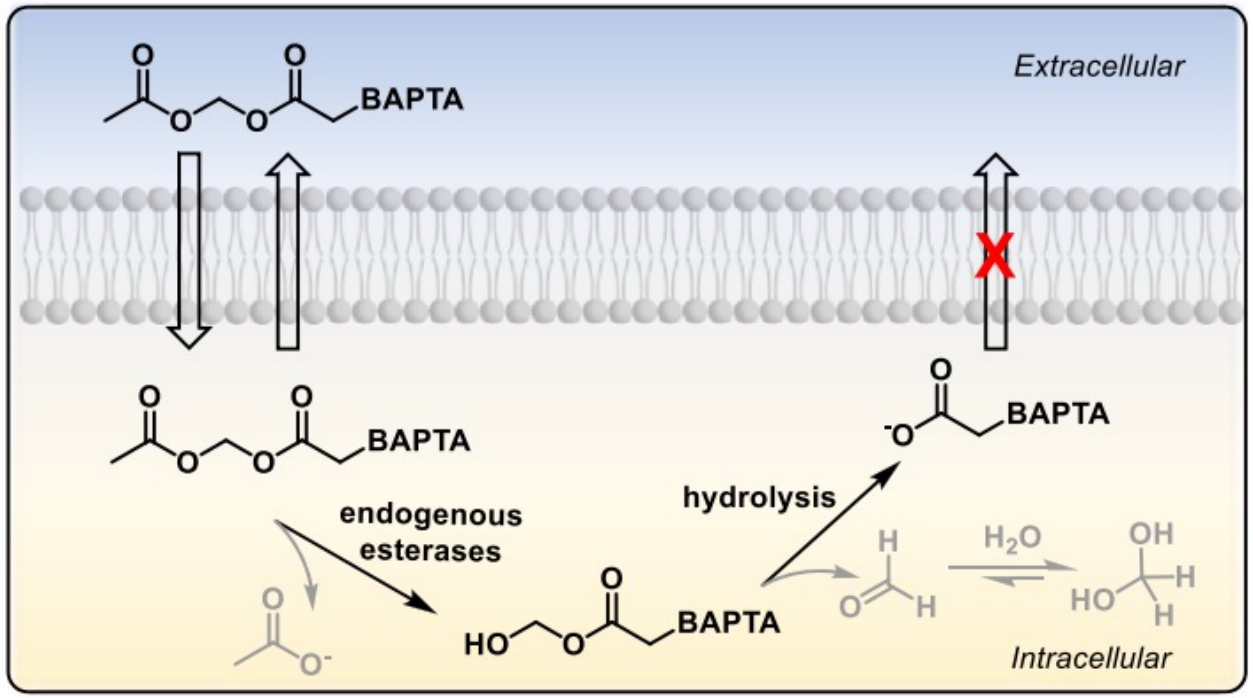
Zhou *et al.*, *Biochemistry*, 2021

Delivering calcium fluorophores

HOW DO CALCIUM FLUOROPHORES GET INTO CELLS?

Issues with this mechanism?

AM esters create cell-reversible lipid solubility



Zhou *et al.*, *Biochemistry*, 2021

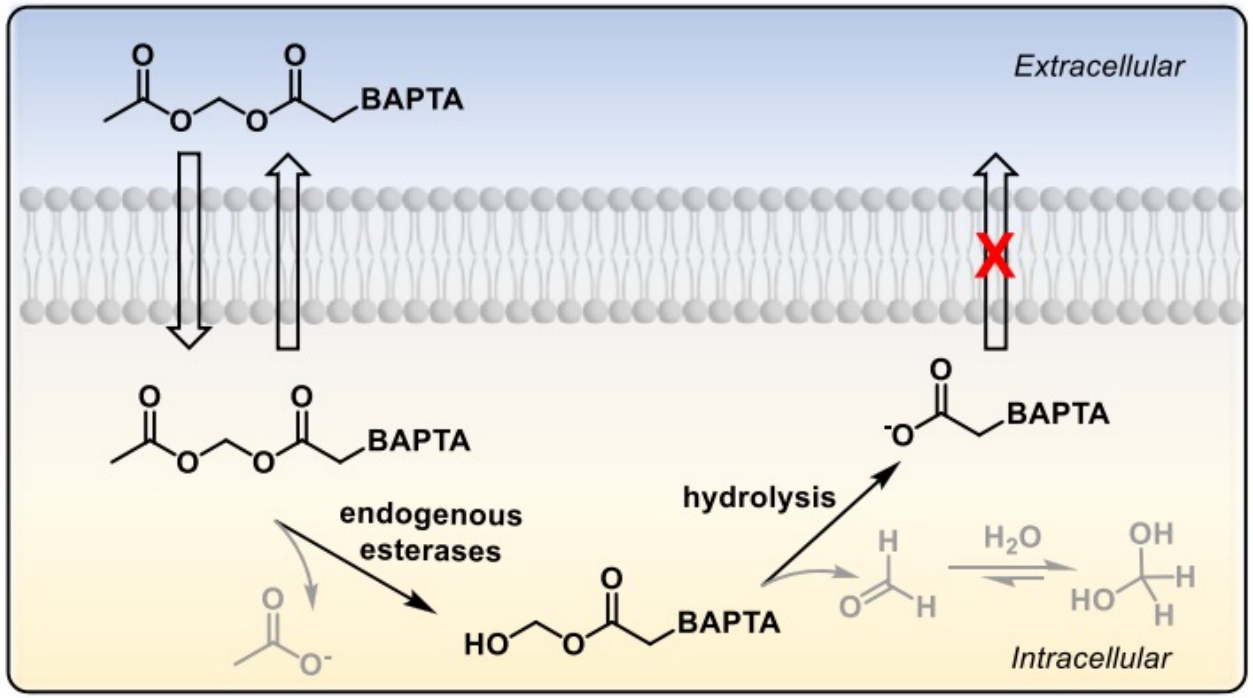
Delivering calcium fluorophores

CELL SOLUBLE CALCIUM FLUOROPHORES

1. Cell permeant

1. Adsorption to lipophilic substrates
2. Variable/regional fluorophore loading

AM esters create cell-reversible lipid solubility



Zhou *et al.*, *Biochemistry*, 2021

Designing fluorescence

RATIOMETRIC CALCIUM IMAGING

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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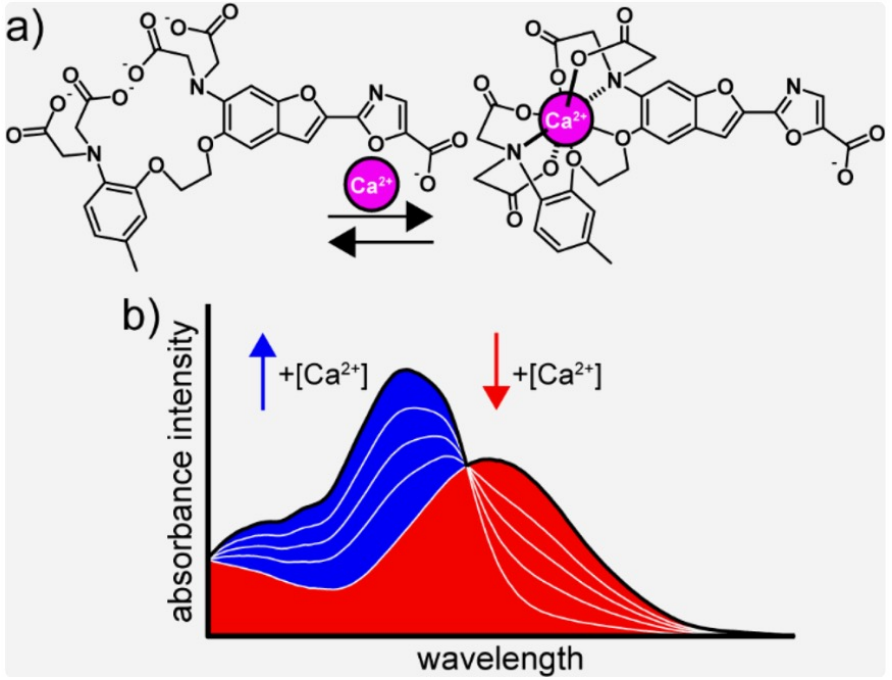
Vol. 260, No. 6, Issue of March 25, pp. 3440–3450, 1985
Printed in U.S.A.

A New Generation of Ca^{2+} Indicators with Greatly Improved Fluorescence Properties*

(Received for publication, August 23, 1984)

Grzegorz Grynkiewicz‡, Martin Poenie, and Roger Y. Tsien§
From the Department of Physiology-Anatomy, University of California, Berkeley, California 94720

Benzofuran fluorophore



Zhou *et al.*, *Biochemistry*, 2021

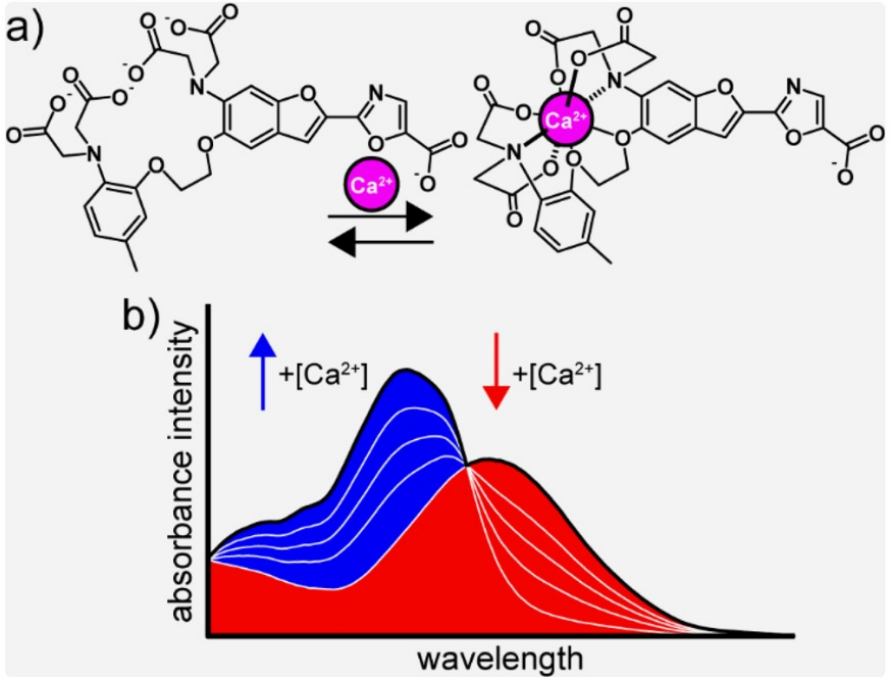
Ratiometric calcium fluorophores

RATIOMETRIC CALCIUM IMAGING

1. Cell permeant
2. Ratiometric Ca^{2+} imaging (Fura-2: F_{340}/F_{380})
3. Visible emission
   - λ_{em} = 510 nm

1. Modest quantum yield (0.5-0.6)
2. High energy UV excitation is toxic
3. Variable/regional fluorophore loading

Benzofuran fluorophore



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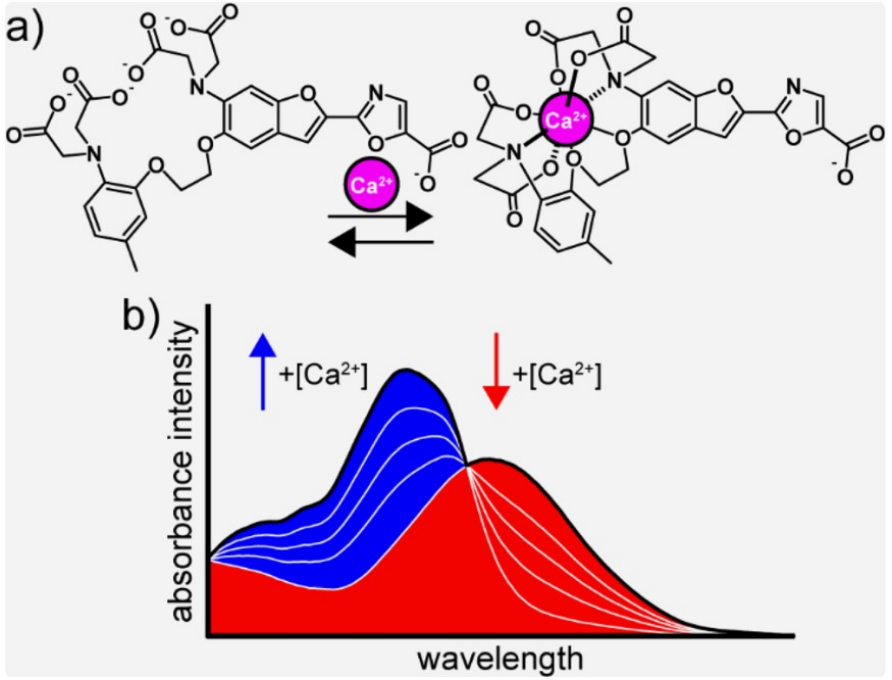
Question: Advantage of ratiometric imaging?

Benzofuran fluorophore



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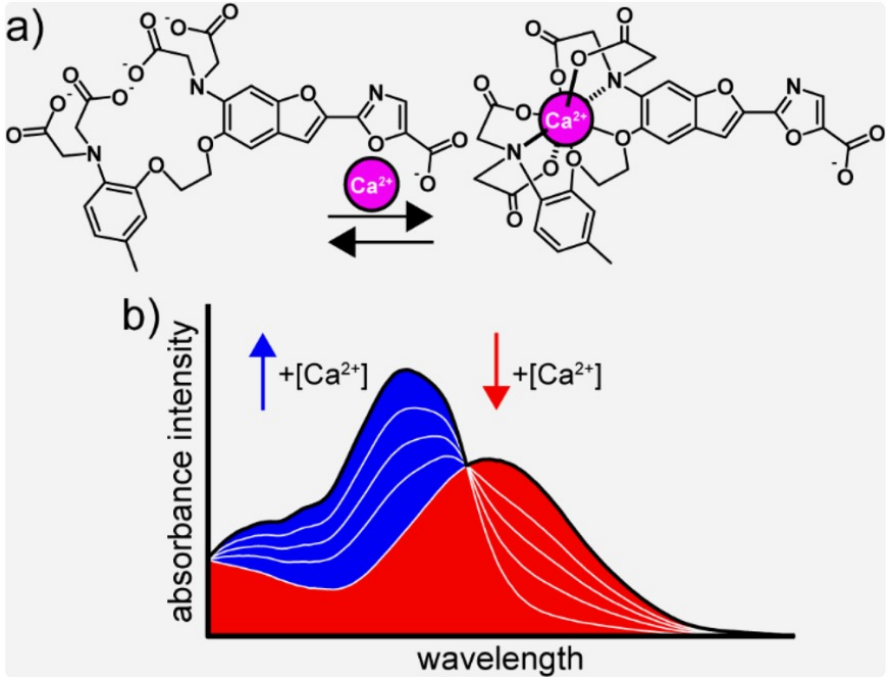
3. Variable/regional fluorophore loading

Question: Advantage of ratiometric imaging?
Answer: Calibration

Benzofuran fluorophore

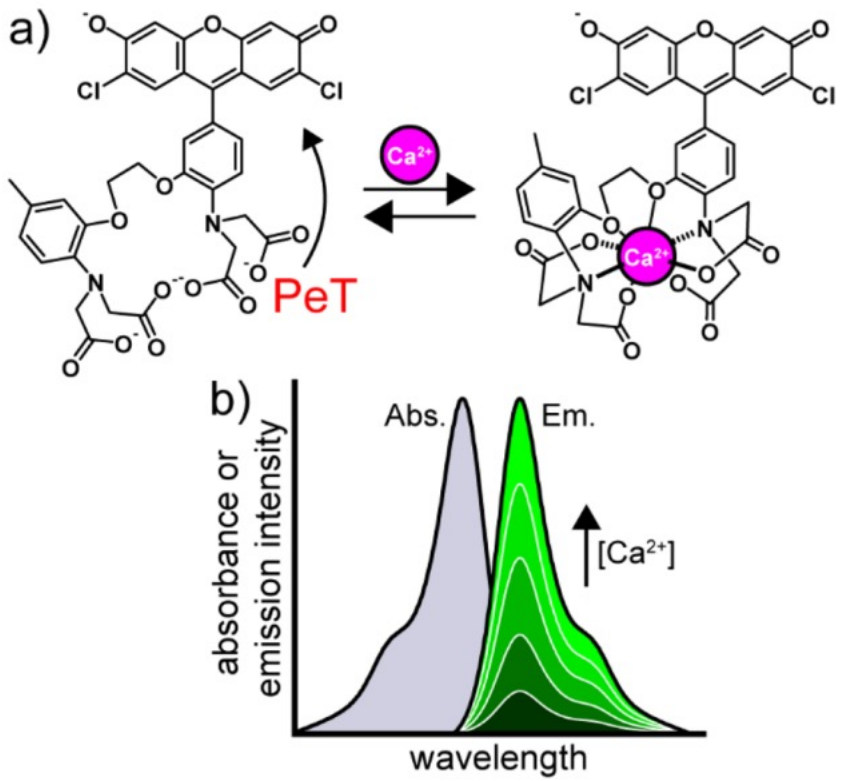


Zhou *et al.*, *Biochemistry*, 2021

Intensity-based calcium imaging

CALCIUM-DEPENDENT BRIGHTNESS

Photoinduced electron transfer (PeT) dyes



Zhou *et al.*, *Biochemistry*, 2021

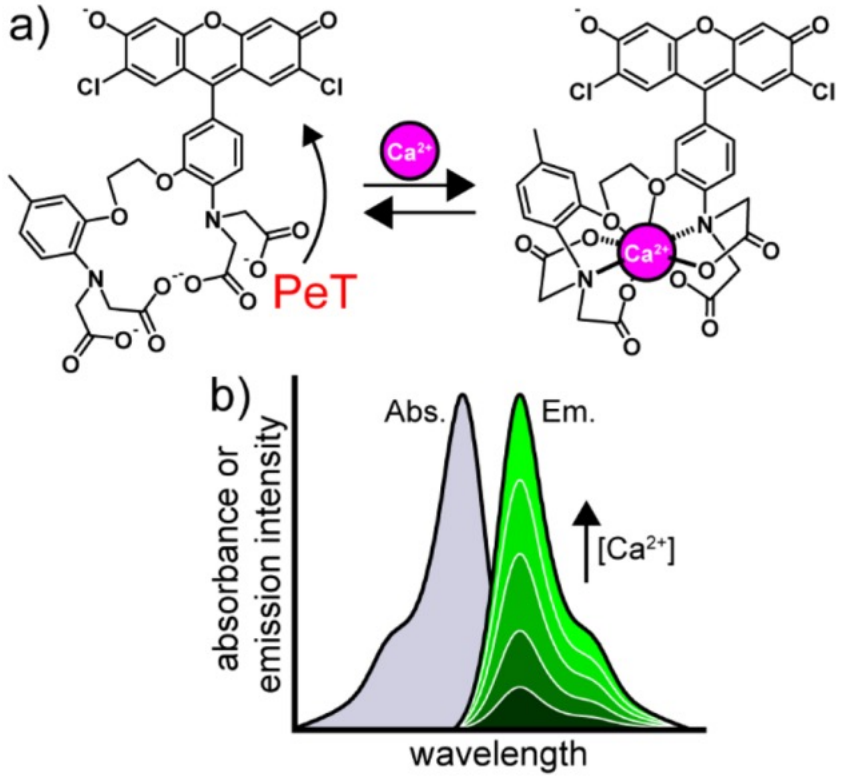
Intensity-based calcium imaging

CALCIUM-DEPENDENT BRIGHTNESS

1. Visible-range excitation
2. High quantum yield – bright
3. Multicolor emission

1. Direct calibration not possible
2. Variable/regional fluorophore loading

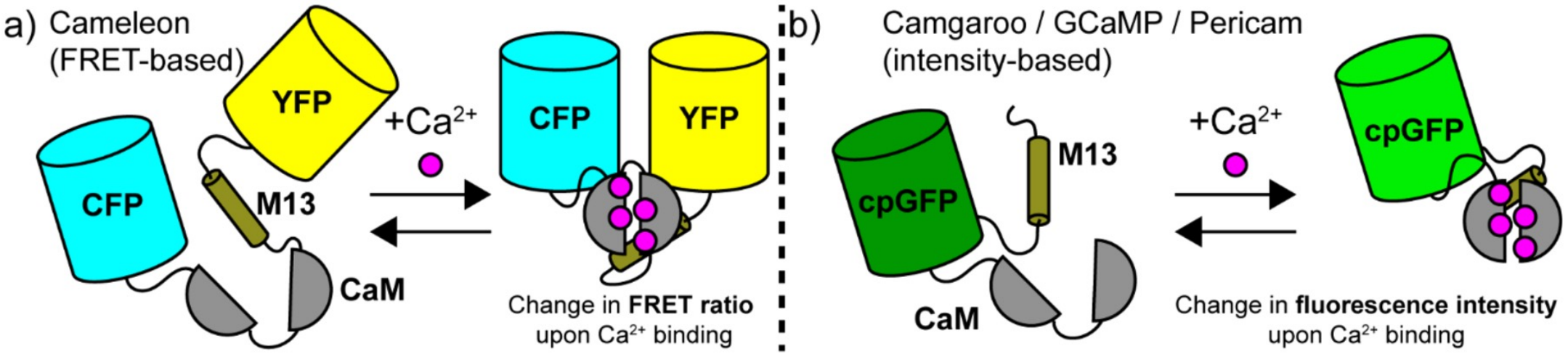
Photoinduced electron transfer (PeT) dyes



Zhou *et al.*, *Biochemistry*, 2021

Leveraging protein fluorescence

GENETICALLY ENCODED CALCIUM INDICATORS (GECIS)



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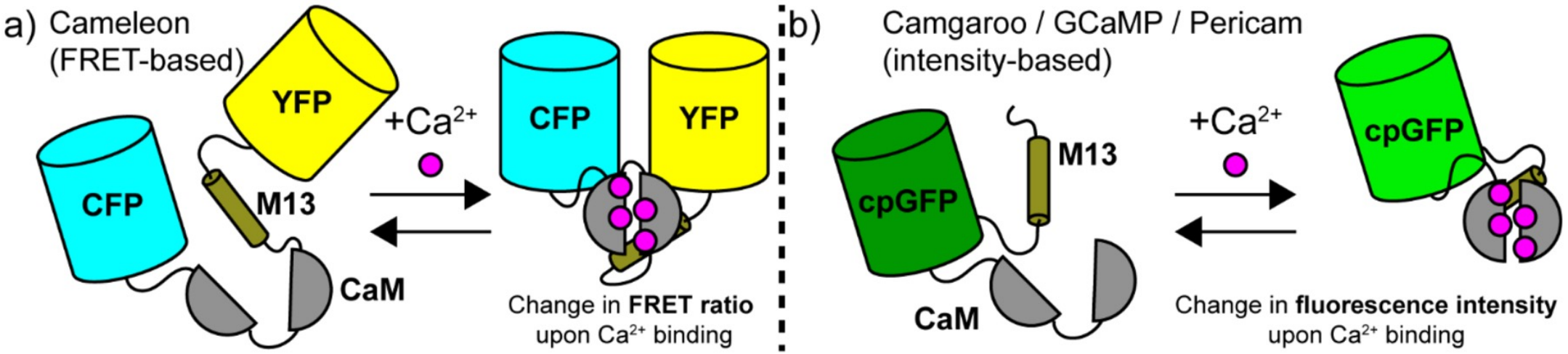


Zhou *et al.*, *Biochemistry*, 2021

Pros

1. Much less variable fluorophore content
2. No spatial concerns with loading in tissue
3. Acute viral loading possible, but challenging

Cons

1. Direct calibration still not possible
2. Dedicated cell lines required for reproducible *in vitro* designs

Summary: Calcium fluorescence

STATE OF THE ART FOR MPS MEASUREMENTS

1. Modern Ca^{2+} dyes

- Bright
- Excellent dynamic range
- Quick loading (synthetic), or dedicated GECI cell lines
- Many colors (multiplexing)

2. Ongoing challenges

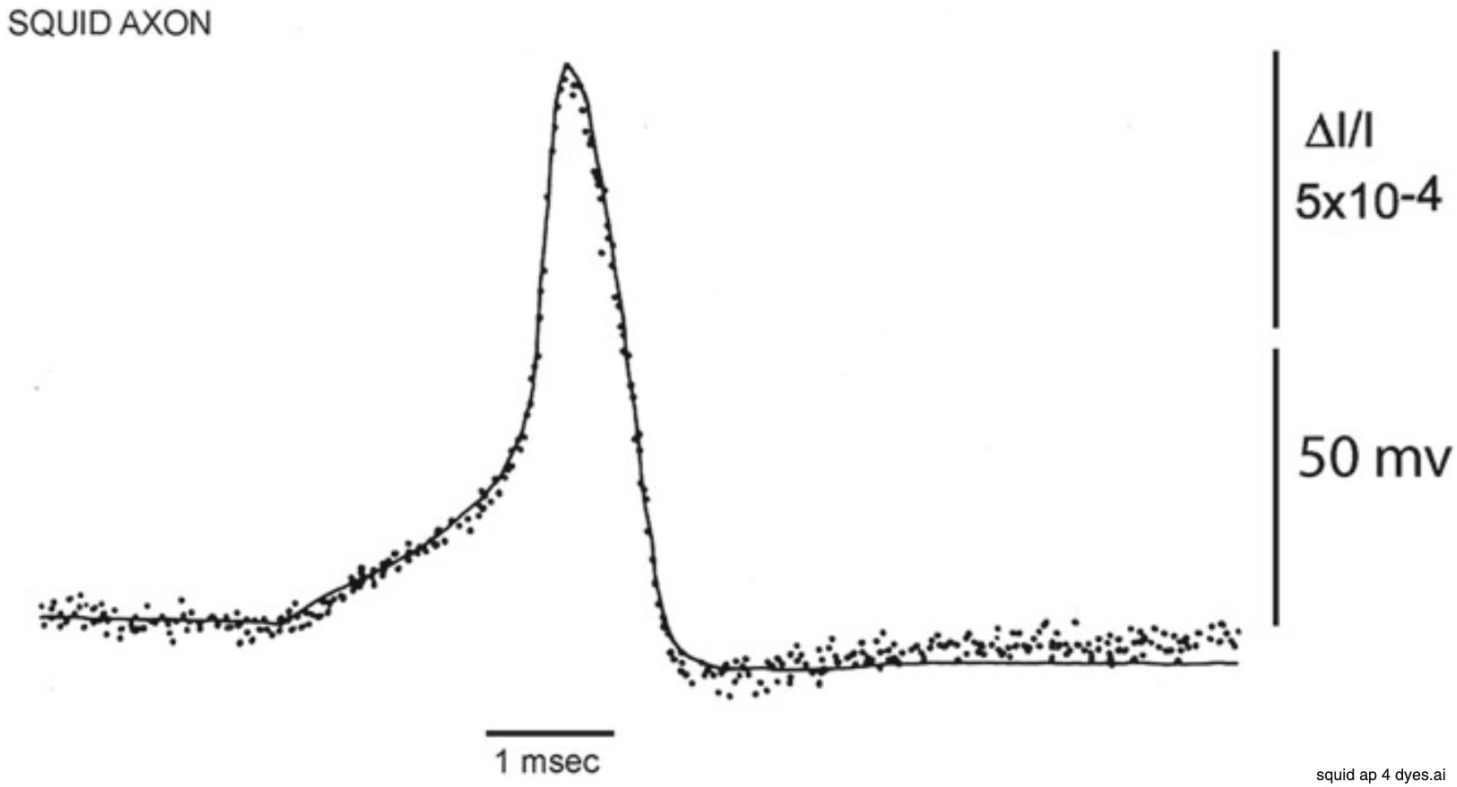
- Variable loading (synthetic dyes)
- Calibrated [Ca^{2+}] largely infeasible
- GECIs limited to specific engineered cell lines

Optical voltage sensors

ADDED CHALLENGES FOR OPTICAL VOLTAGE SENSORS

—— 10 kHz electrode recording

∴∴ 1 kHz optical recording (50 sweeps)



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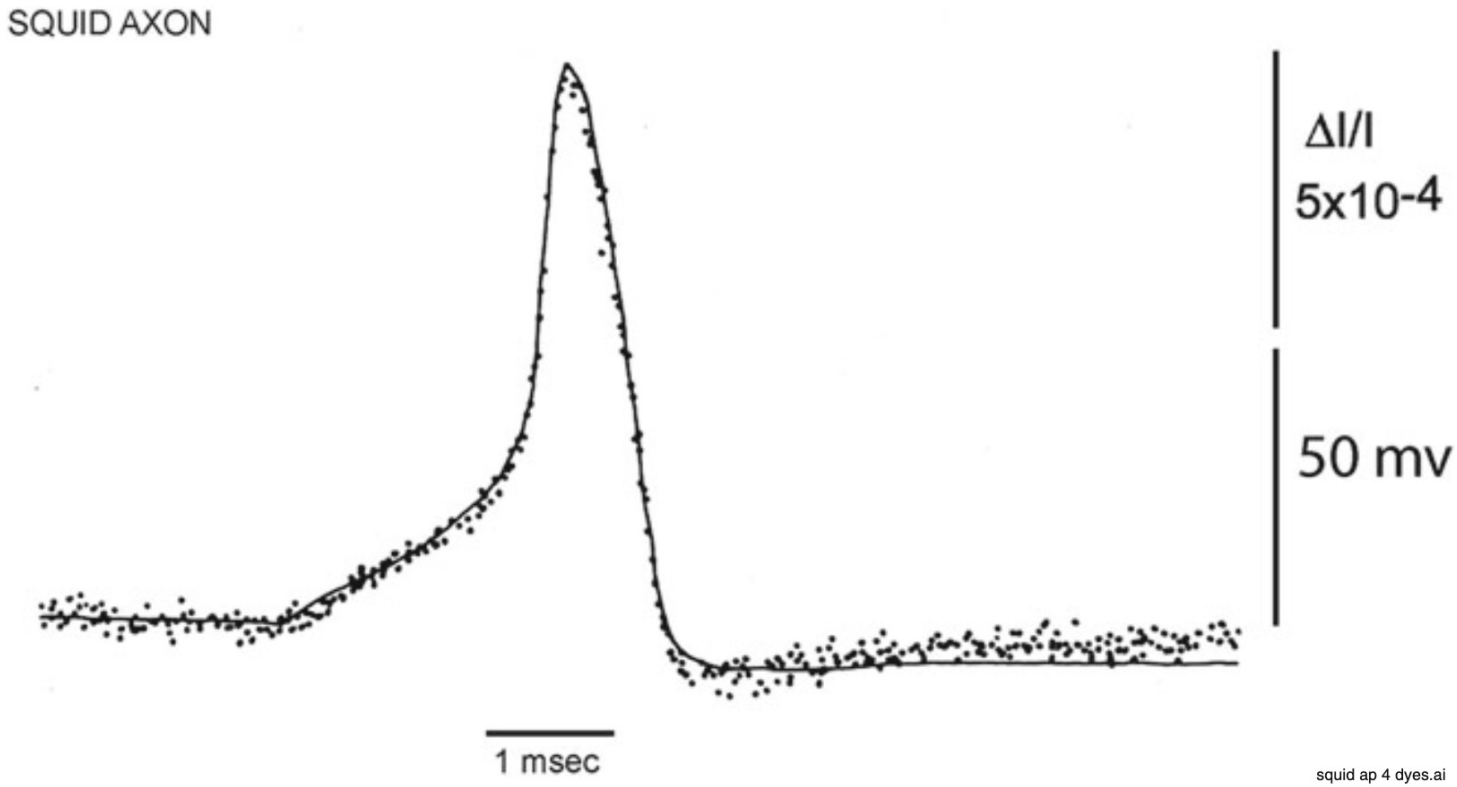

Question: Any fundamental differences compared to a calcium recording?

Optical voltage sensors

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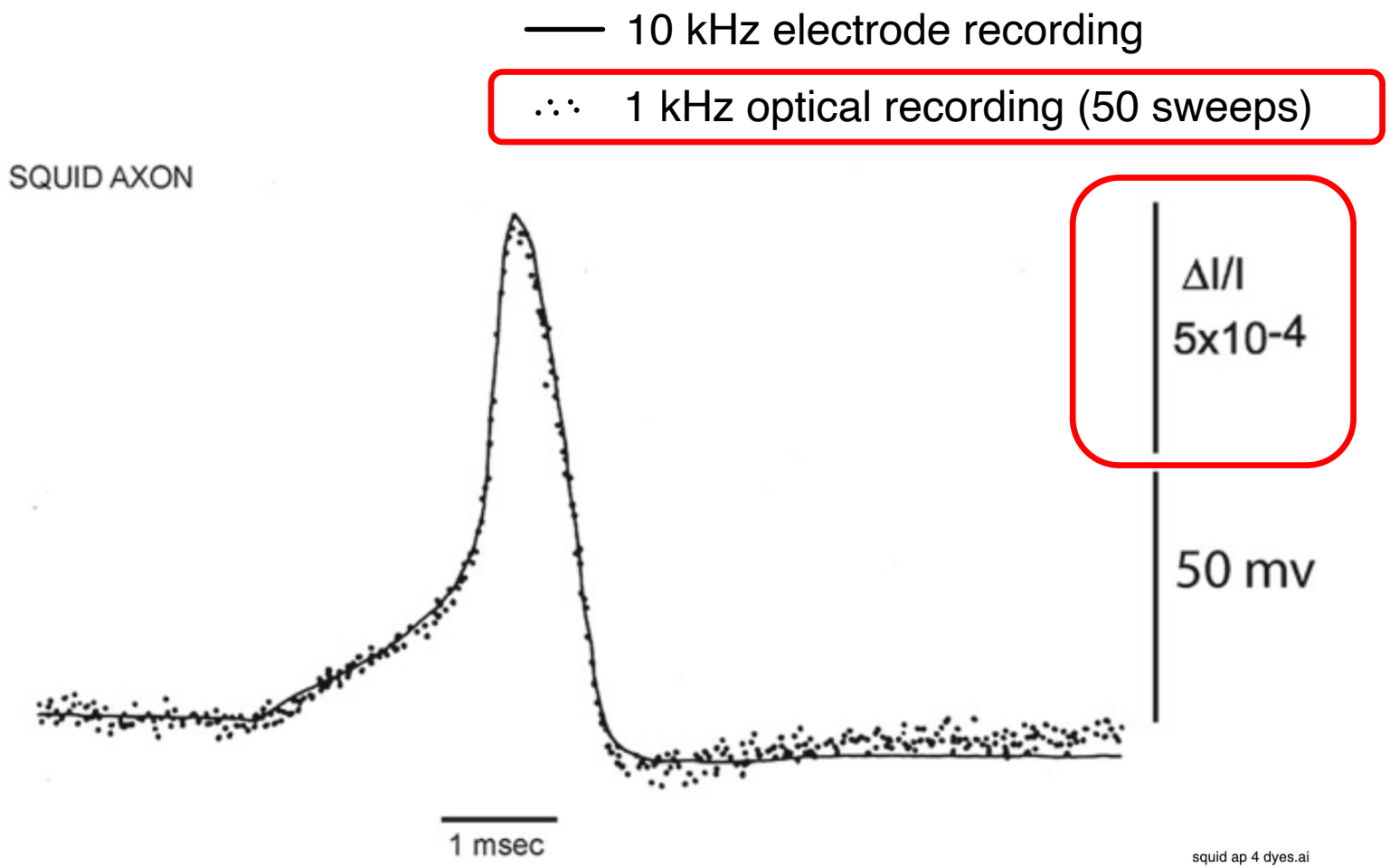

Question: Any fundamental differences compared to a calcium recording?

Answer: Fast voltage responses and low fluorophore density challenge signal-to-noise

Optical voltage sensors

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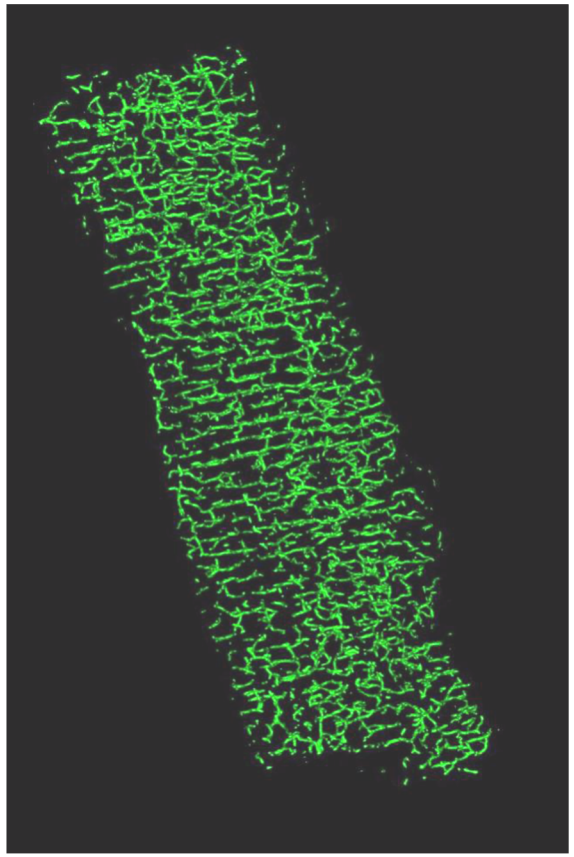


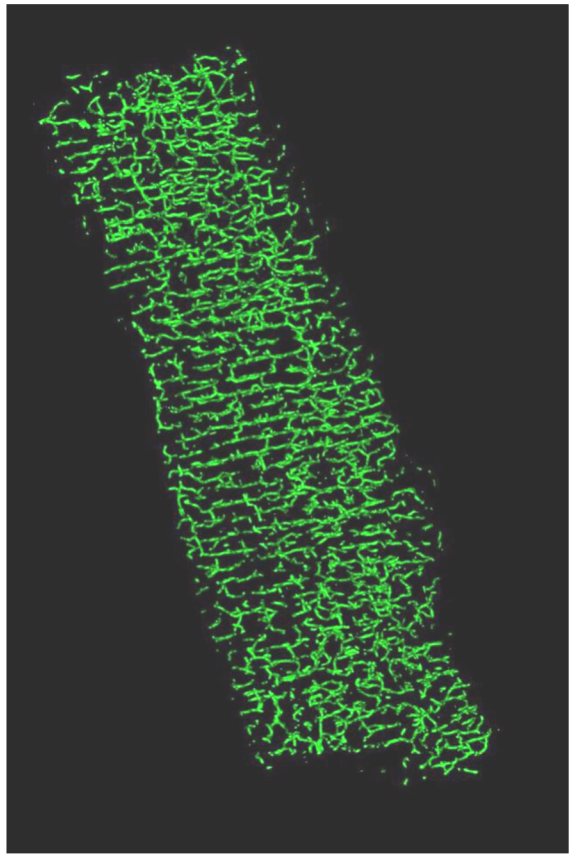
Cardiac cell cytosol volume:

$$W \times H \times L = 10 \times 20 \times 100\ (\mu m^3) = 20\ pL$$

Question: Typical intracellular concentration for calcium fluorophores?

Optical voltage sensors

ADDED CHALLENGES FOR OPTICAL VOLTAGE SENSORS



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Question: Typical intracellular concentration for calcium fluorophores?

Answer: ~ 200 µM

Optical voltage sensors

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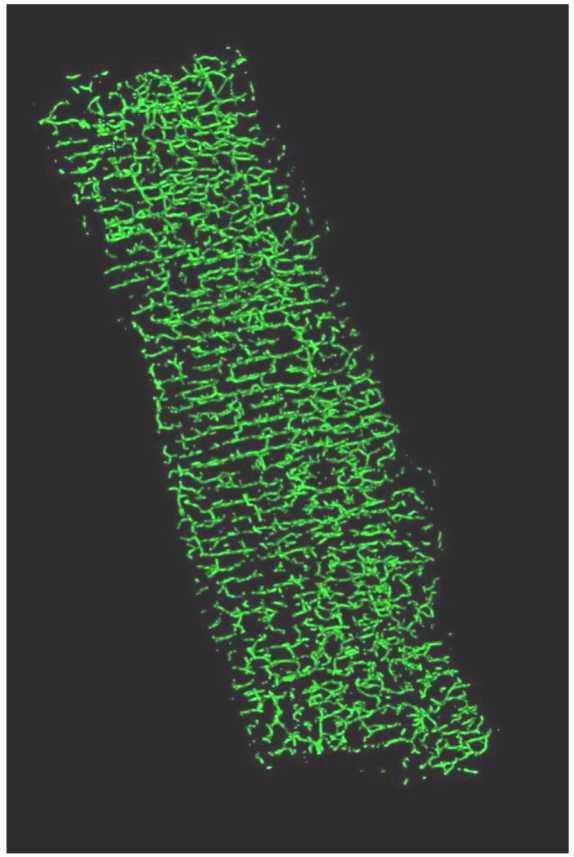

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Cardiac cell membrane volume:

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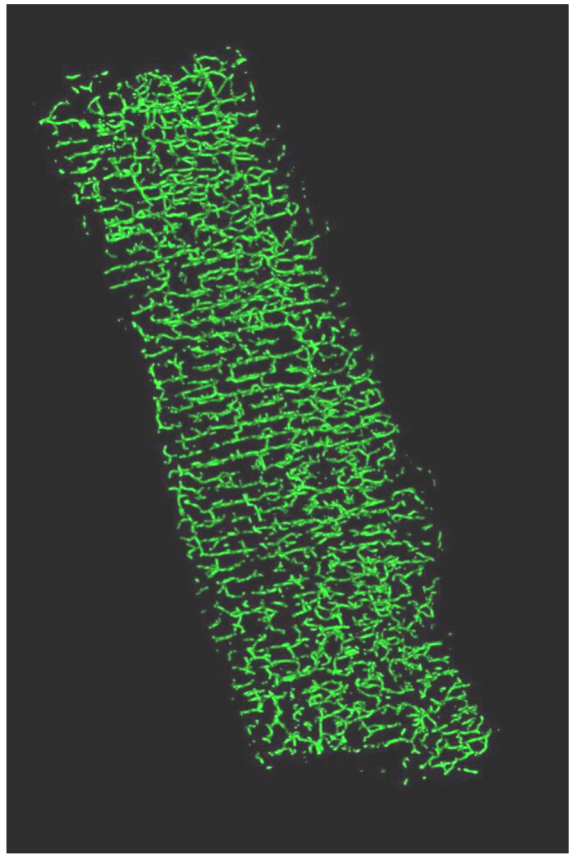
Optical voltage sensors

ADDED CHALLENGES FOR OPTICAL VOLTAGE SENSORS



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Question: Implication for simultaneous V_m-Ca^{2+} imaging ($I_f = N_f P_{abs} P_{em}$)?

Optical voltage sensors

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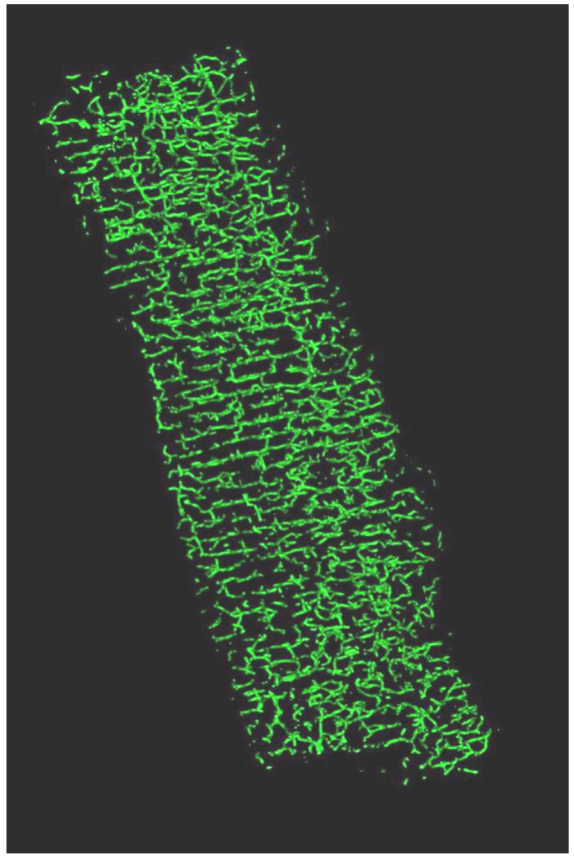

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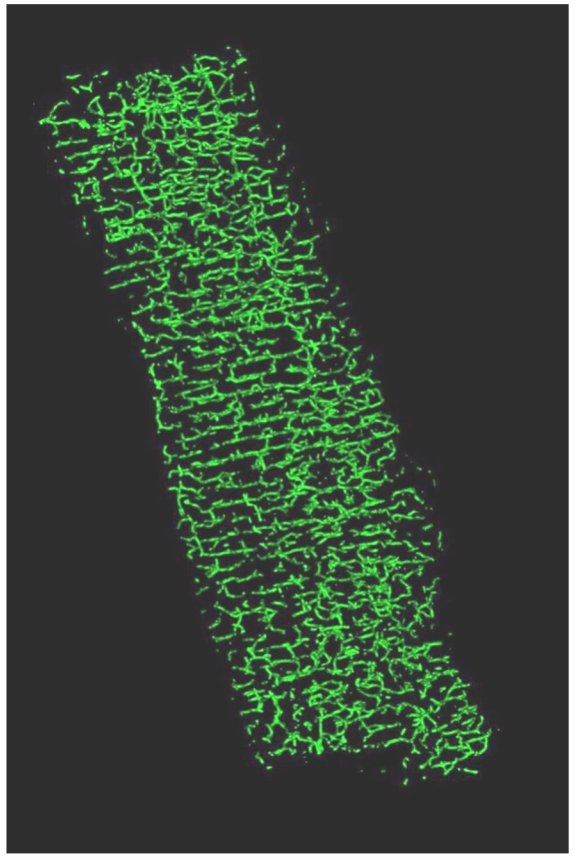
Question: Implication for simultaneous V_m-Ca^{2+} imaging ($I_f = N_f P_{abs} P_{em}$)?

Answer: I_0 required for high fidelity V_m imaging is much more than for Ca^{2+}

= major constraints on experimental design

Optical voltage sensors

ADDED CHALLENGES FOR OPTICAL VOLTAGE SENSORS



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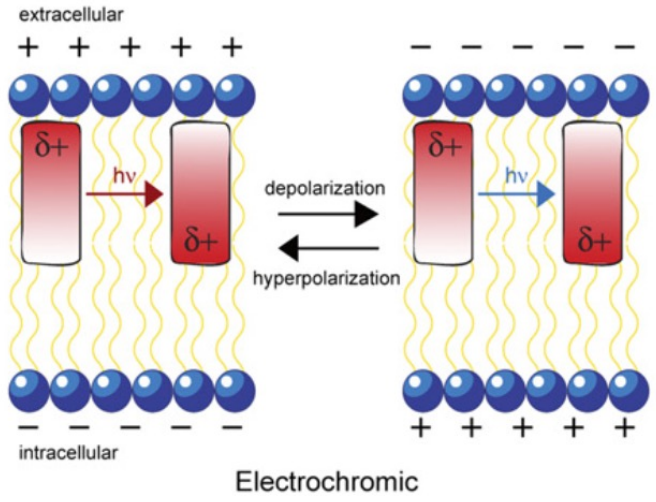
Question: Can we simply enrich the membrane with V_m fluorophores?

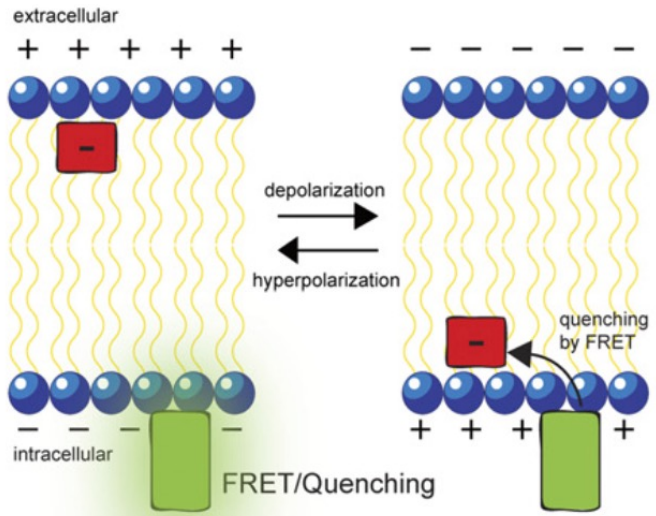
Optical voltage sensors

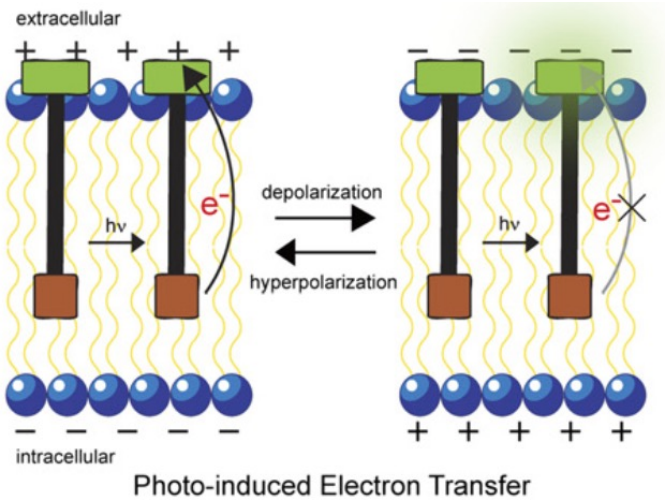
STRATEGIES FOR DEVELOPING OPTICAL VOLTAGE SENSING

Question: Can we simply enrich the membrane with V_m fluorophores?

Answer: Not really, all V_m fluorophores embed in membranes and act as detergents



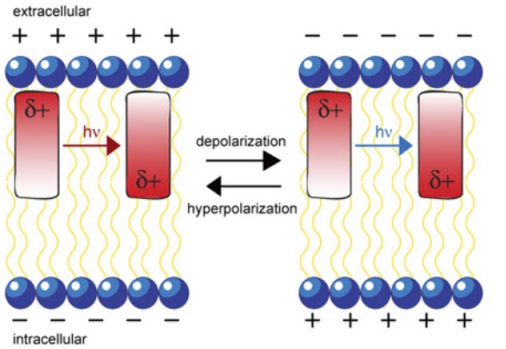




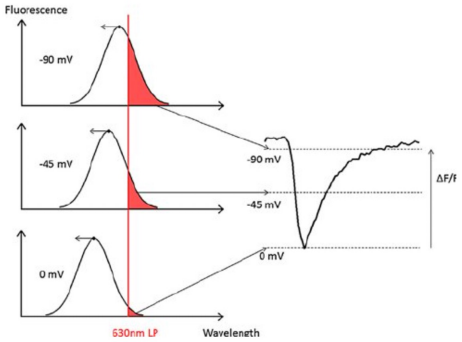
Miller *et al*. 2011

Optical voltage sensors

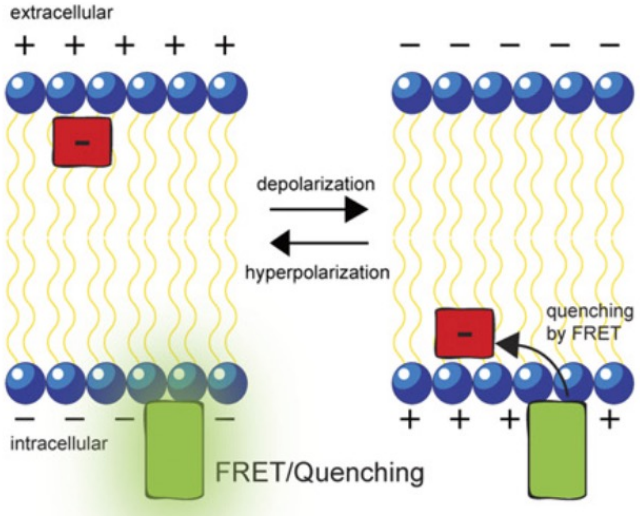
STRATEGIES FOR DEVELOPING OPTICAL VOLTAGE SENSING



Electrochromic



- Used extensively
- Calibration possible in principle
- Spectral shifting less bright (note GEVIs)



FRET/Quenching

- High quantum yield
- Calibration not possible
- Slow kinetics

BeRST

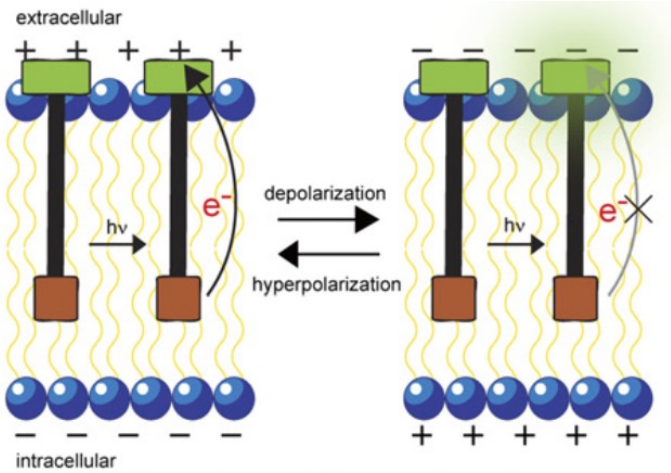


Photo-induced Electron Transfer

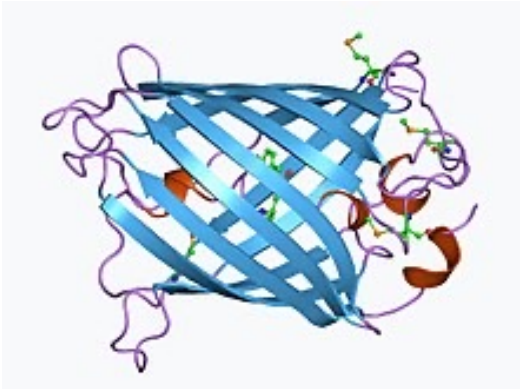
Miller *et al.* 2011

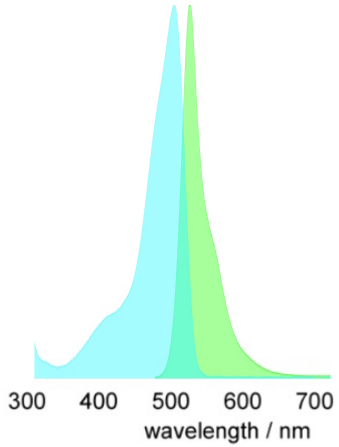
- High quantum yield
- Fast
- Far-red shifted
- Calibration not possible
- Not yet broadly distributed

Combining V_m and Ca^{2+} sensors

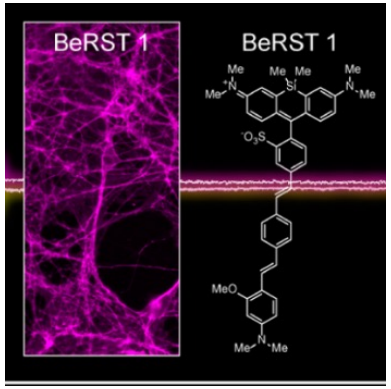
SPECTRAL DISTINCTION AND PHOTOSTABILITY

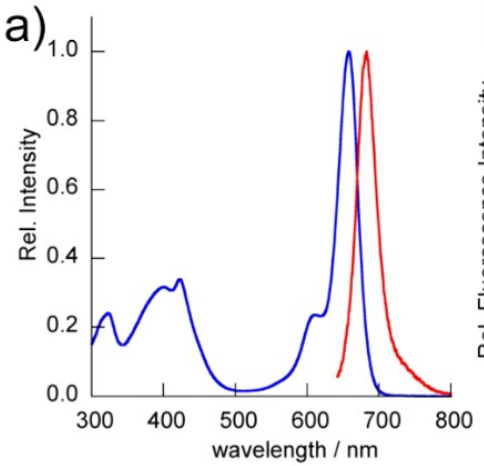
GFP

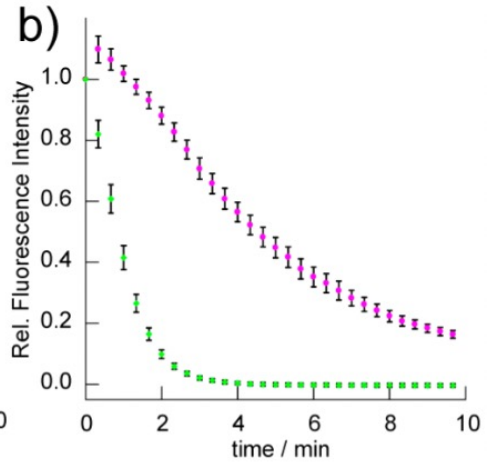




BeRST







Huang *et al.* 2015

Summary: Voltage fluorescence

STATE OF THE ART FOR MPS MEASUREMENTS

1. Modern V_m dyes

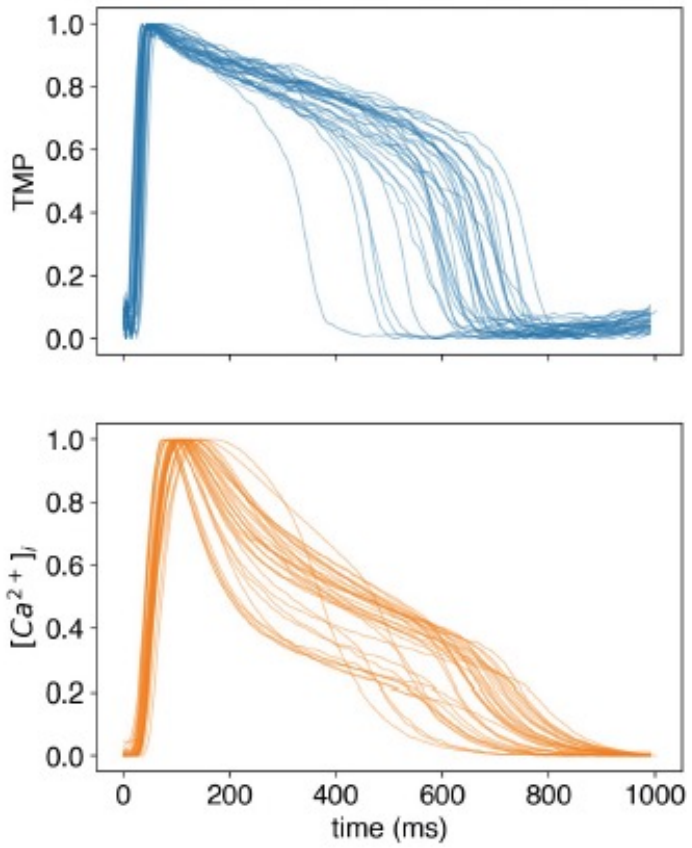
- Red-shifted spectra available
- Fast
- Improved Φ through PeT and electrochromic GEVIs

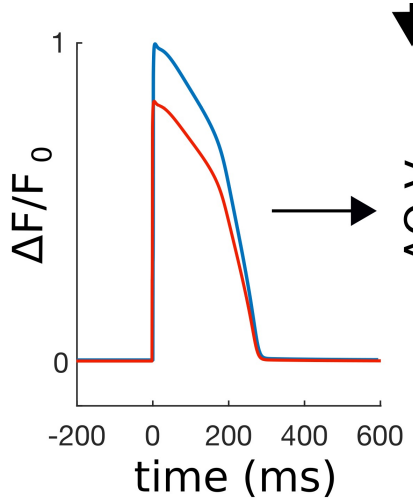
2. Ongoing challenges

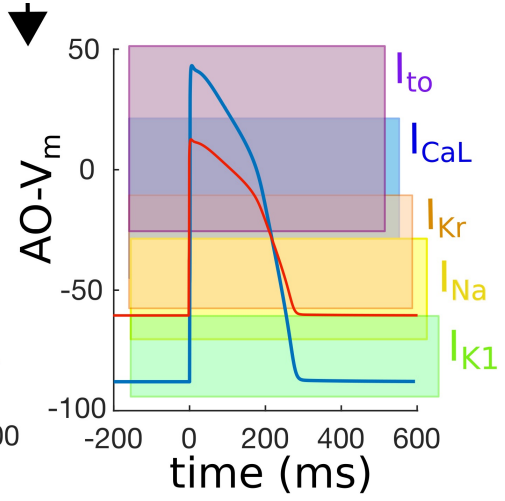
- Reconciling temporal constraints and signal strength
- High illumination intensity light required
- Calibration remains a challenge

Closing arguments: Calibration

STATE OF THE ART FOR MPS MEASUREMENTS

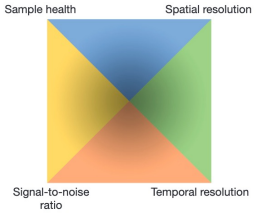






Rich protocols:

- Multiple [drug]
- Frequency
- Extracellular [ion]



Calibrated signals