

Transbilayer Coupling of Lipids in Cells Investigated by ImFCS

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The Plasma Membrane

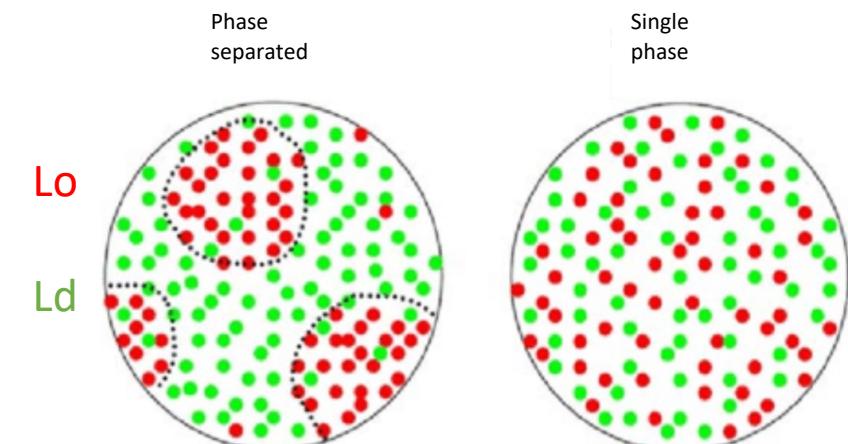
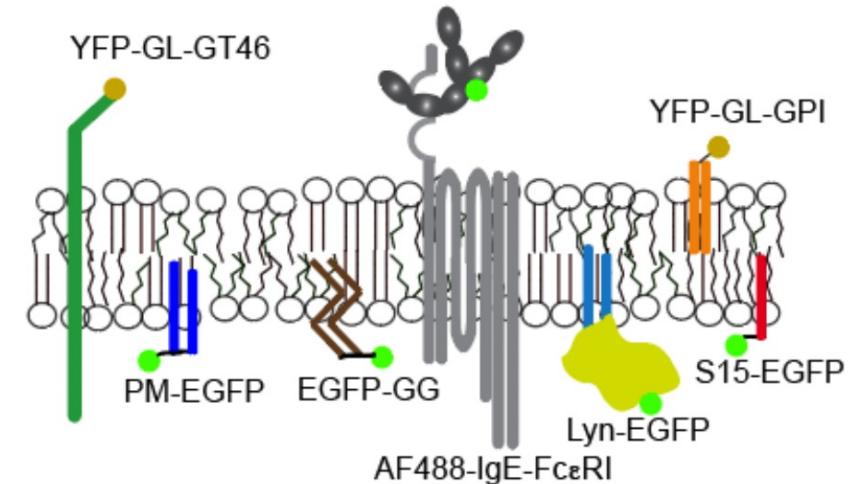
- Composition
- Function (Signal transduction, secretion)

- Phase separation:

Lo = Liquid-ordered phase (lipid rafts)

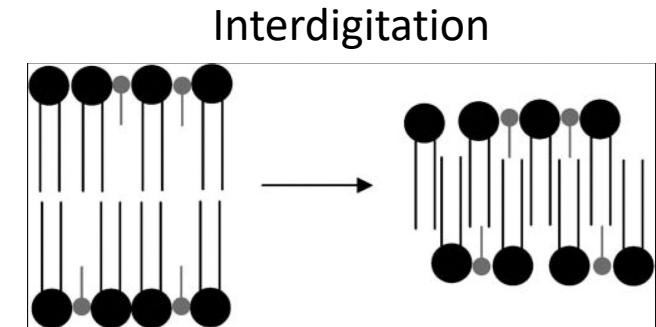
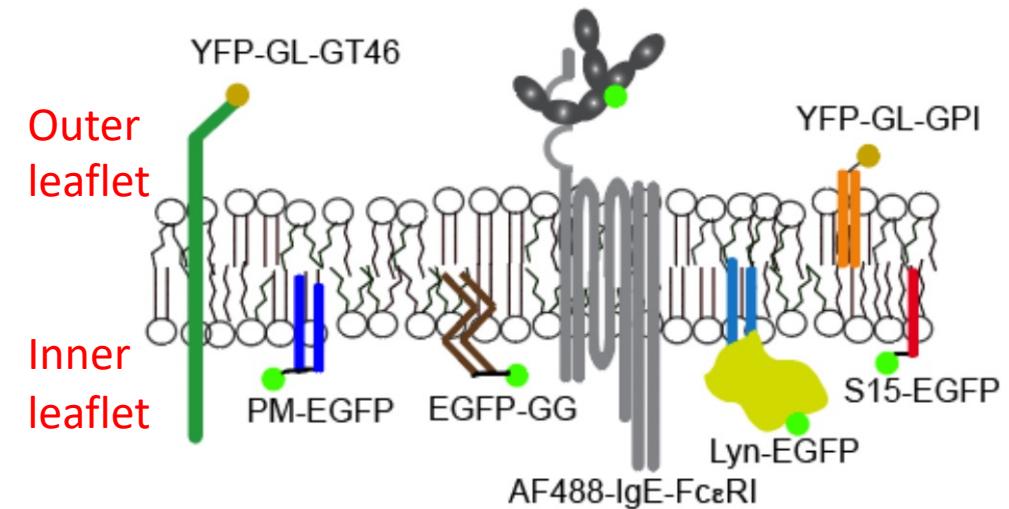
Ld = Liquid-disordered phase

- Some molecules are Lo-preferring and some are Ld-preferring



Membrane asymmetry

- Inner and outer leaflets
- Different amounts of different types of molecules
- Outer: GSL, SM, Inner: PE, PS, PIP's
- PC, cholesterol on both leaflets
- Flip-flop between leaflets
- Asymmetry is important for cellular activity 'outside-in' and 'inside-out': change in one leaflet affects the other
- Leaflets are believed to affect each other through interdigitation (interlocking of lipids)



<https://doi.org/10.1529/biophysj.104.045005>

Motivation

- **Previous evidence** for nanoscopic domains in lipid bilayers
- For example, one group used STED-FCS to characterize transmembrane coupling in live cells and identified Lo-like region confinement.
- Current diffraction-limited microscopy (FRAP, FCS, SPT) of transbilayer interactions is **very challenging** with live cells and **results vary broadly**.
- Imaging in live cells requires specialized microscopes and difficult sample preparation, which limits accessibility

FCS

Fluorescence Correlation Spectroscopy:

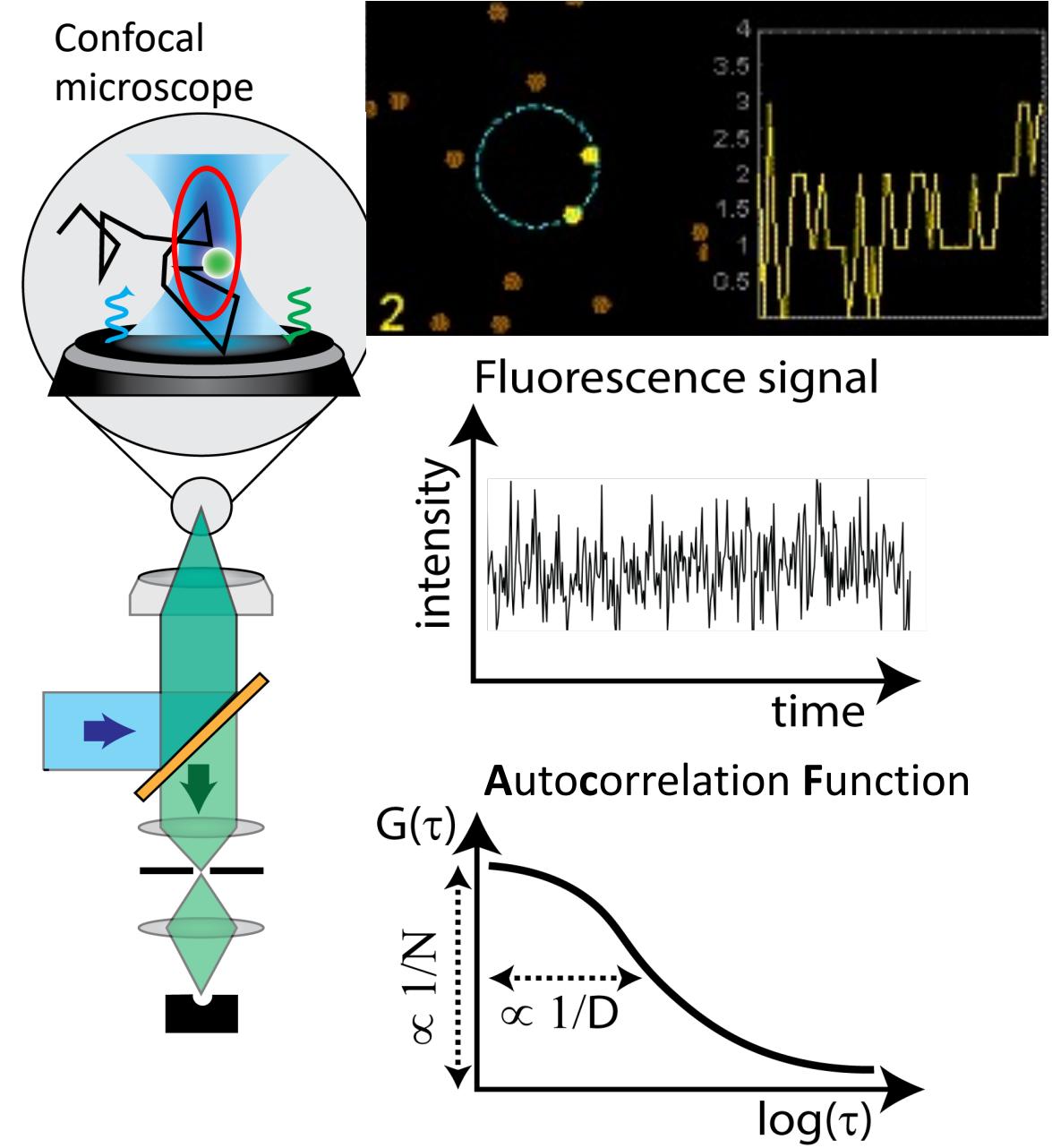
Monitoring fluorescence of a small region in the sample

Fluorescence signal over time

Auto-correlate signal at different time offsets

ACF gives characteristics such as concentration and diffusion coefficients

Disadvantages: small region, long acquisition time, misses biological phenomena which occur on bigger length scales



ImFCS on Total Internal Reflection microscope = TIRF

Imaging FCS: multiple spatial point at the same time

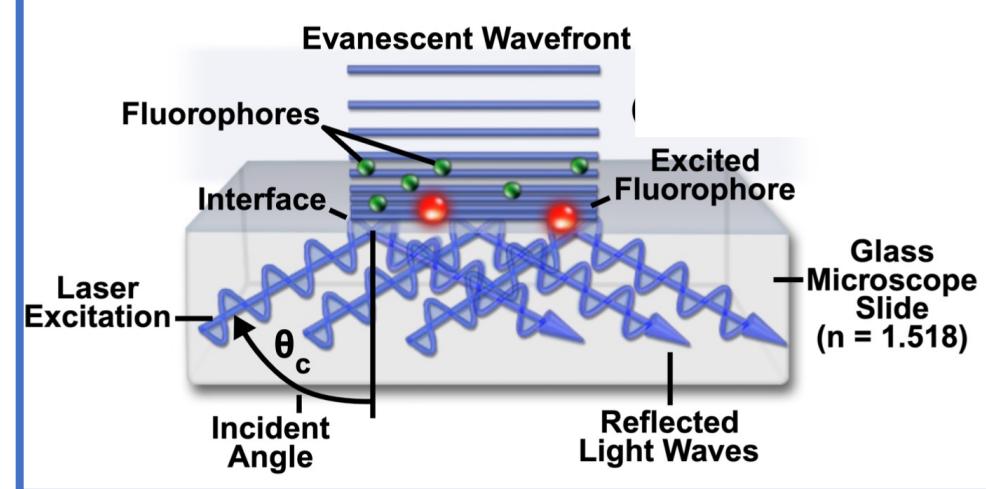
Monitoring fluorescence of a large region (plane)

Record images with a camera

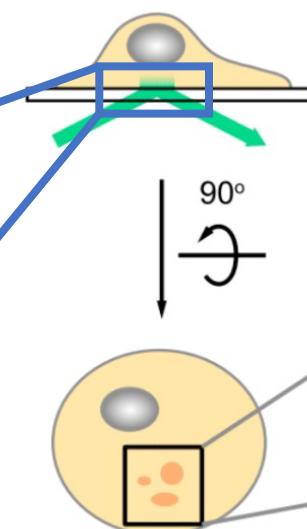
Auto-correlate signal at each pixel

Advantages:

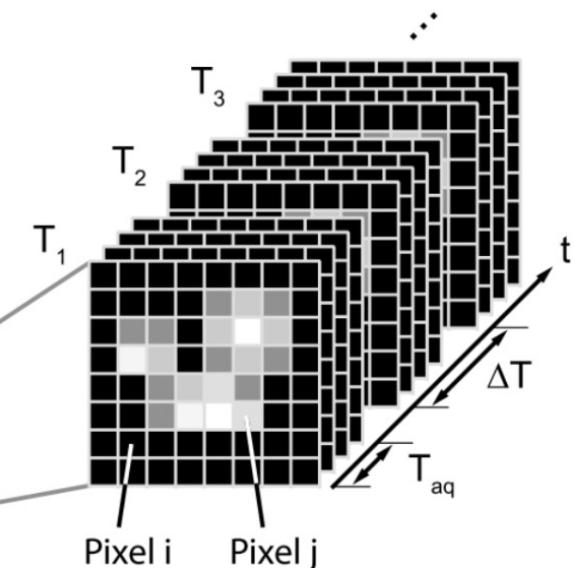
Large region, fast data acquisition



Live cell TIRF imaging



Raw TIRF movies



Experimental setup

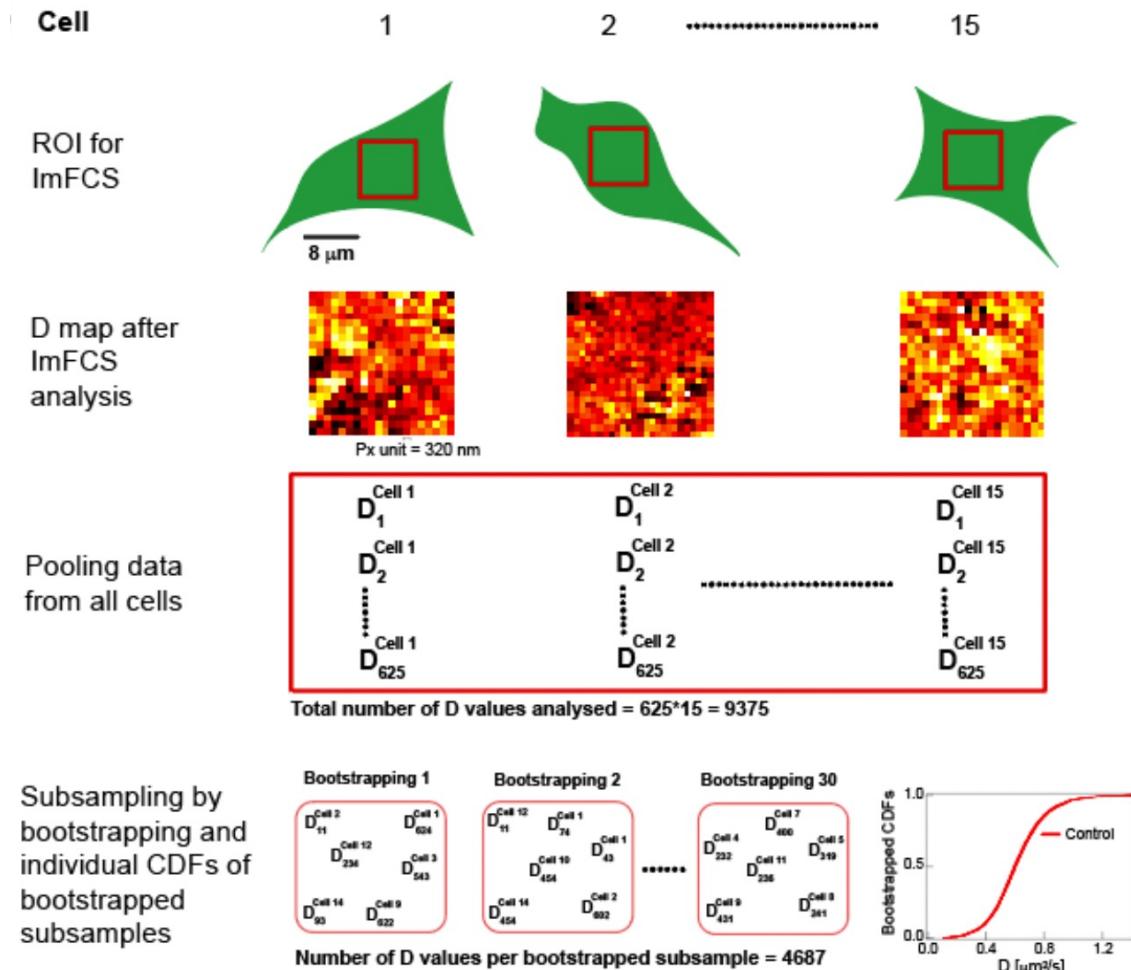
- Compiled D values for various probes
- Tested ‘outside-in’ coupling mode in rat basophilic leukemia cells
- Labelled multiple inner leaflet, outer leaflet, and transmembrane probes

- Labelled two known inner leaflet probes:

EGFP-GG (Ld-preferring)

PM-EGFP (Lo-preferring)

- Imaged an $8 \times 8 \mu\text{m}^2$ region with $320 \times 320 \text{ nm}^2$ pixels, yielding 625 ACF’s with one observation (280 seconds), repeated with 15 cells (over 9000 D values), for each probe.



Bootstrapping, CDF's

- Re-sampling method in which all observed data is aggregated into one set, from which points are then subsampled with replacement and equal weighing, ‘simulating’ the true unknown CDF.

Empirical Cumulative Dist. Function:

$$F_n(x) = \frac{1}{n} \sum_{i=1}^n I(X_i \leq x)$$

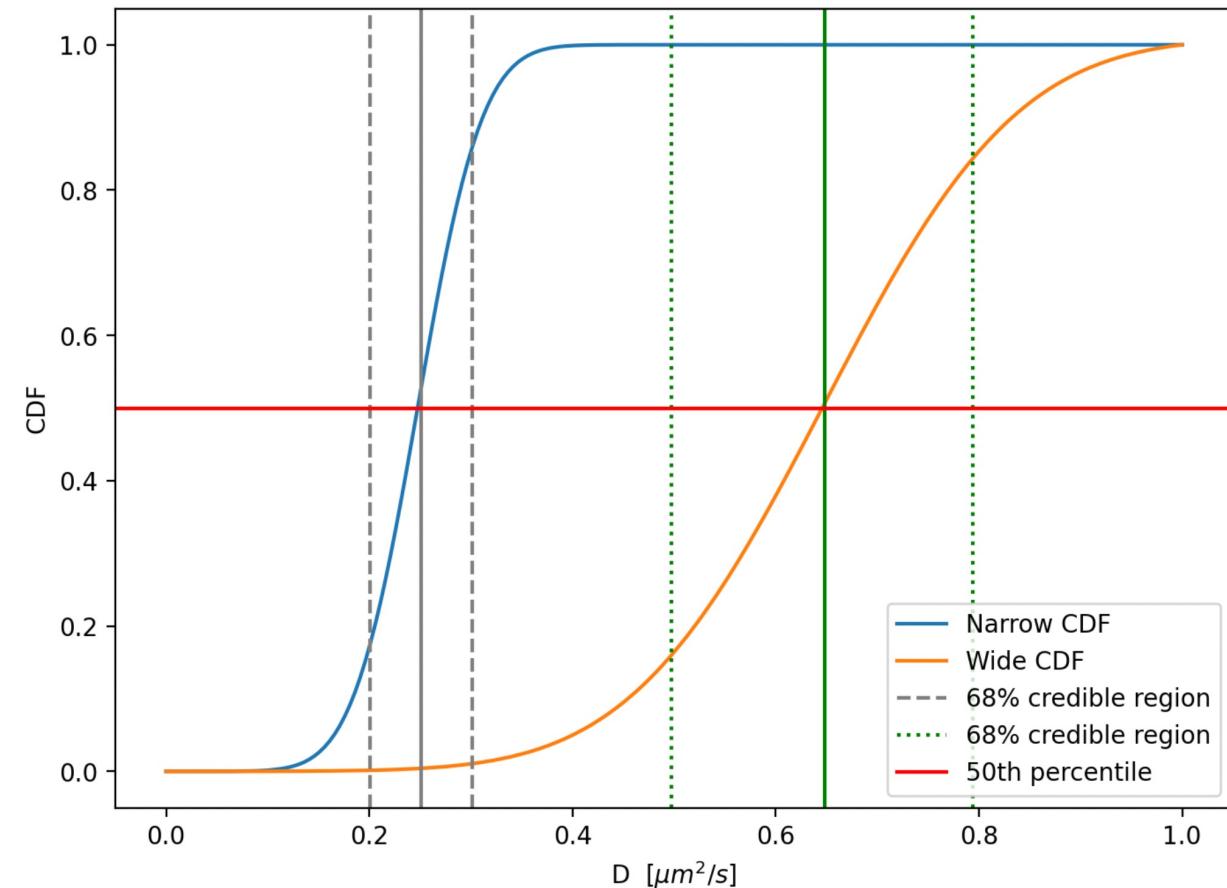
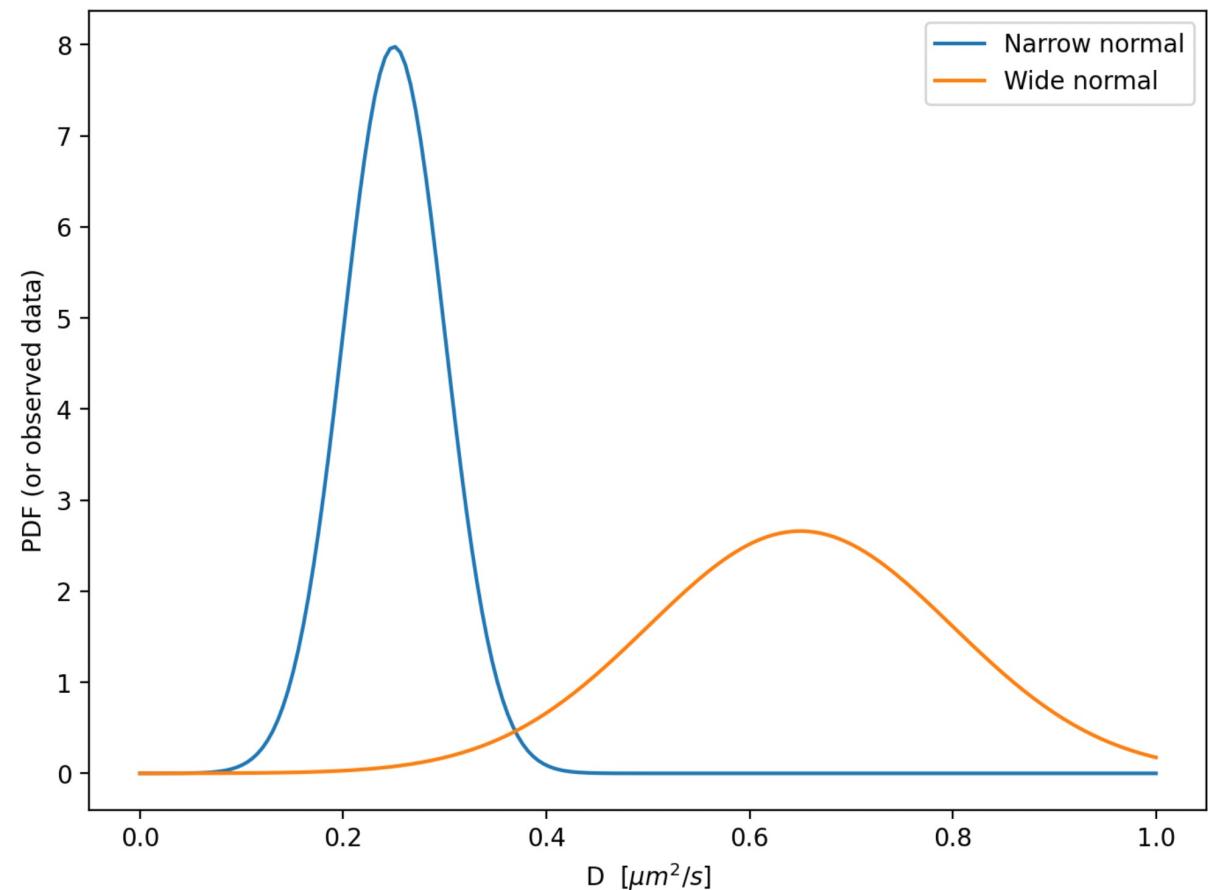
Glivenko-Cantelli Theorem:

$$F_n(x) \xrightarrow{\text{as}} F(x) \text{ for all } x \text{ as } n \rightarrow \infty$$

$$I(X_i \leq x) = \begin{cases} 1 & \text{if } X_i \leq x \\ 0 & \text{otherwise} \end{cases}$$

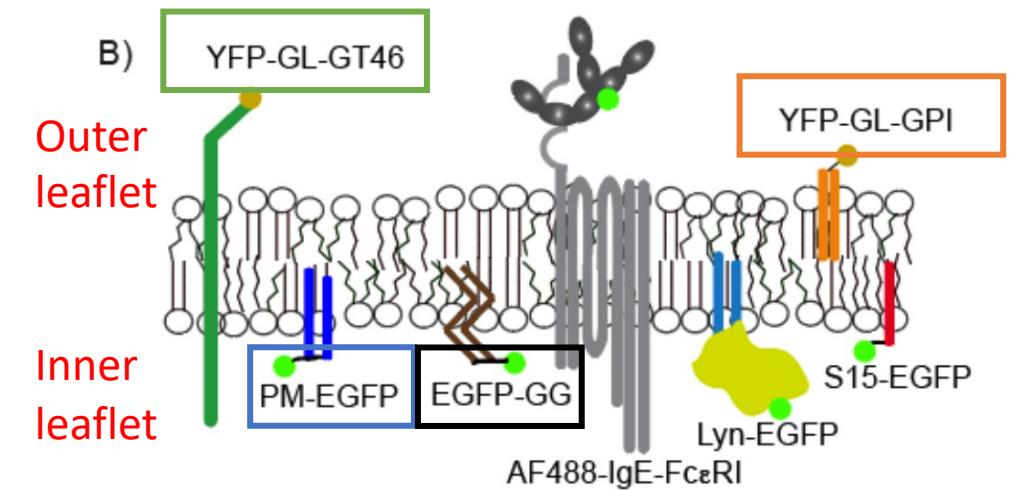
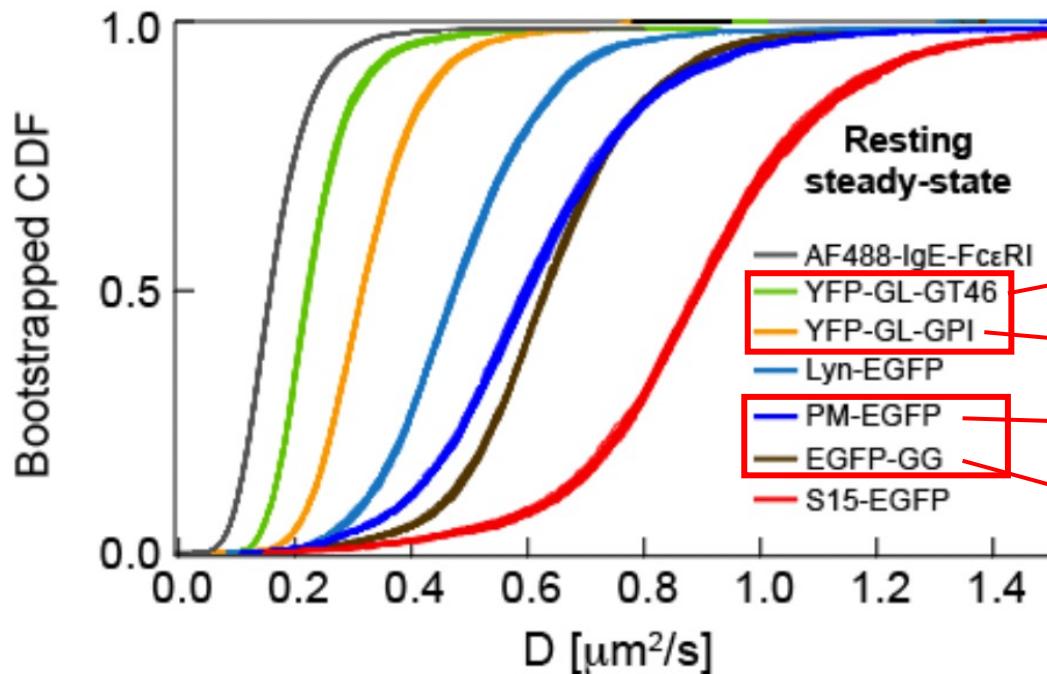
All CDF's for each probe are then averaged over to produce one final CDF.

Observed data to CDF



Results: Compiled D values

- General D values, no perturbation



Transmembrane probe

Outer leaflet Lo-preferring, Lipid-anchored probe

Inner leaflet Lo-preferring probe

Inner leaflet Ld-preferring probe

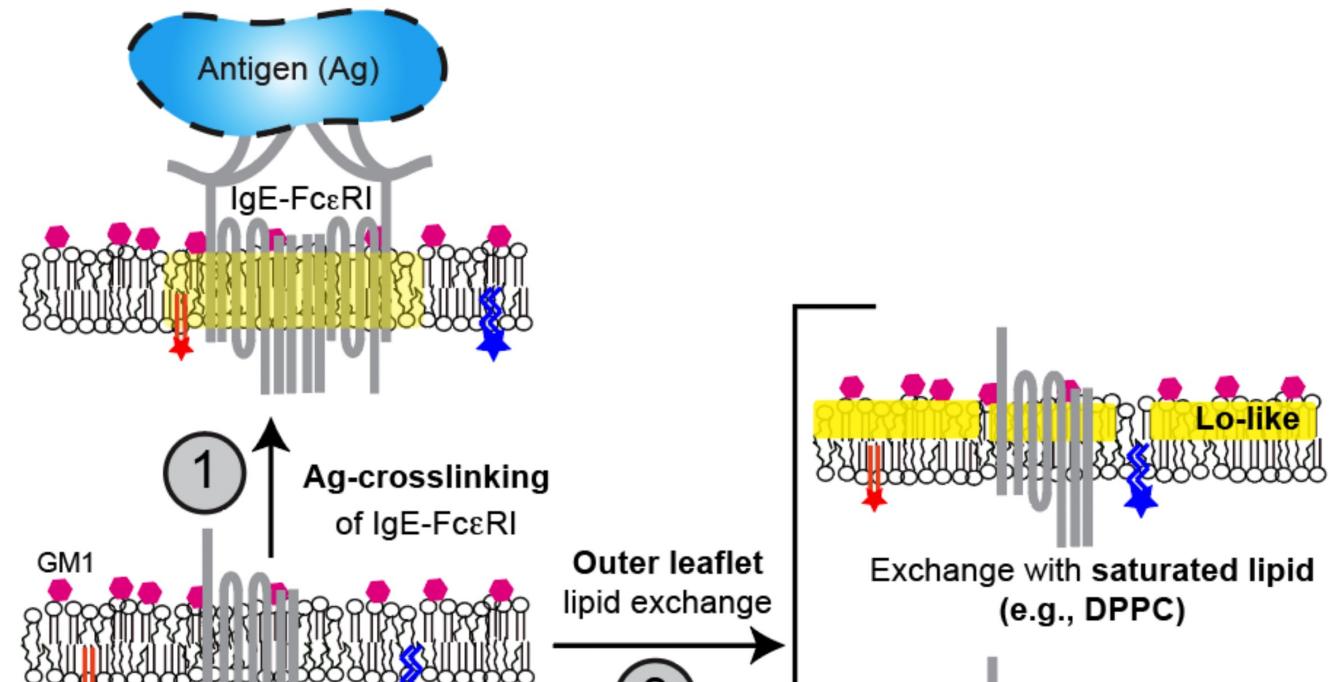
Error in D_{av} values is < 1%

Method demonstration on 2 well-established model systems

- Outside-in coupling (inner-leaflet probes PM-EGFP and EGFP-GG)
- 1. Ag crosslinking of transmembrane IgE-Fc ϵ RI
- 2. Antibody (Ab)-crosslinking of glycosphingolipid complex CTxB-GM1

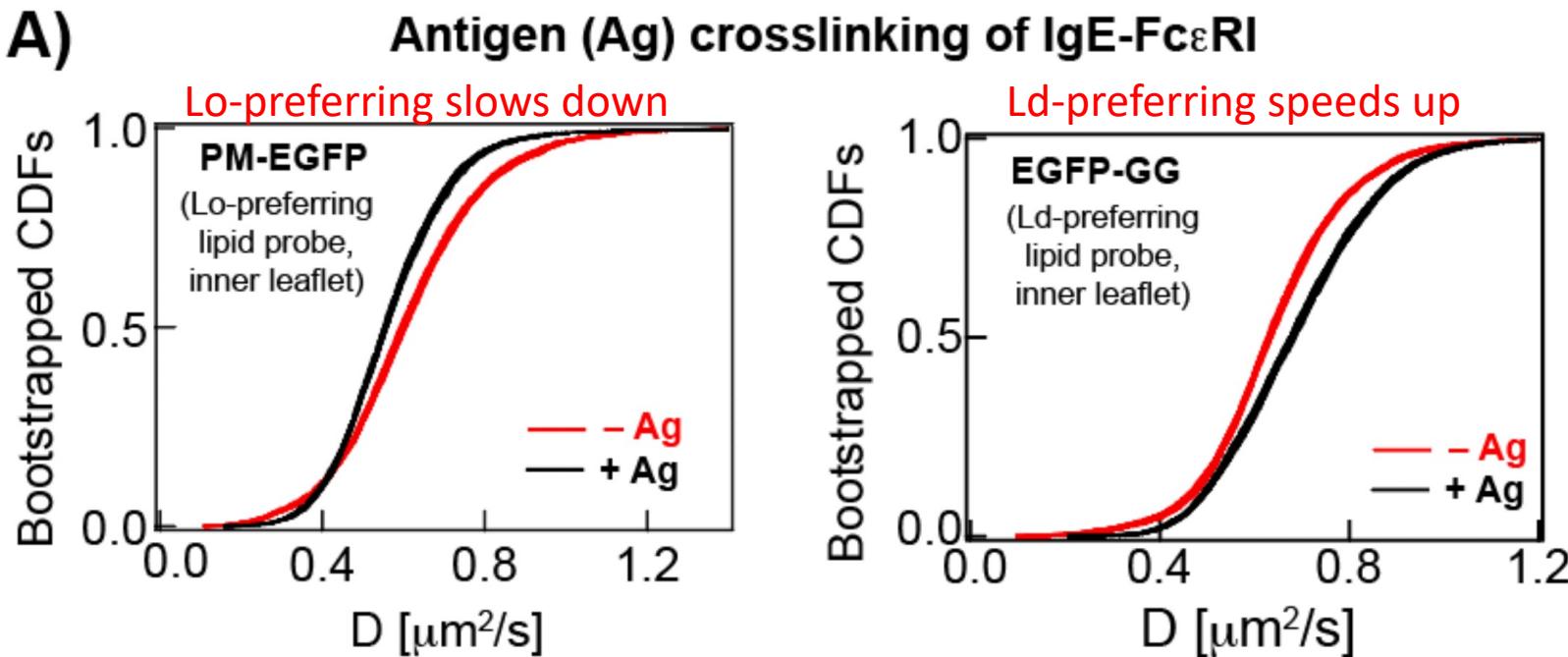
1. Ag crosslinking of transmembrane IgE-Fc ϵ RI

- Transmembrane protein-mediated coupling
- Immune system pathway
- Triggers a signaling cascade which initiates allergic and inflammatory responses.
- Known to locally stabilize Lo-like nanodomains.



Ag crosslinking of transmembrane IgE-Fc ϵ RI

A)



Results:

Lo-preferring probe slows down

Ld-preferring probe speeds up

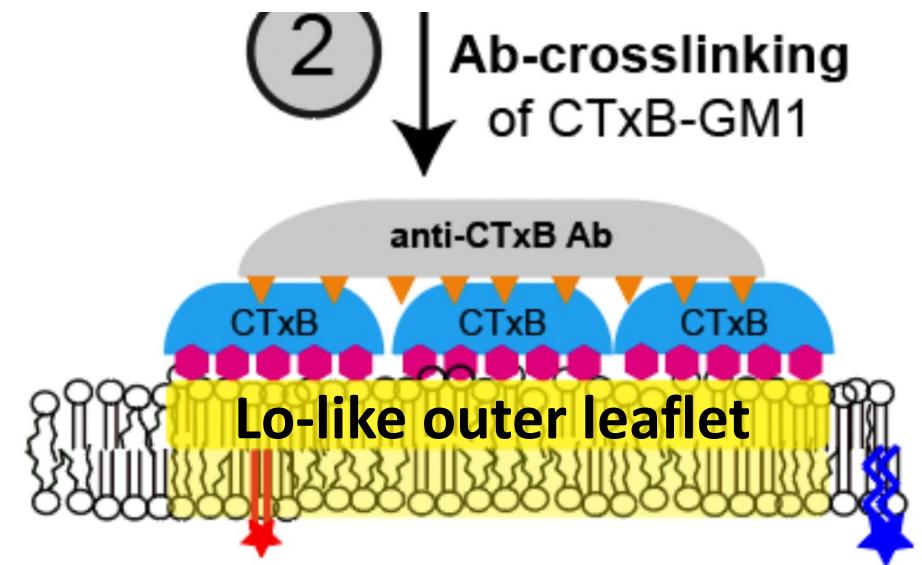
Conclusion:

Lo-like phase stabilization around clustered receptor after crosslinking

Ld-like probes can diffuse faster in absence of Lo-like probes

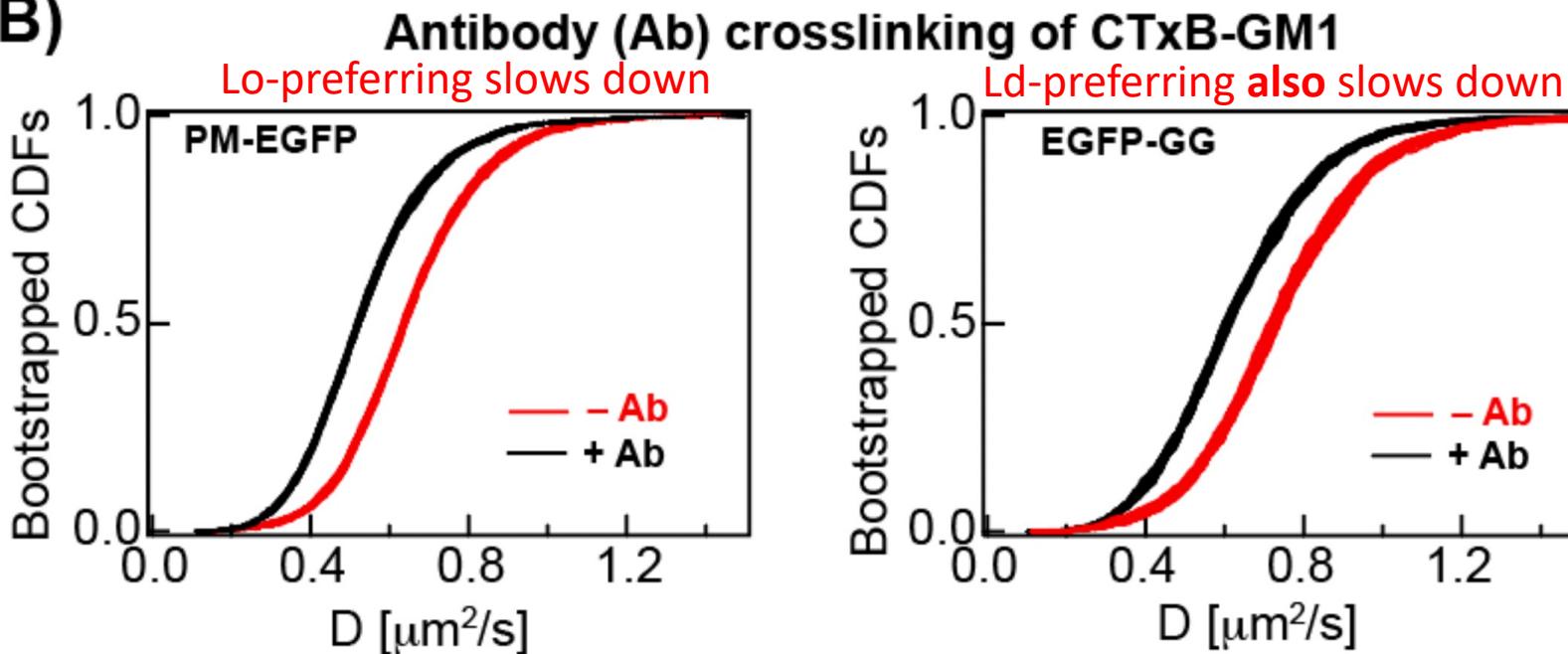
2. Ab-crosslinking of CTxB-GM1 complex

- Lipid-mediated transbilayer coupling
- GM1 is an outer leaflet Lo-preferring lipid
- Ab-crosslinking causes Lo-like phase stabilization in outer leaflet



Antibody (Ab)-crosslinking of CTxB-GM1 complex

B)



Contrasting results
compared to ag-IgE-Fc ϵ RI
crosslinking!

Results:

- Observed no probe clustering on inner leaflet
- Both probe types slowed down

Hypothesis:

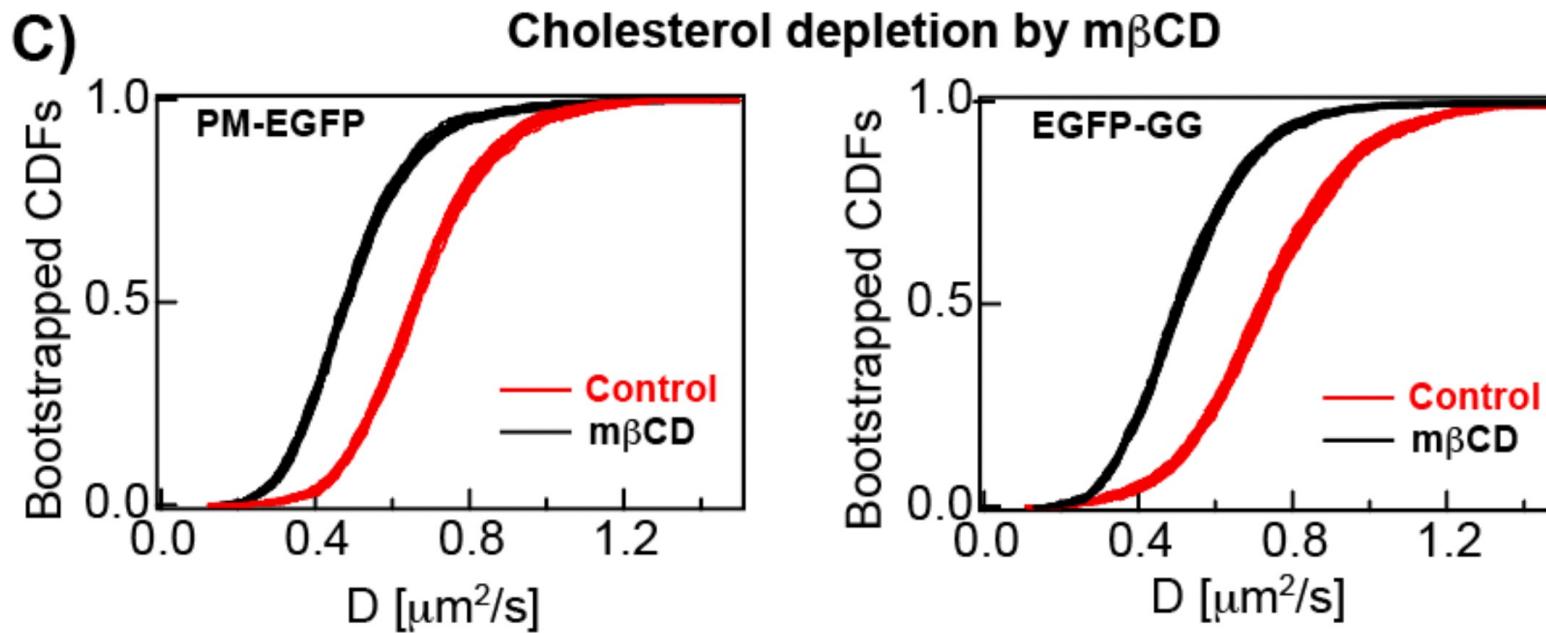
- May be due to transmembrane coupling mediator (lipid vs. transmembrane protein)
- Could be actin-engagement, effects of membrane curvature...

Conclusion:

- Ab-crosslinking of CTxB-GM1 **globally** affects inner leaflet diffusion properties

Methods to systematically perturb membrane lipid composition

- Cholesterol is a key component in Lo domain formation
- Cholesterol uptake by m β CD (less free cholesterol in membrane)



Results:

- Both probe types slowed down

Hypothesis:

- Cholesterol extraction causes a more ordered state due to formation of gel-like nanodomains

Conclusion:

- Cholesterol extraction **globally** affects inner leaflet diffusion properties

Methods to systematically perturb membrane lipid composition

- Endogenous outer leaflet lipid exchange with exogenous lipids
- LEX method that allows exchange of 80-100% of endogenous lipids of outer leaflet in both model vesicles and live cells, and remains stable for several hours
- Many perturbation possibilities can be tested

Drawbacks of membrane perturbation methods

- Cholesterol and other molecules flip-flop at various rates
- Results mentioned are from model membranes which lack a cytoskeleton and may have different asymmetry.

Conclusions

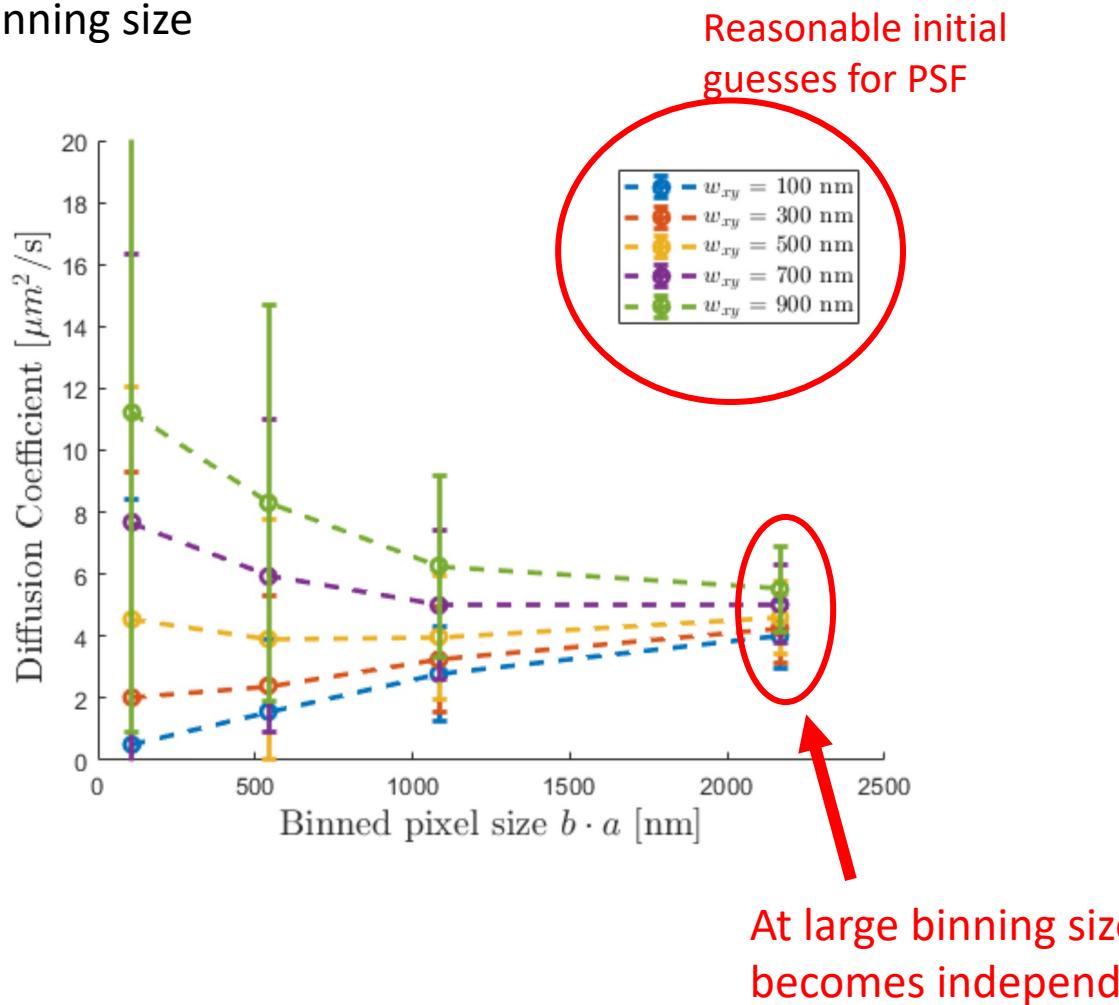
- Diffusion coefficients of various membrane probes have been compiled with very high precision from live cells
- Effect of ‘Outside-in’ coupling mode has been demonstrated on well-established systems
- Established a method to measure much smaller differences in D, previously not measurable.
- ImFCS is a quantitative approach to studying transmembrane coupling with very good statistics, allowing for smaller effects to be observed

What my project is about

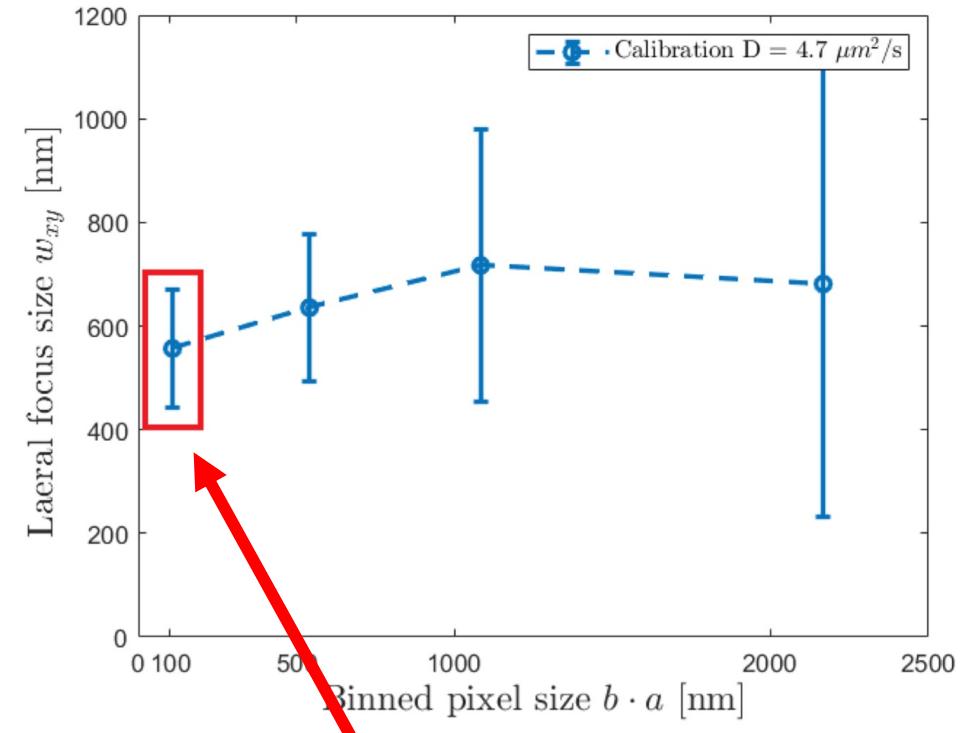
- Characterize PSF of TIRF microscope
- Validate measurements against a confocal microscope
- Write protocol for performing ImFCS on TIRF microscope

PSF calibration

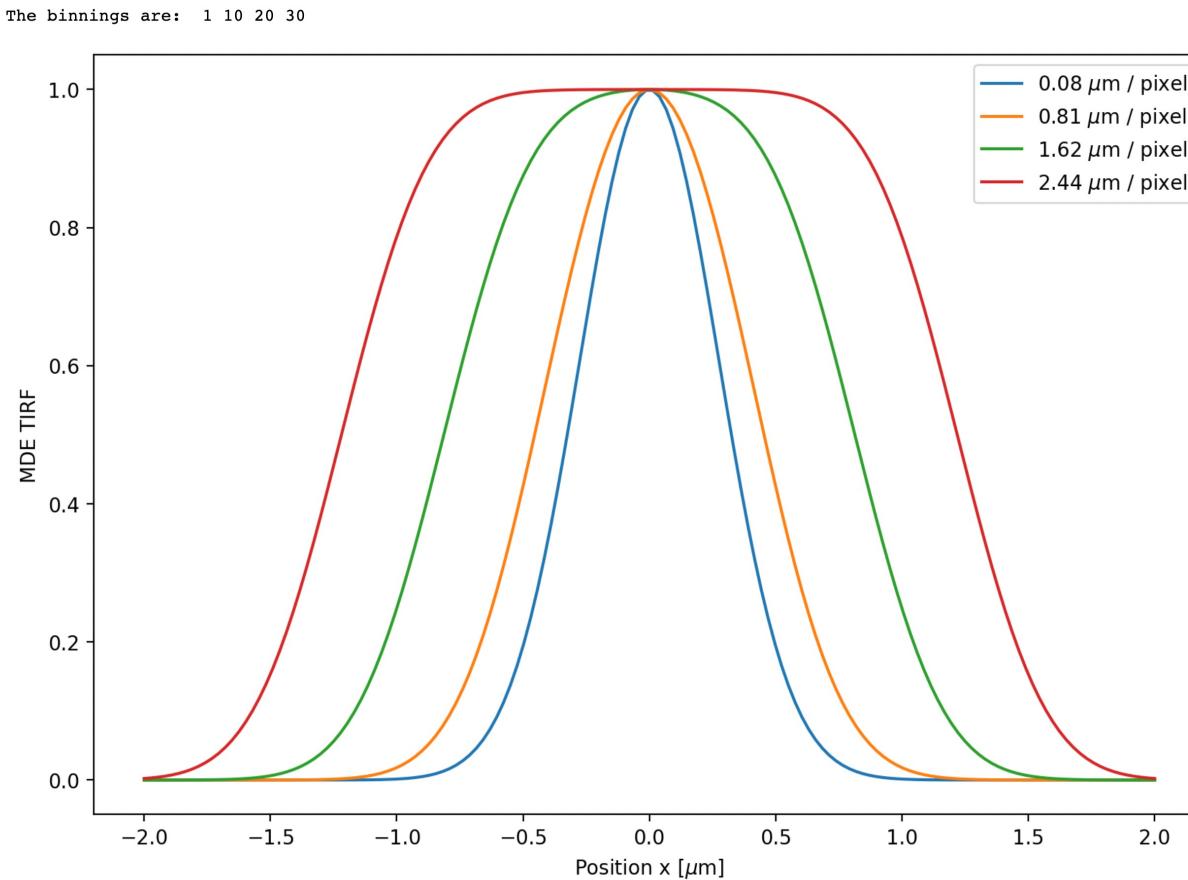
1. Plot D as function of binning size



2. Plot PSF as function of binning size, with now known reference D value

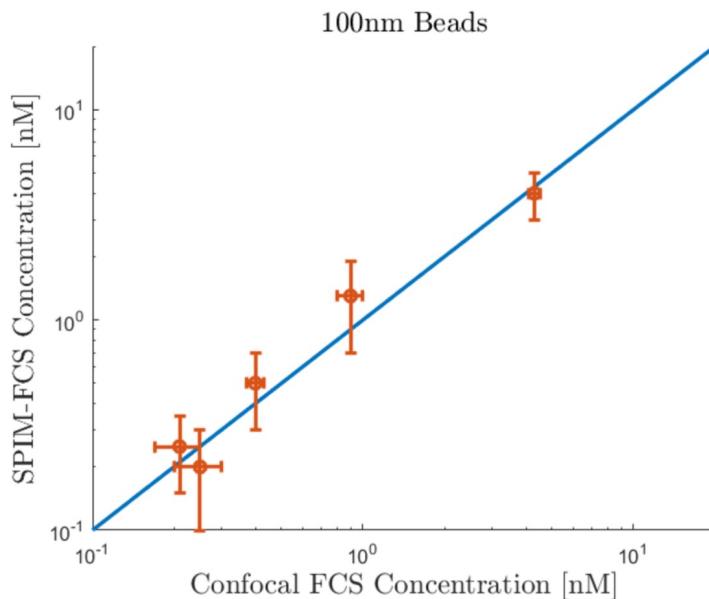


MDE function for PSF calibration



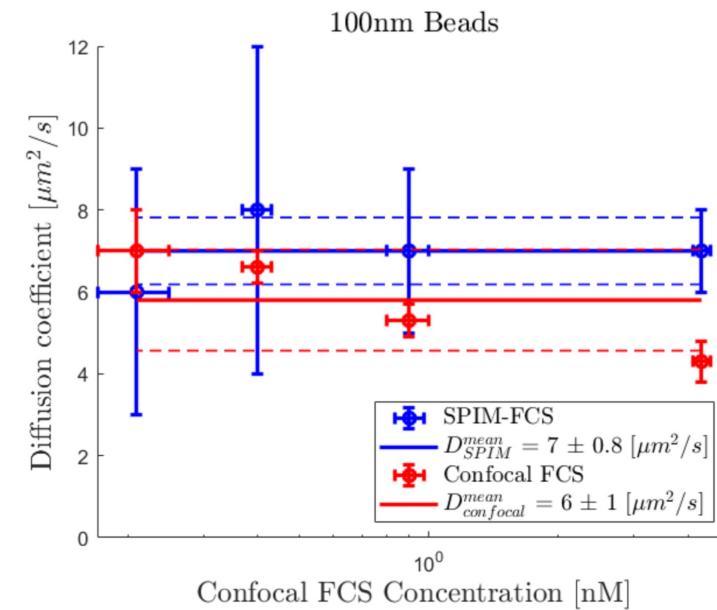
Validation against confocal microscope

1. Plot sample concentration measurements from TIRF vs. Confocal FCS



A linear plot will indicate agreement

2. Plot D vs. sample concentration measurements for both TIRF and confocal FCS



Measurements from each microscope within error bars of each other will indicate agreement

Overview of project

- what has been done:
 - Reading lit., learning how to use software, analyzed previous data
 - Image 100nm, 200nm beads (preliminary data)
- what's left to do:
 - SLB protocol, image SLB
 - Calibrate PSF with SLB data
 - Write protocol