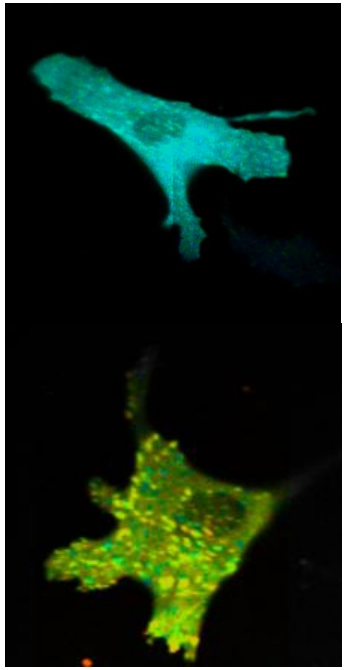
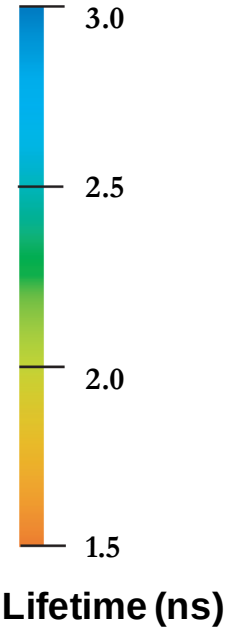
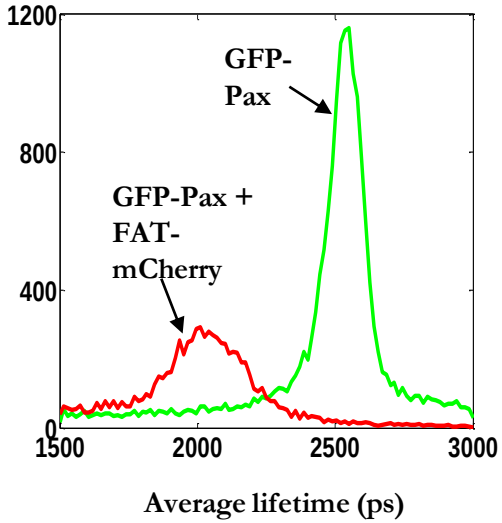
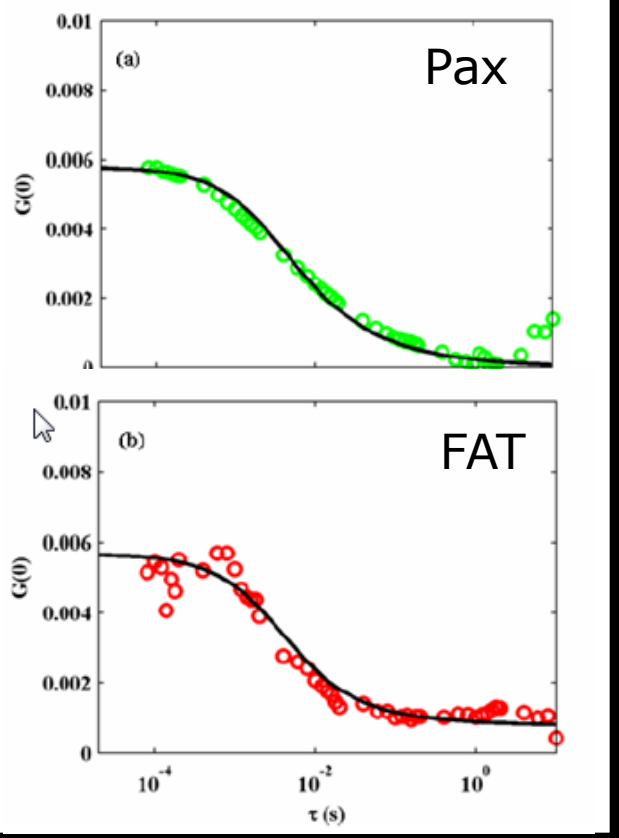
Advanced Fluorescence Microscopy I: Fluorescence (Foster) Resonance Energy Transfer

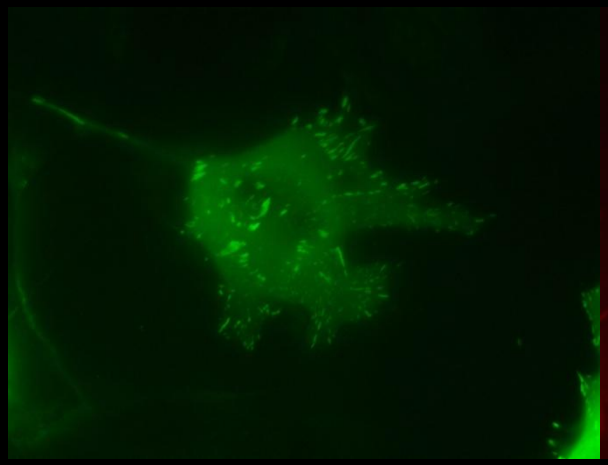




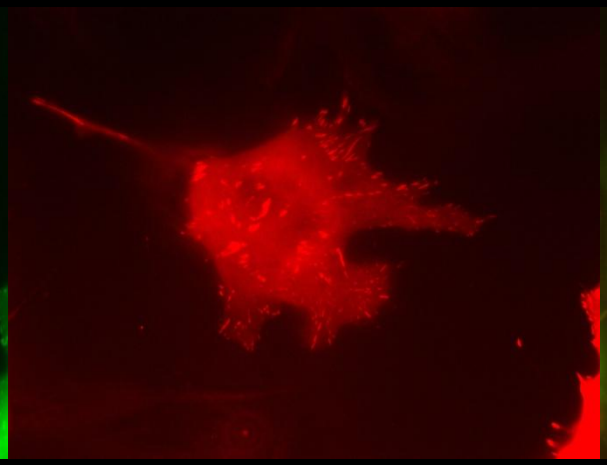




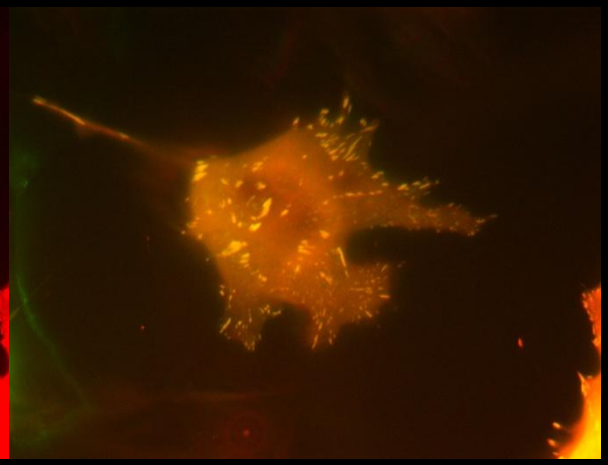
Paxillin-FAT in endothelial cells



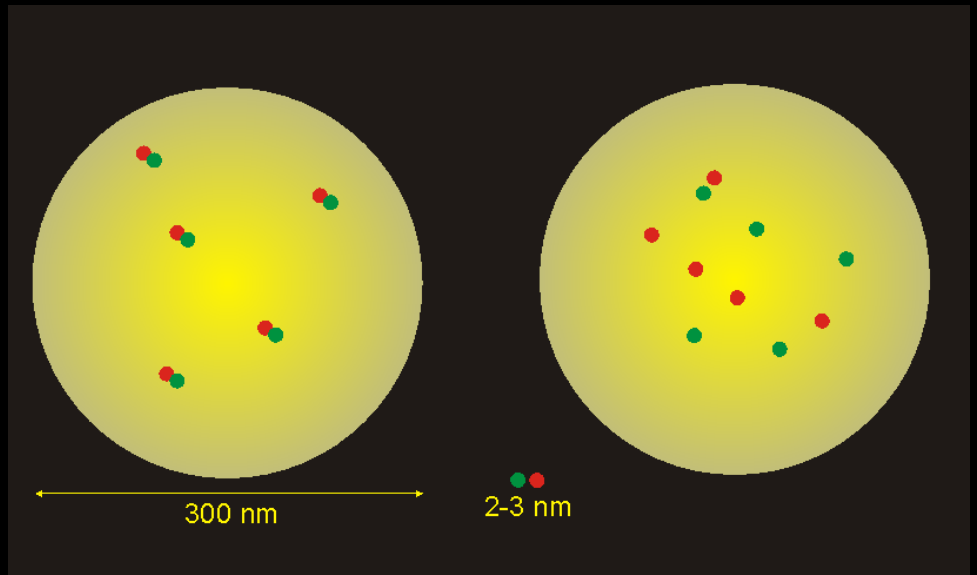
GFP-Paxillin



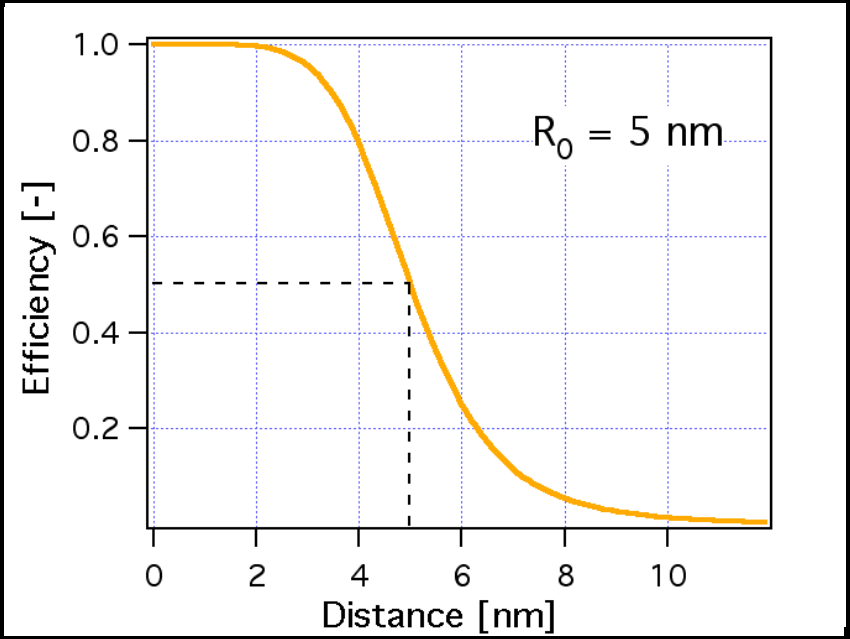
FAT-mCherry



Spectral overlap



Fluorescence Resonance Energy Transfer (FRET)



Dipole - dipole interaction

r^6 dependence

Efficiency

50% energy transfer

Förster distance

R_0 = 40 to 70 Å

Decrease donor intensity

Increase acceptor intensity

Decrease donor lifetime

$$E = \frac{R_0^6}{R_0^6 + r^6} = 1 - \frac{F_{DA}}{F_D}$$

$$E = \frac{R_0^6}{R_0^6 + r^6} = 1 - \frac{\tau_{DA}}{\tau_D}$$

$$R_0^6 = \frac{9000 \ln(10) \kappa^2 \phi_D}{128 \pi^5 N_A n^4} J$$

where, $J = \frac{\int F_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda}{\int F_D(\lambda)}$

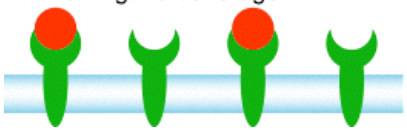
“Quantify” Signaling Pathway Using t-FRET

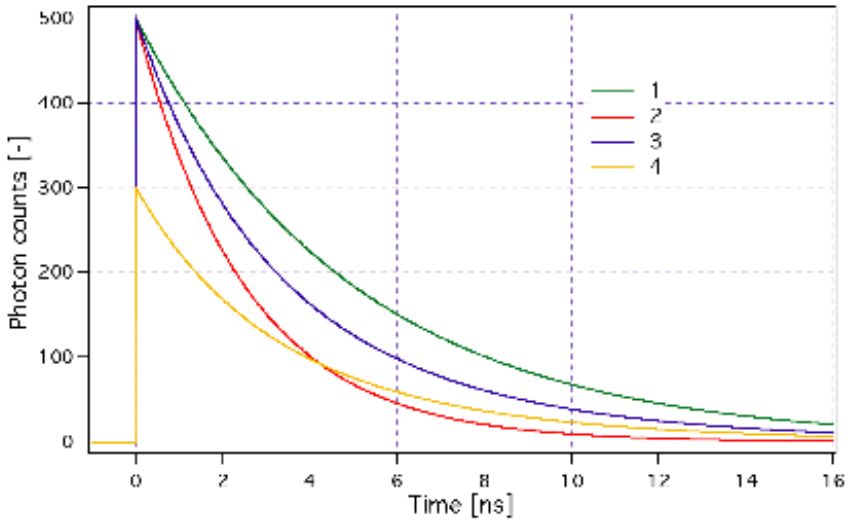
1. High receptor concentration, No ligand

2. High receptor concentration, Full ligand coverage

3. High receptor concentration, low ligand coverage

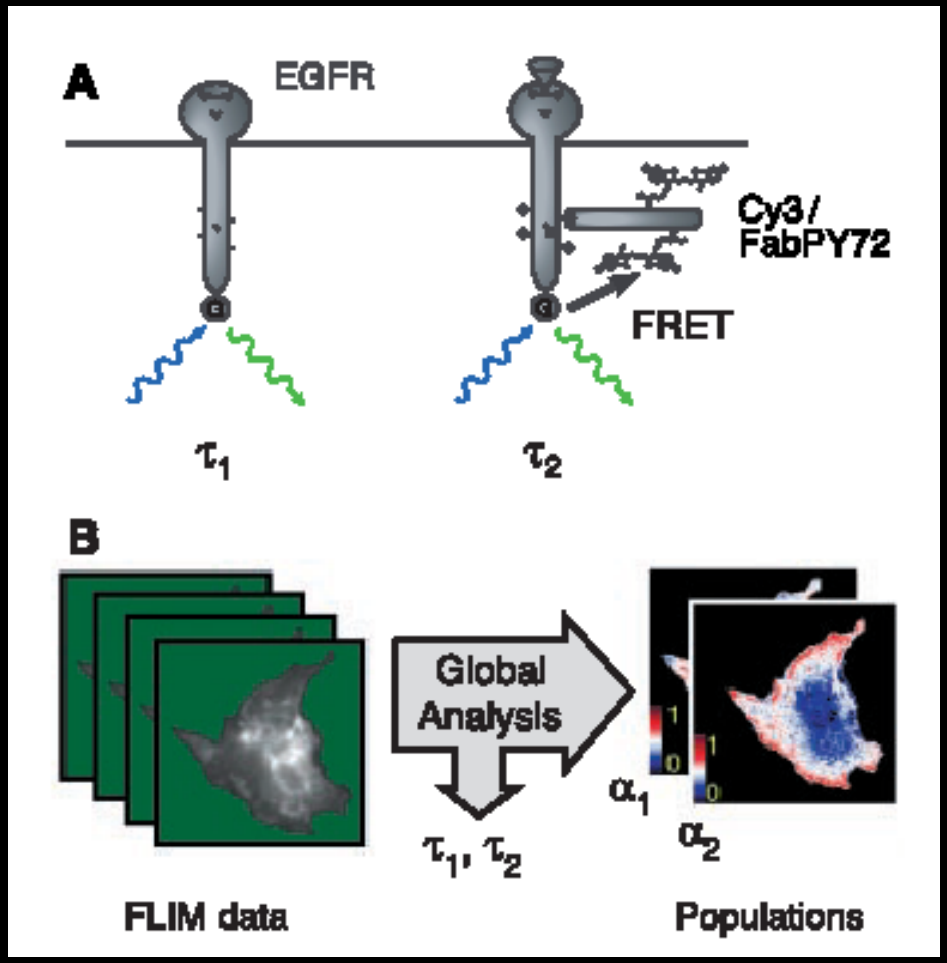
4. Low receptor concentration, low ligand coverage





$$I_{\mathrm{i}}^{\mathrm{model}}(t) = \int_0^{\mathrm{t}} G(t-T) \times c_{2\mathrm{i}+1} \left(c_{2\mathrm{i}+2} \exp\left(-\frac{T}{c_1}\right) + (1 - c_{2\mathrm{i}+2}) \exp\left(-\frac{T}{c_2}\right)\right) \mathrm{d}T.$$

Apply Lifetime Resolved FRET to Study Receptor Mediated Signaling



Verveer, Science 2000

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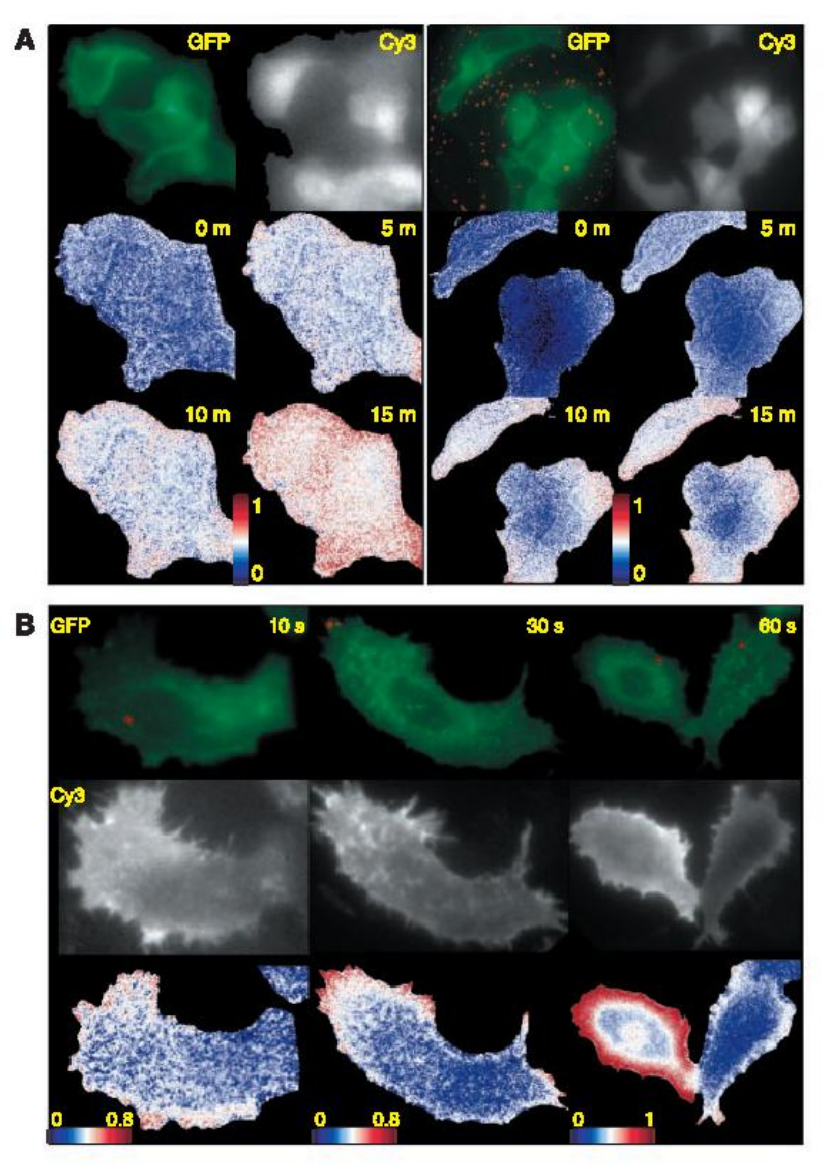

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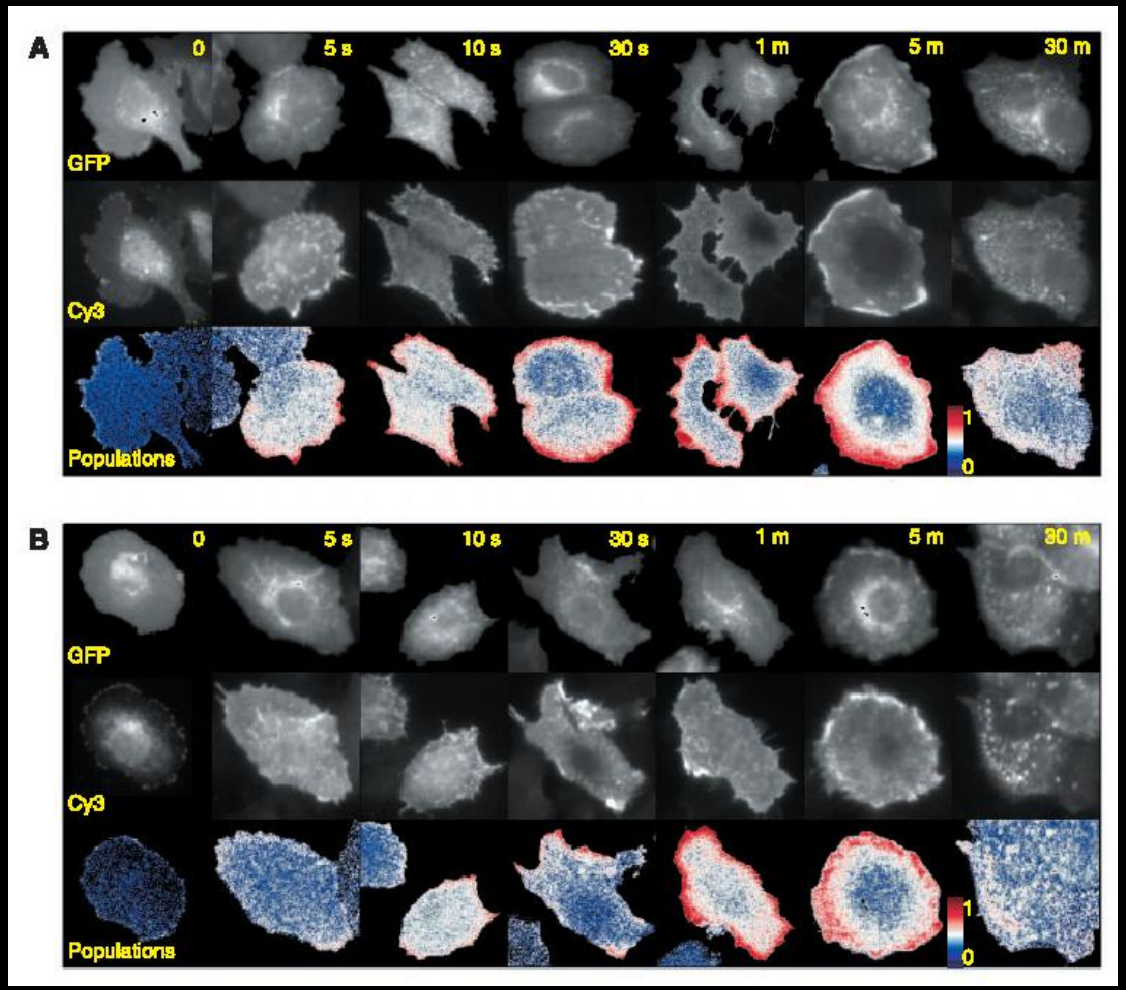

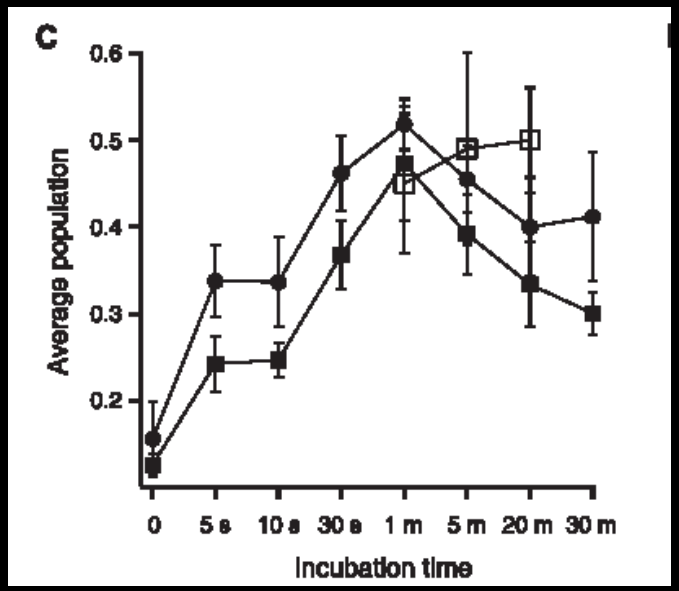

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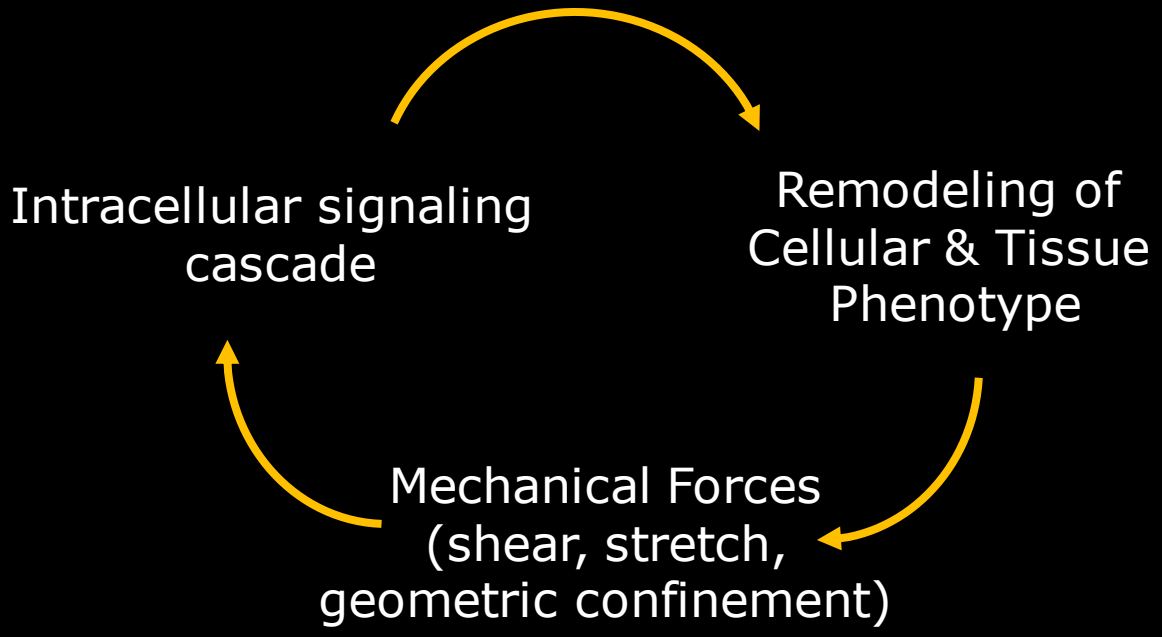
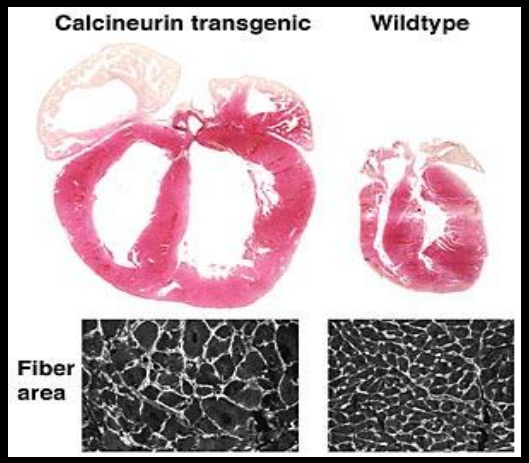
Mechanotransduction

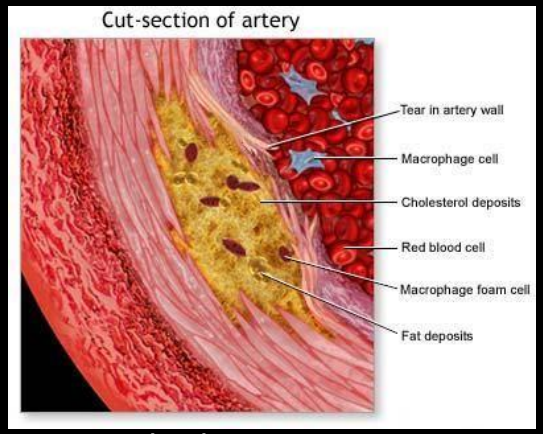


Cardiac Hypertrophy



http://www.cincinnatichildrens.org

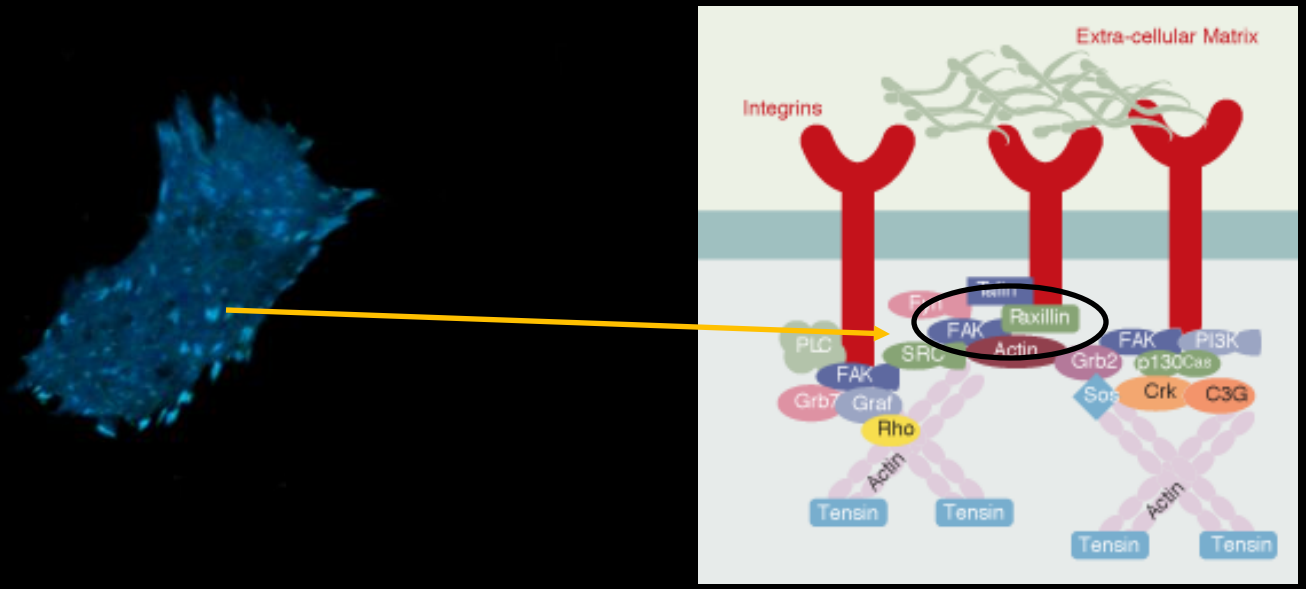
Arteriosclerosis



www.bodyrepairstore.com

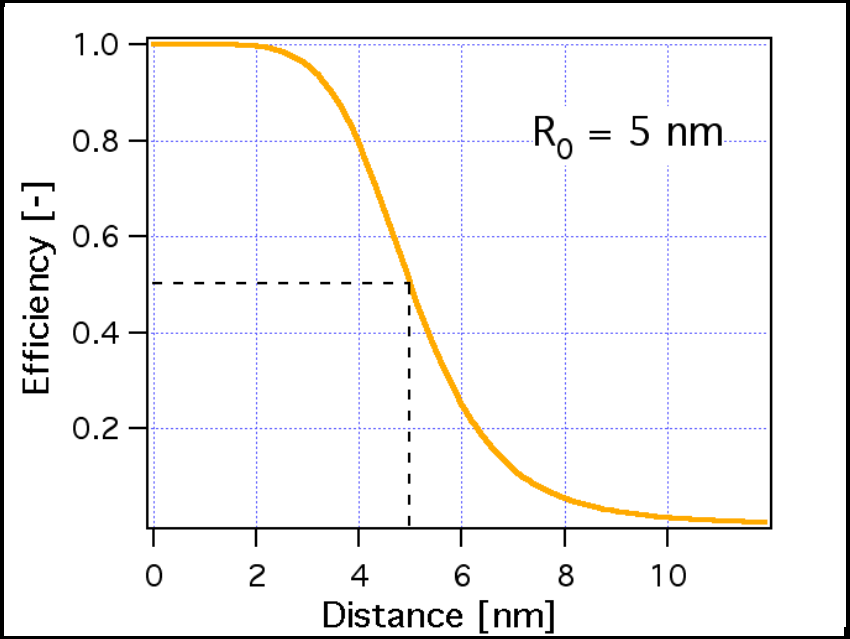
Focal adhesion complex

Focal adhesion complex serves as the adhesion sites of cells and mechano-signal transduction center of the cell



Quantification of Paxillin-Focal adhesion kinase interaction

Fluorescence Resonance Energy Transfer (FRET)



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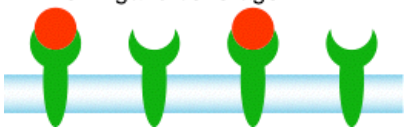

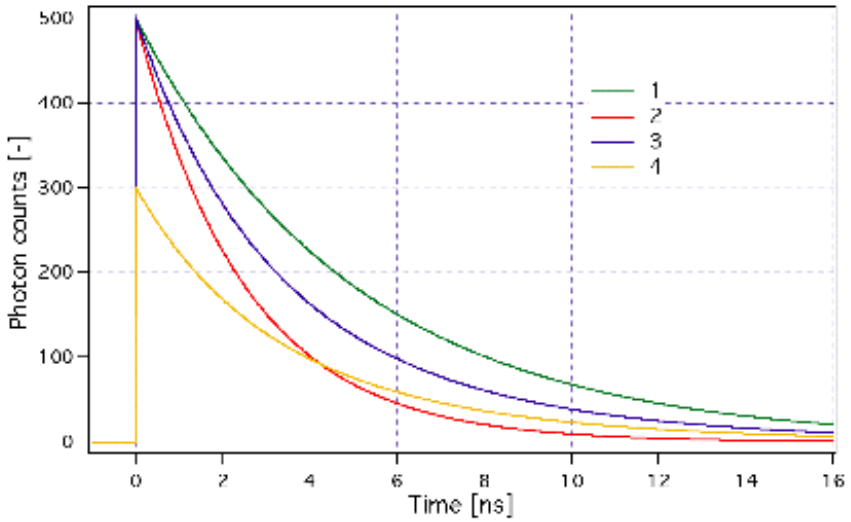

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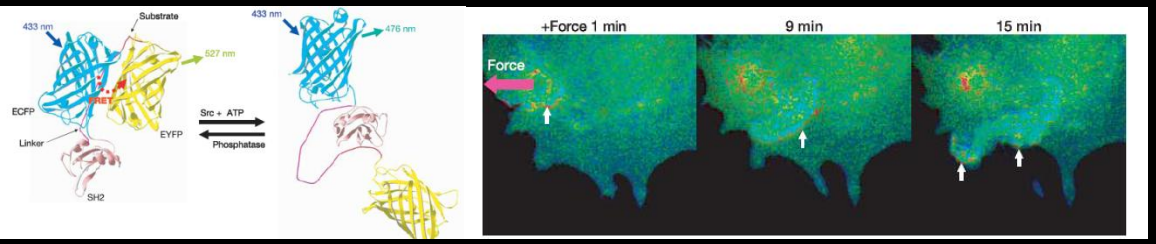
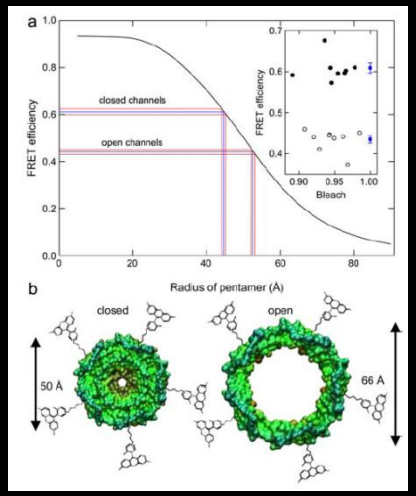
Quantification of Mechanotransduction with Foster resonance energy transfer (FRET)

Src phosphorylation dynamics

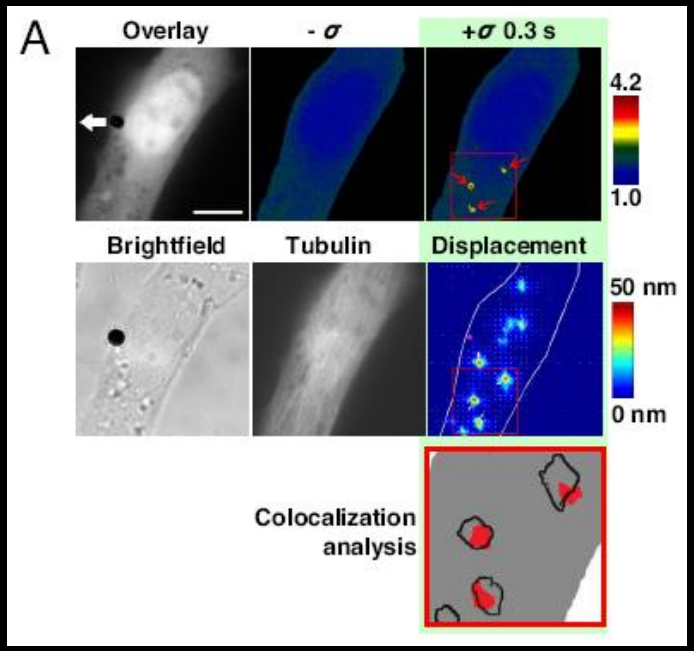


Wang et al., Nature 2005

MscL activation

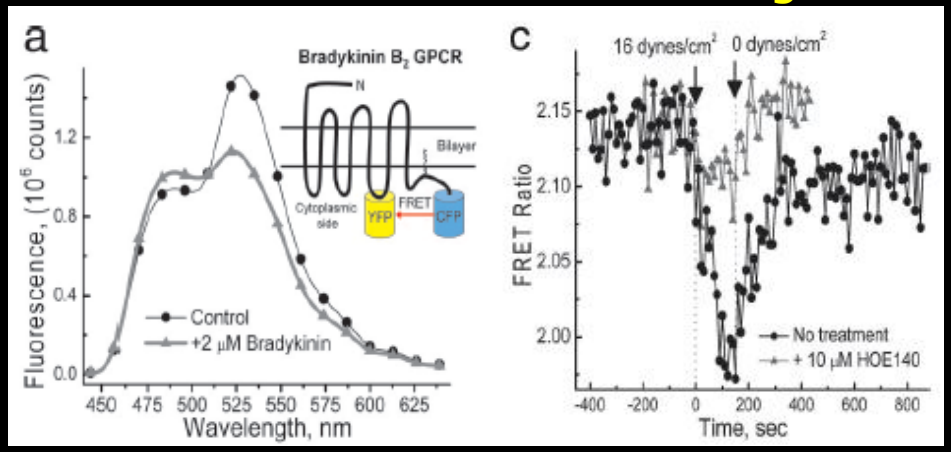


Corry et al., BJ 2005



Na et al., PNAS 2008

GPCR conformation change



Chachivilis et al., PNAS 2008

What can we quantify?

- Is there binding?

  Presence or absence of FRET

- What is the conformation of the bound molecule?

  FRET Efficiency: $E = \frac{R_0^6}{R_0^6 + r^6} = 1 - \frac{\tau_{DA}}{\tau_D}$

- What is the fraction of molecule bound?

  FRET ratio: $[P-F]/[P] = I_{P-F} / I_P$

- What is the thermodynamic constants of binding?

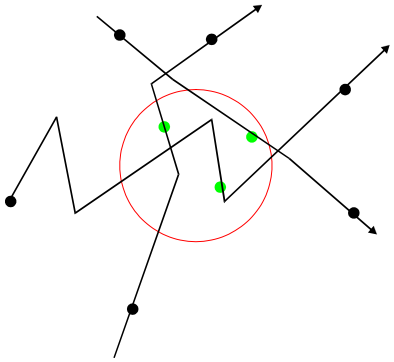
  Dissociation constant & Gibb's free energy

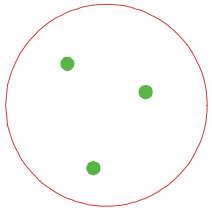
$$\ln K = -\frac{\Delta G}{kT} = \frac{[P][F]}{[P-F]}$$

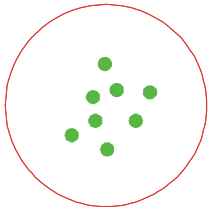
Use fluorescence correlation spectroscopy to get [F]

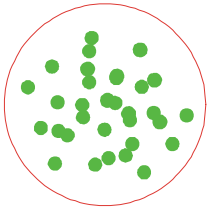
Fluorescence Correlation Spectroscopy (FCS)

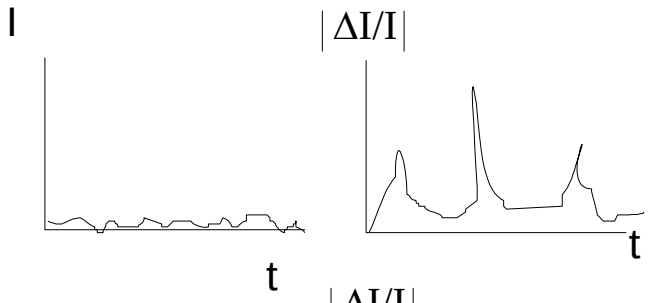
Poisson statistics: $\sigma_n^2 = \bar{n}$

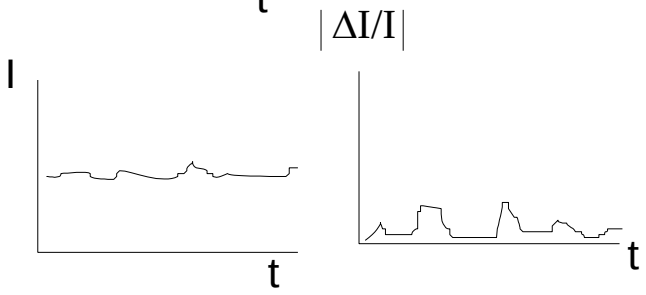


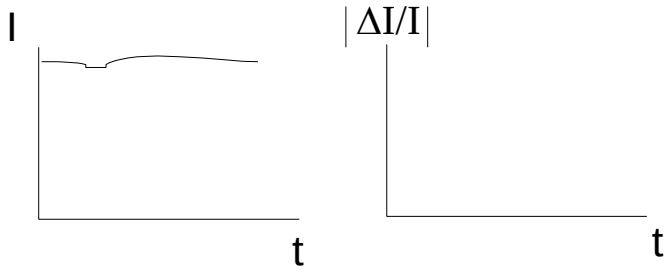




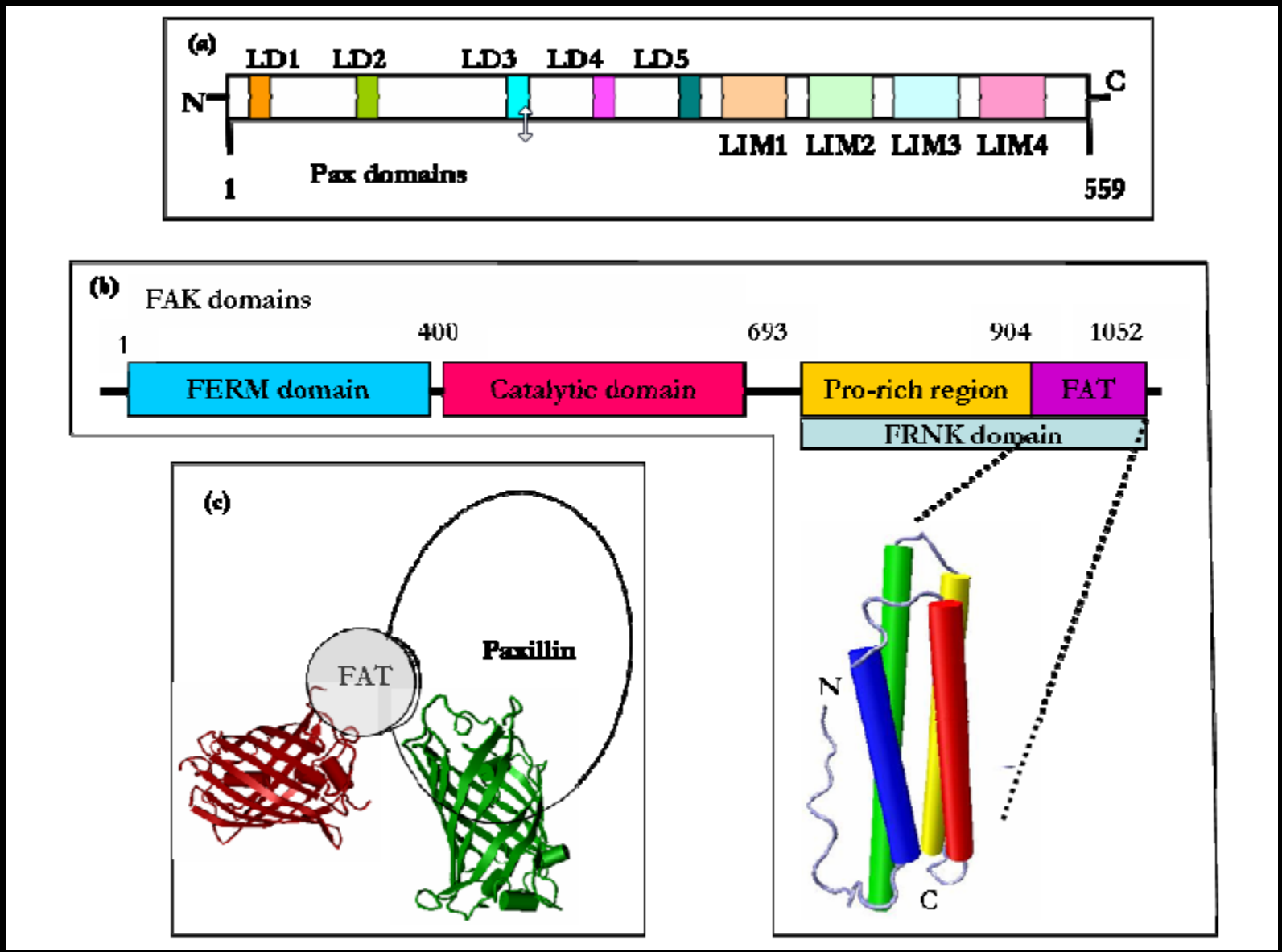








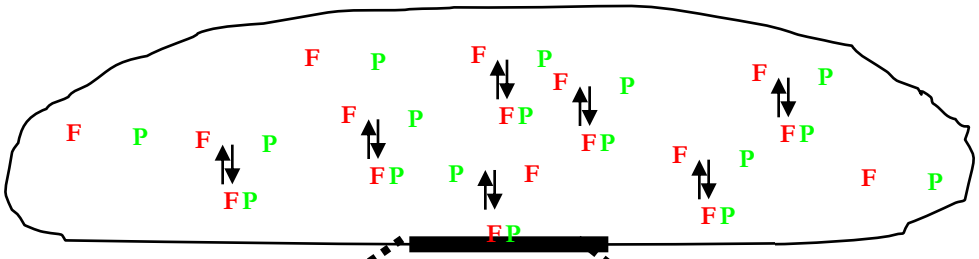
FAT and Paxillin Binding

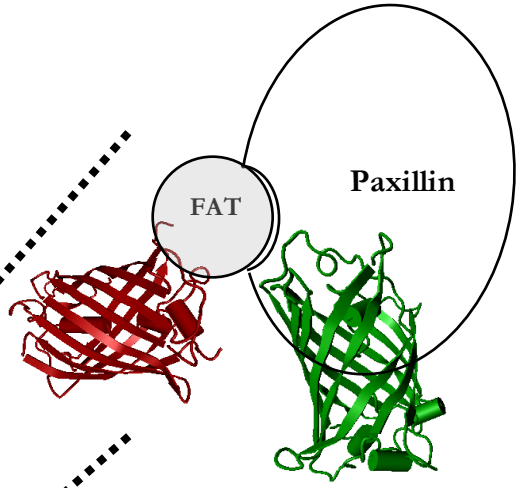


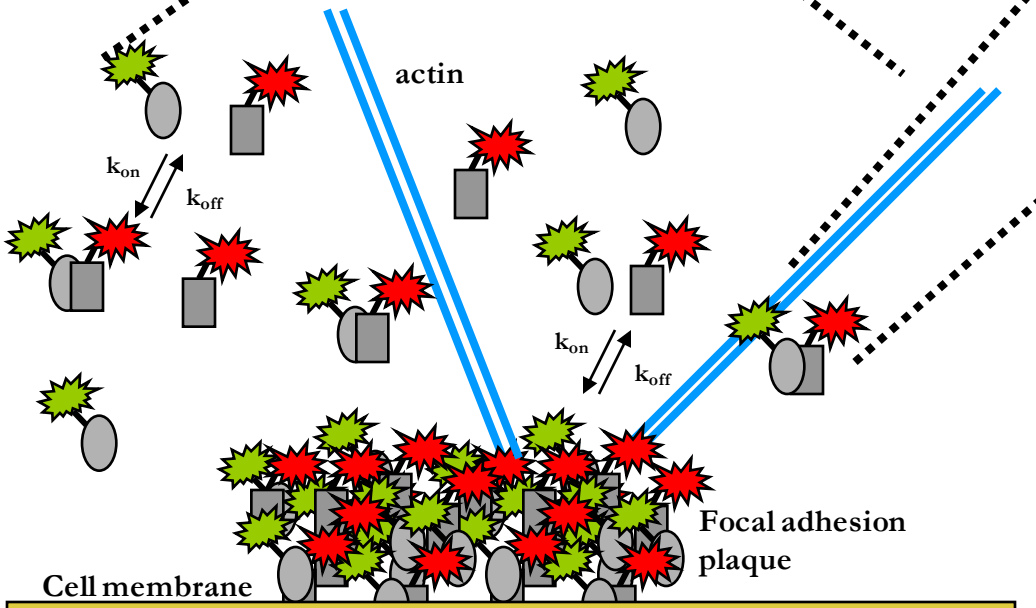
Thermodynamics of Pax/FAT Interaction

Bovine aortic endothelial cells (BAECs)

Co-transfected with Pax and FAT plasmids







$$\text{FAT-mCh}_{\text{cyto}} + \text{GFP-Pax}_{\text{cyto}} \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} \text{FAT-mCh---GFP-Pax}_{\text{cyto}}$$

$$K_d = \frac{[\text{FAT}] * [\text{Pax}]}{[\text{FAT-Pax}]}$$

How to measure k_d & ΔG spectroscopically

$$K_d = \frac{[\text{FAT}] * [\text{Pax}]}{[\text{FAT-Pax}]}$$

For a given cell, measure concentrations or ratio of concentrations

$$\rightarrow \quad \frac{[\text{Pax}]}{[\text{FAT-Pax}]} = \frac{1}{1 - \text{FRETratio}}$$

$$\rightarrow \quad \eta = 1 - \frac{\text{FRETlifetime}}{\text{non-FRETlifetime}}$$

$\rightarrow$ B = Green molecule intensity/C_{gfp} = [Pax] +(1-η)[FAT- Pax]

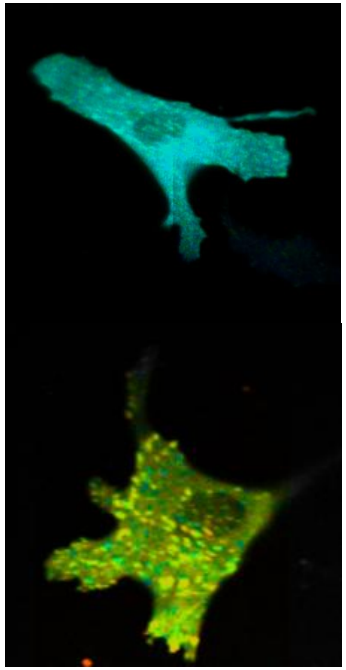
$\rightarrow$ C = Red molecule intensity/C_{mc} = [FAT] + [FAT-Pax] + B/γ

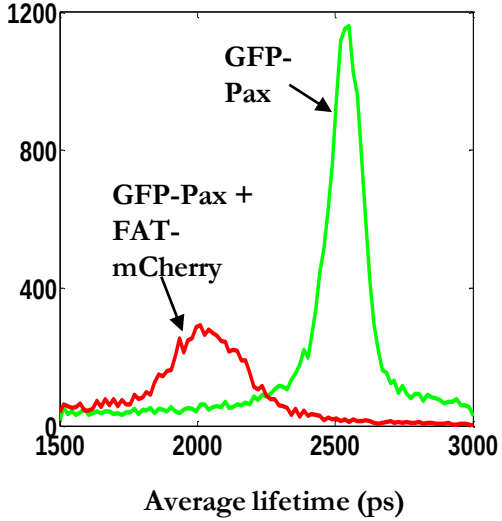
C_{gfp} is the brightness of gfp, C_{mc} is the brightness of m-cherry,
γ is a parameter characterizing bleedthrough from the green to the red channel

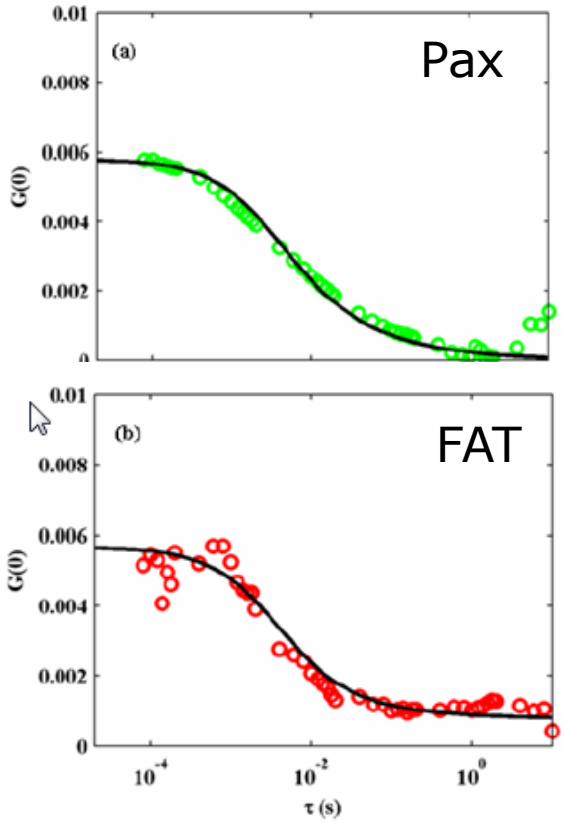
Solve simultaneous equations to obtain K_d. Calculate Gibbs free energy, $\Delta G = RT \ln K_d$

In vitro systems exist to measure K_d for purified protein pairs e.g. isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR) but no *in vivo* methods exist.

Typical FLIM-FRET & FCS data

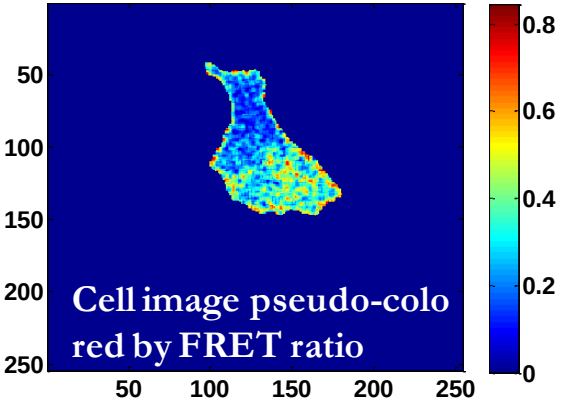






Quantification of a single cell

FRET



Fixed τ_1 = 2.6ns, fit τ_2 = 1.9ns

⇒ R ~ 56Å

$\eta = 1 - \tau_2/\tau_1 = 0.2692$

Solve simultaneous equations to obtain K_d

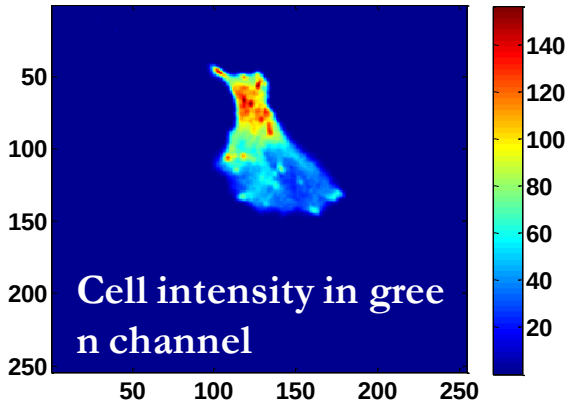
FRET / FLIM: $\dfrac{[\text{FAT-Pax}]}{[\text{Pax}] + [\text{FAT-Pax}]} = A$

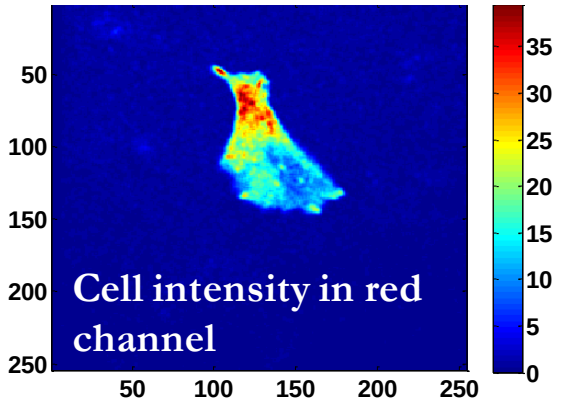
FCS @ 890nm: [Pax] +(1-η)[FAT- Pax] = B

FCS @ 780nm: [FAT] + [FAT-Pax] + B/17 = C

FCS

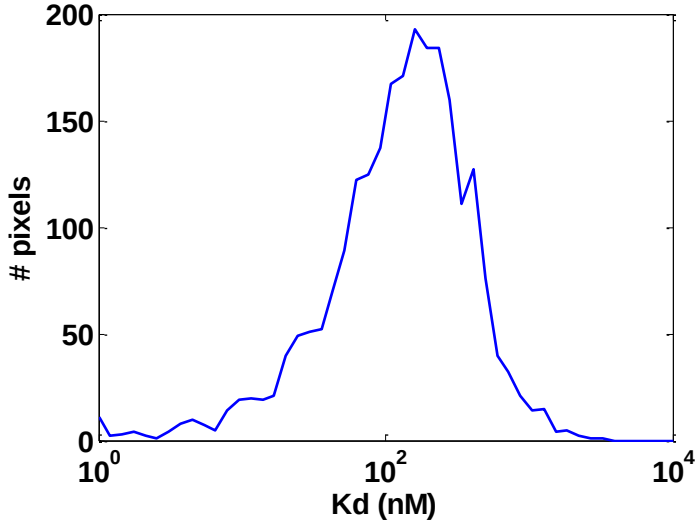
Calibration	Red ch	Green ch
Intensity	0.3	5.2
Concentration	18.2 nM	21.8 nM





Thermodynamics of Pax/FAT Interaction in a single cell

Histogram of K_d for cytosolic region

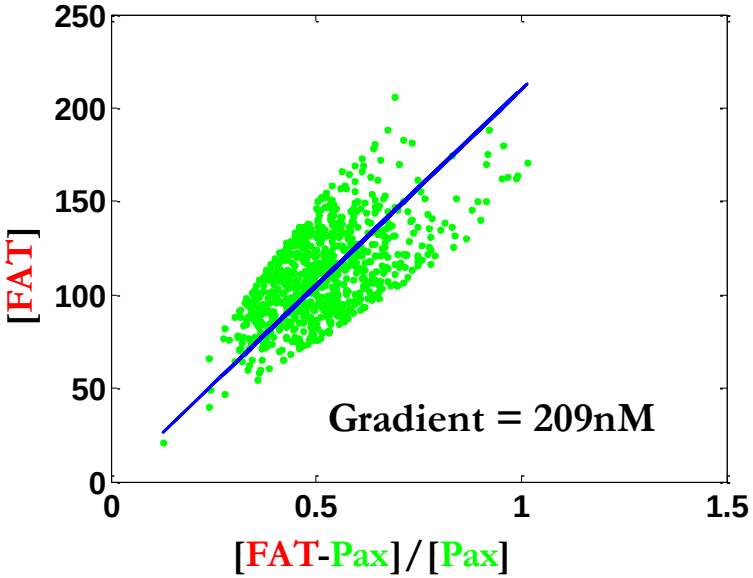


- Histogram peaks at K_d value ~200nM

$$K_d = \frac{[\text{FAT}] * [\text{Pax}]}{[\text{FAT-Pax}]}$$

$$[\text{FAT}] = K_d \frac{[\text{FAT-Pax}]}{[\text{Pax}]}$$

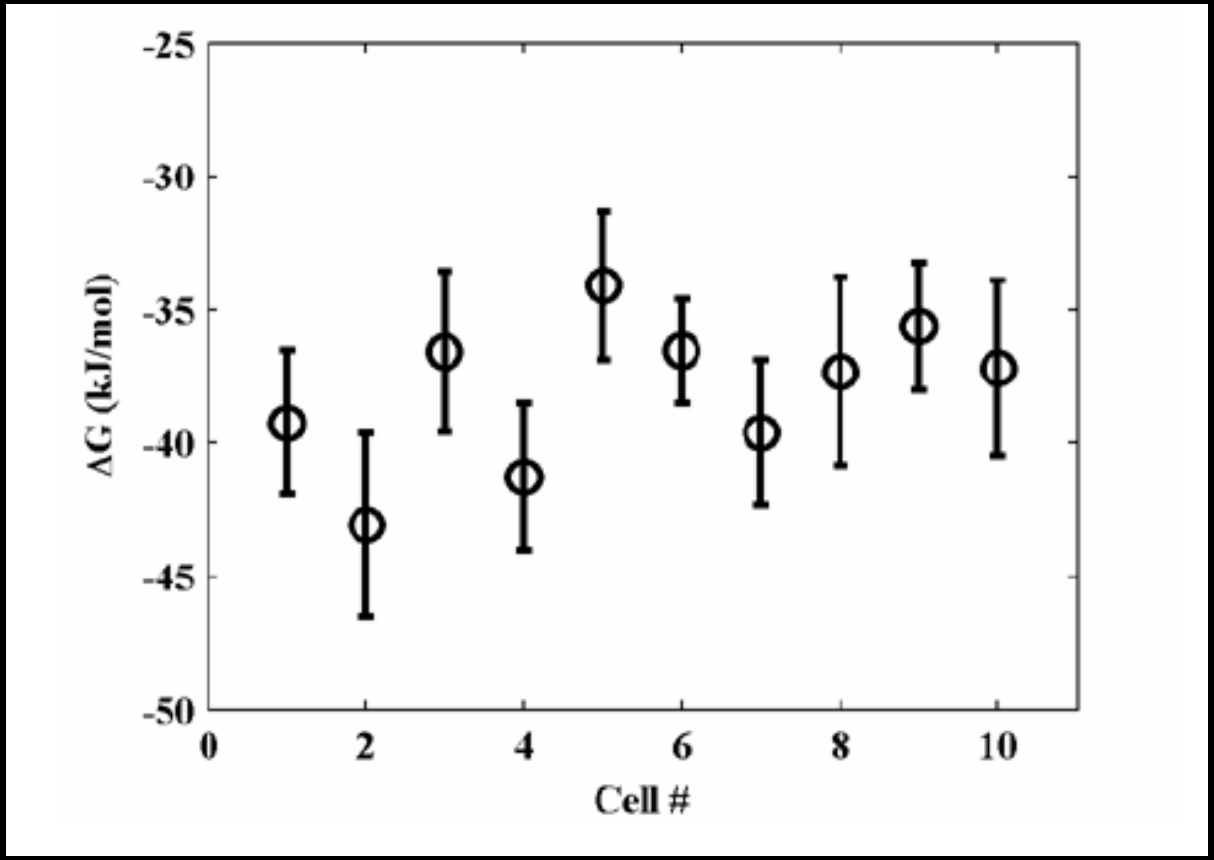
[FAT] vs [FAT-Pax]/[Pax]



Pixels within 3 bins on either side of histogram peak

Linear fit result

Variation of ΔG across different cells



Measurement of 10 distinct cells over three days
Error bars are std dev in one cell

Compare k_d & ΔG with in vitro system

Spectroscopic measurement: K_d = 367 ± 33 nM (S.E. 10 cells)

In vitro results:

- Isometric Titration Calorimetry (ITC) *Gao et. al. J. Biol Chem. 2004*

K_d ~ 10 μM for FAT + 1 LD domain of Pax

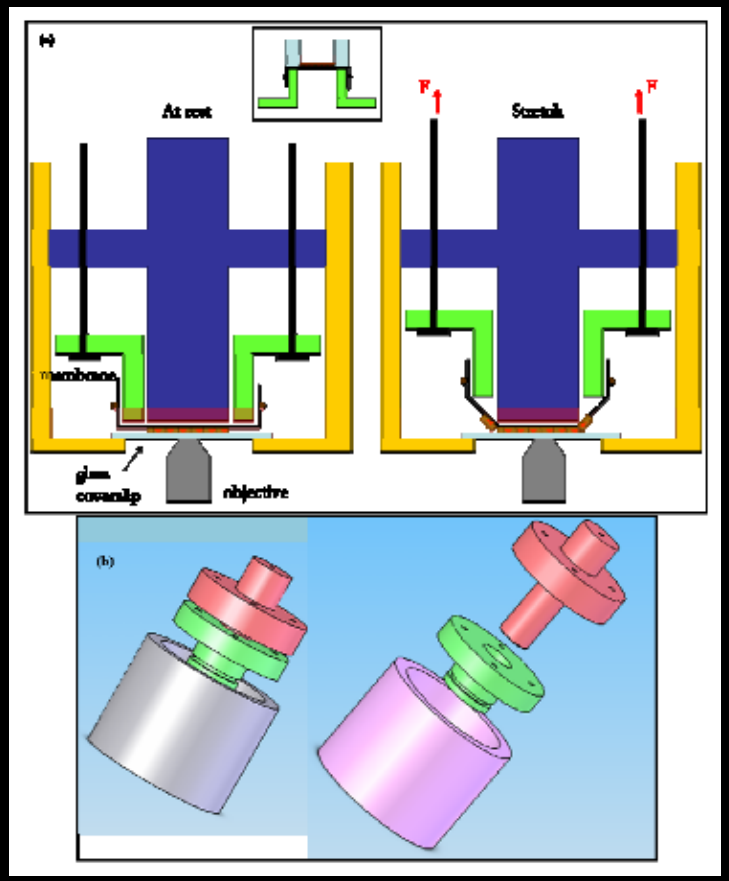
- Surface Plasmon Resonance (SPR): *Thomas et. al. J. Biol Chem. 1999*

K_d ~ 4 μM for FAT + 1 LD domain of Pax

K_d ~ 300 – 600 nM for FAT + both LD domains of Pax that bind FAT

Paxillin-FAT interaction shows significant allosteric effect both in vivo & in vitro

Is paxillin-FAT binding mechno-sensitive?

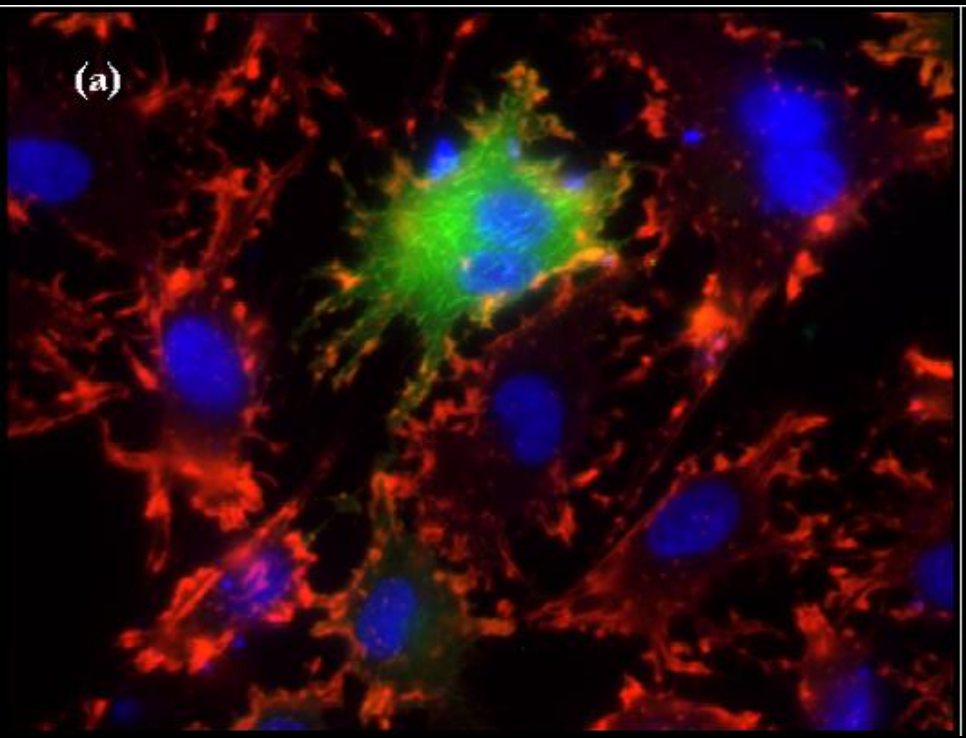


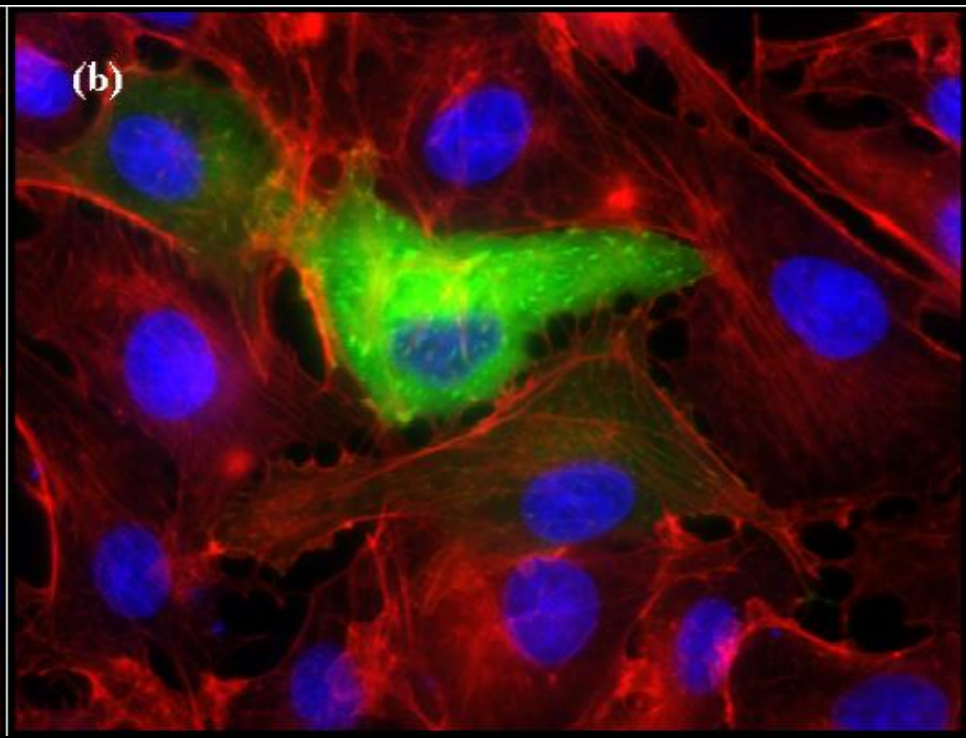
Apply bi-axial stretching (up to 10%)

Chemical disruption to mechanotransduction

Cytochlastin D

Genistein

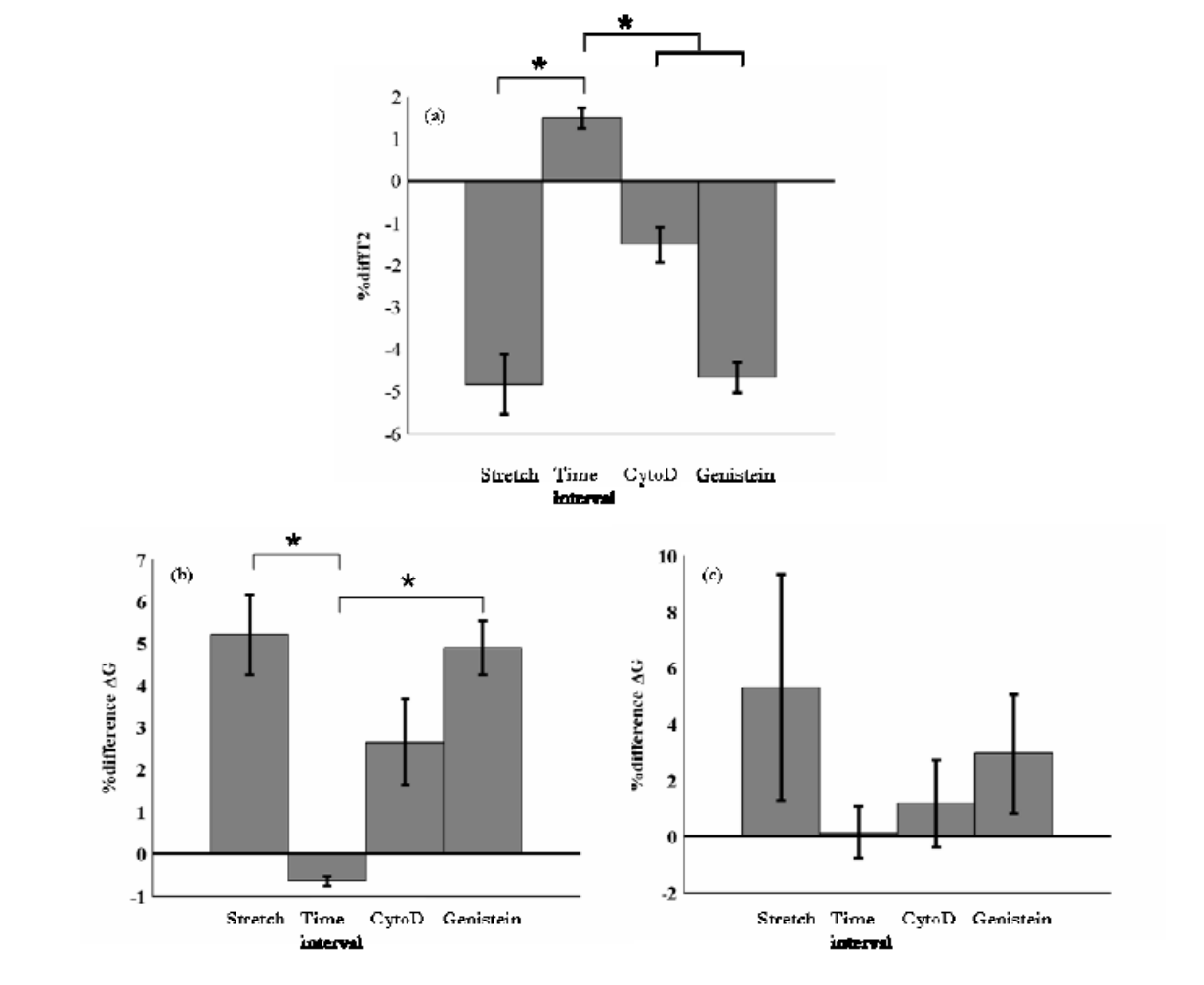




Blocks actin polymerization

Blocks protein tyrosine phosphorylation

Blocking of stretch responses



Disruption of actin cytoskeleton (via cytoD) reduces mechanotransduction
Blocking tryosine phosphorylation does not block mechanotransduction