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Understanding Receptor Regulation for Designing, Targeting and Delivering Novel Therapeutics

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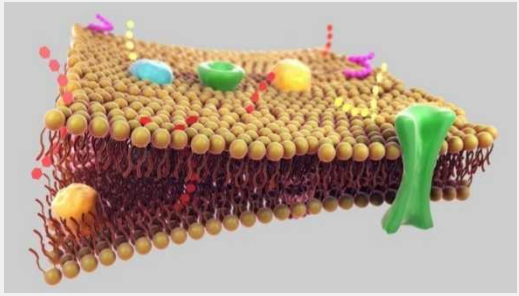
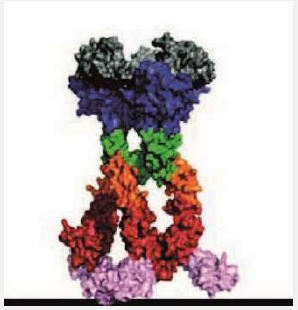
Understanding Regulatory Mechanisms of Biological Systems

Goal: Understand biological systems for applications-based technologies (therapeutic target design, diagnostic tools, biosensing and/or bioengineering technologies)

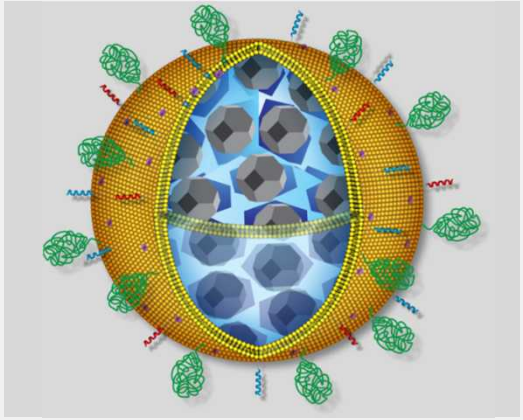
Novel and Effective Therapeutics

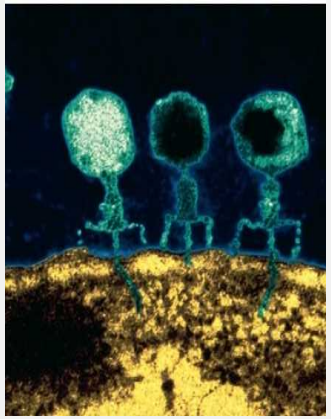
1. Receptor Signal Regulation





2. Targeted Therapeutic Delivery

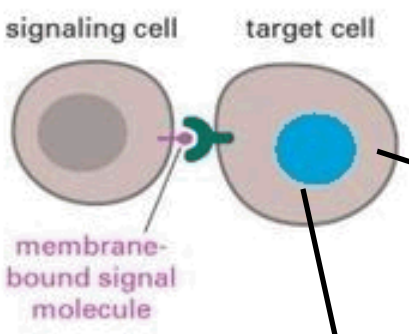




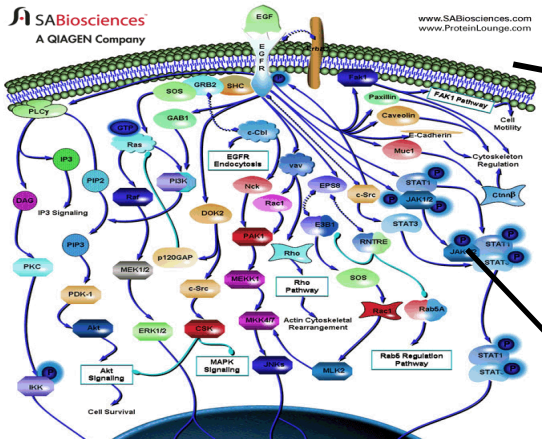
Complex Regulation of Cell Signaling

One mechanism by which cells communicate with each other is through direct physical contact

1. Signal Reception

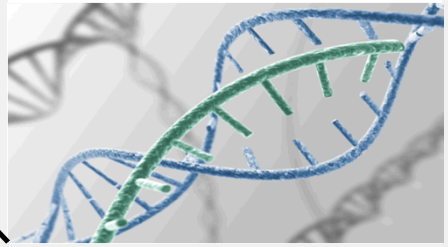


2. Signal Transduction



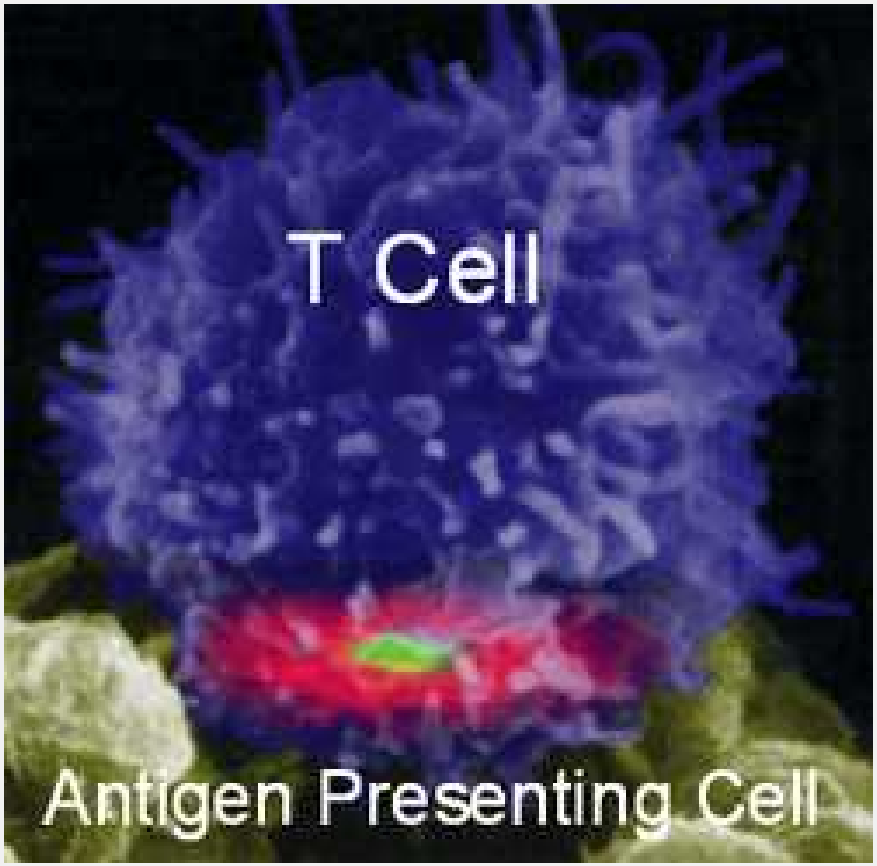
- Cell signaling is “noisy”
  - A single cell input results in a complex cascade of intracellular responses
  - Typically, there are many simultaneous signal inputs, resulting in highly stochastic cell response

3. Activation of Cell Response

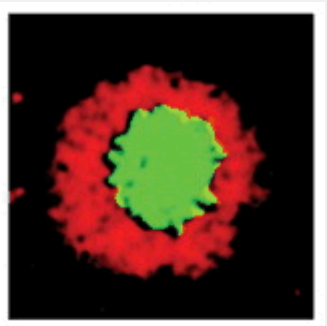


Clustering at a Cell-Cell Interface Regulates Signaling

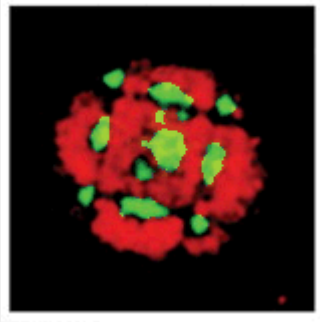
Receptor-ligand organization (clustering) at a membrane is a mechanism for cells to overcome biological noisy signaling environments and impart a downstream signal appropriately



http://www.bme.columbia.edu/~kam/research/research.htm



Correct
Cell Response



Disrupted
Cell Response

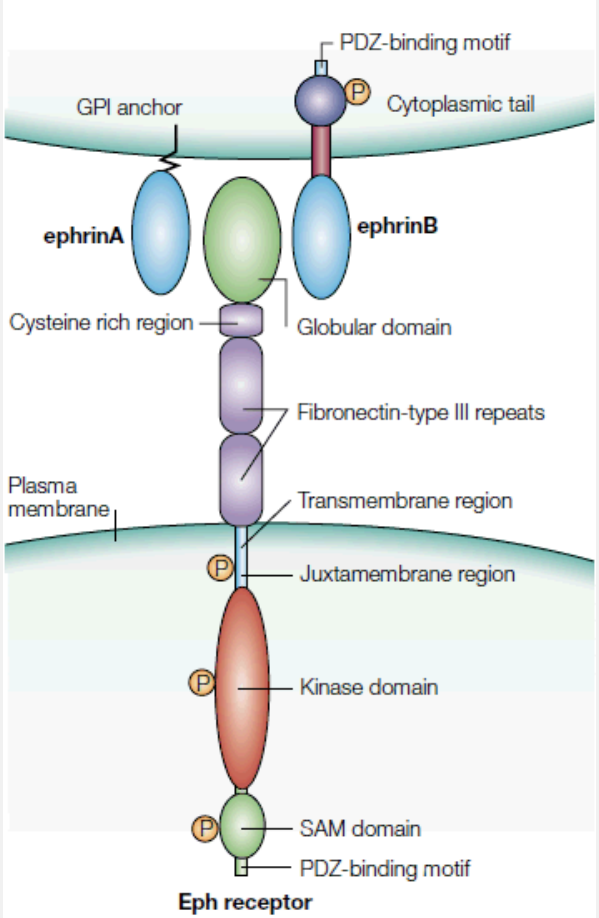
Same # of Signal Inputs!

Mossman, K.D, and Groves, J.T. 2005. *Science*. 18: 1191-3.

Eph Receptor Tyrosine Kinase Signaling

Receptor: EphA2

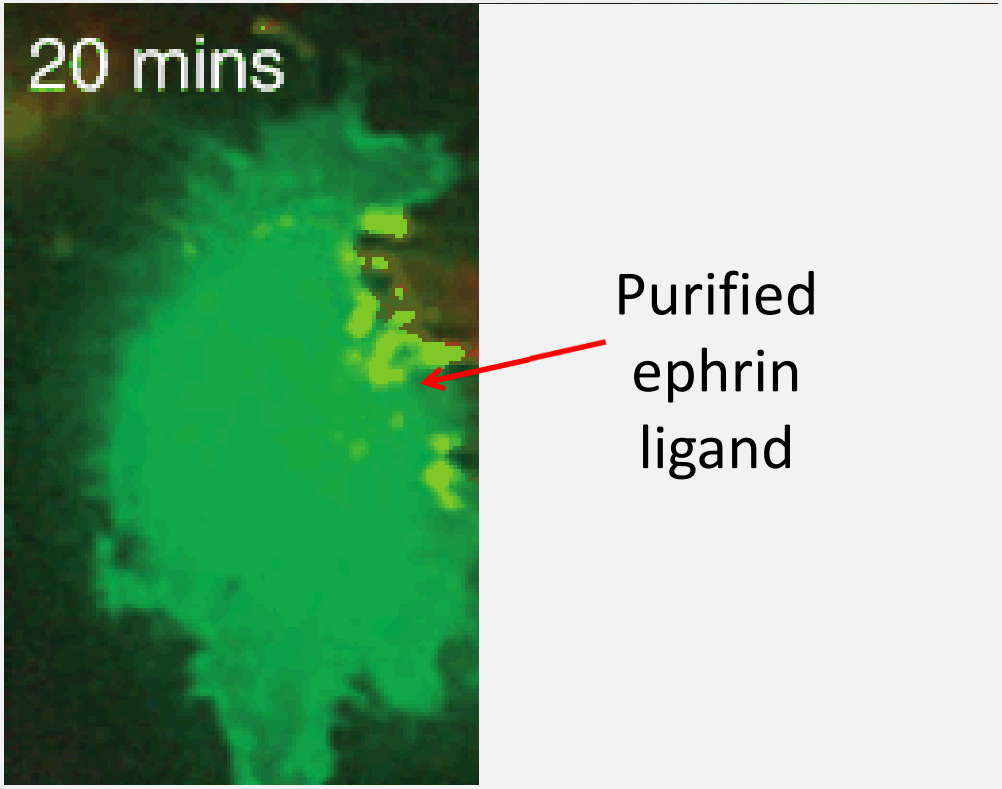
Ligand: ephrinA1



- Bidirectional signaling
  - Bidirectional signaling
- Overexpressed in aggressive cancers
- Highly overexpressed in MDAMB231 cells: invasive and metastatic breast cancer cells
- *Often expressed in triple negative breast cancers (no ER, PR or Her2)*
  - Key therapeutic target

Kullander, K. and Klein, R. 2002. *Nature Reviews*. 3: 475-86.

Activation of Eph Receptors by ephrin Ligands

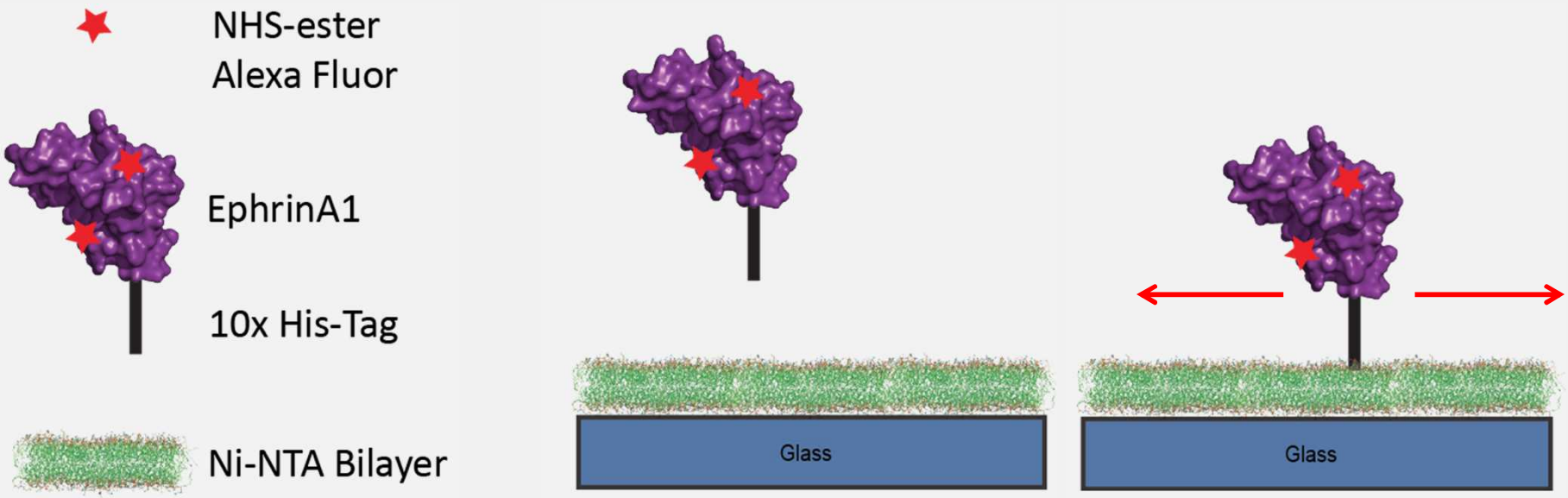


Zimmer, M. *et al*. 2003. *Nature Cell Biol* 5(10): 869-78.

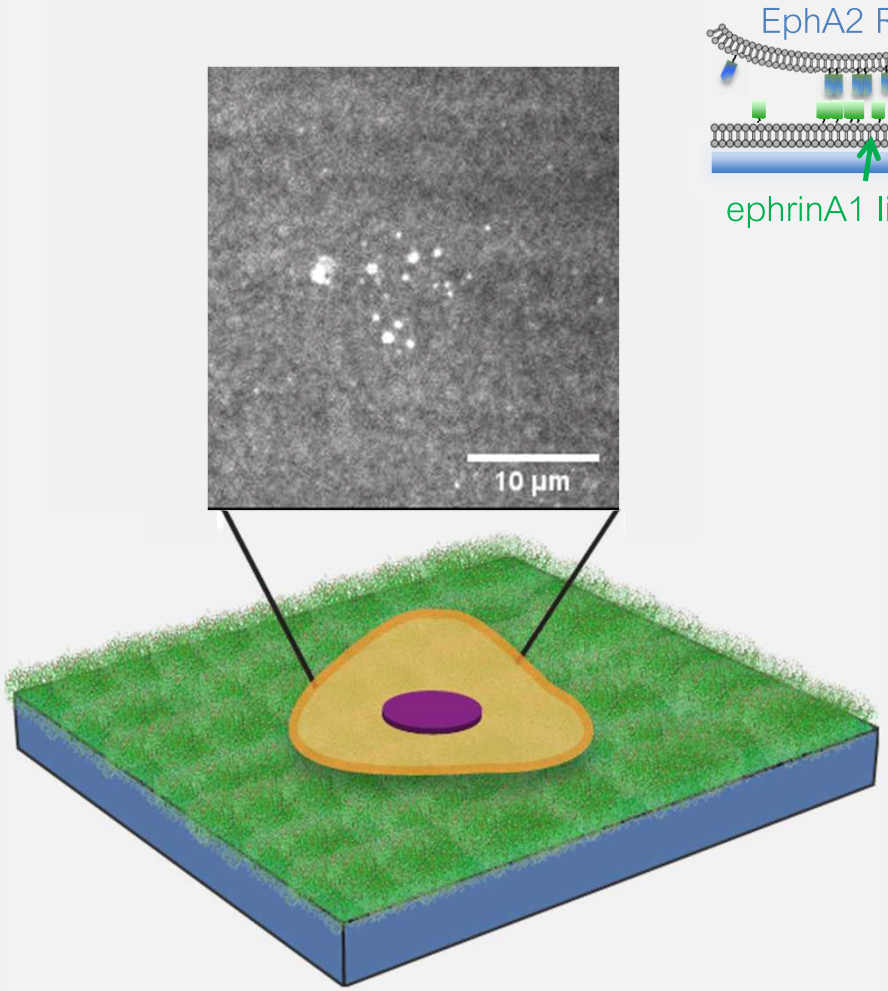
- Typical Eph receptor activation assays use:
  - co-cultures
  - soluble ephrin
- Forward EphA2 signaling (and likely clustering) must be important in the context of a membrane
- Need to use membrane-bound ephrin ligand to more accurately understand Eph receptor forward signaling

Linking ephrin Ligands to a Supported Lipid Membrane

Ephrin ligands can be purified and linked to a supported lipid membrane, creating a synthetic mimic of the ephrin-expressing cell

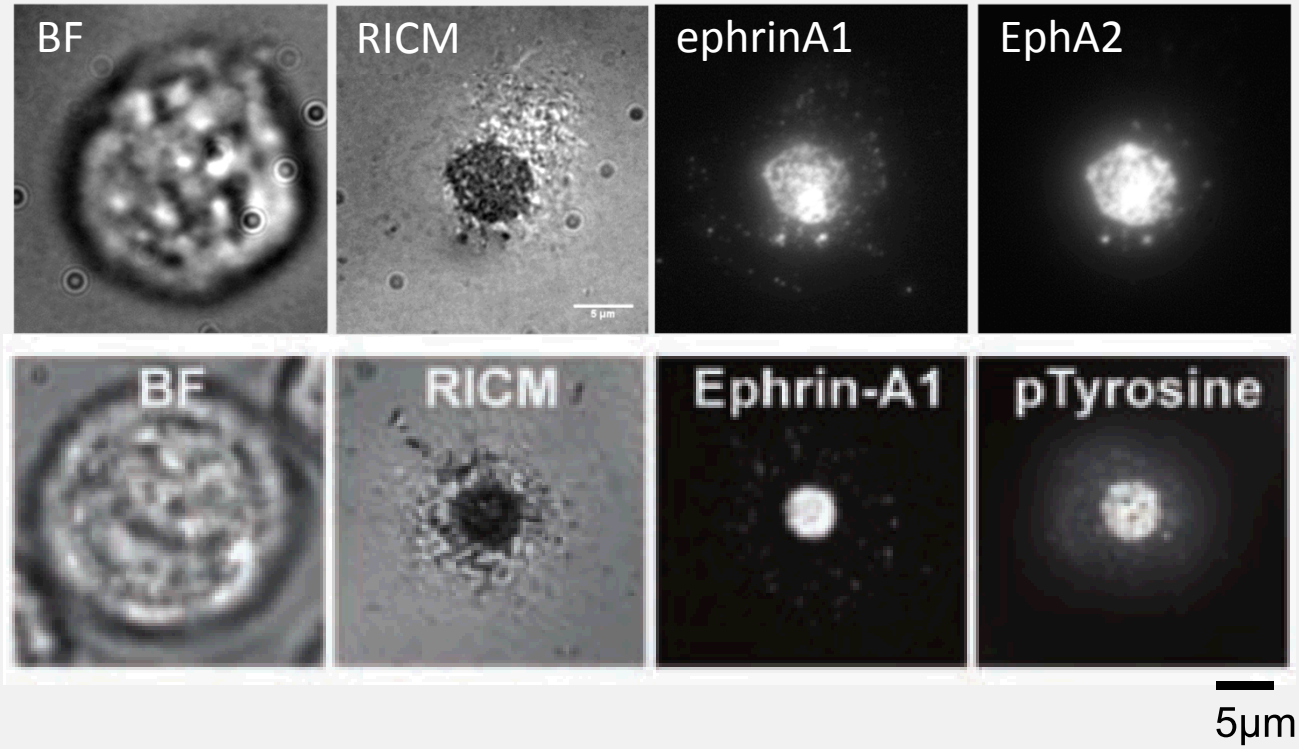


Receptors and Ligands Cluster at the Cell-Membrane Interface

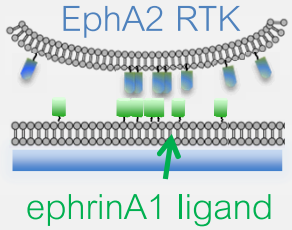


- MDAMB231 cells are seeded onto an ephrinA1 bilayer
- Receptor-ligands undergo higher ordering reorganization at the cell-membrane interface
- High resolution microscopy can be used to probe the importance of spatial organization

Membrane-Bound Ligands Activate Cell Receptors

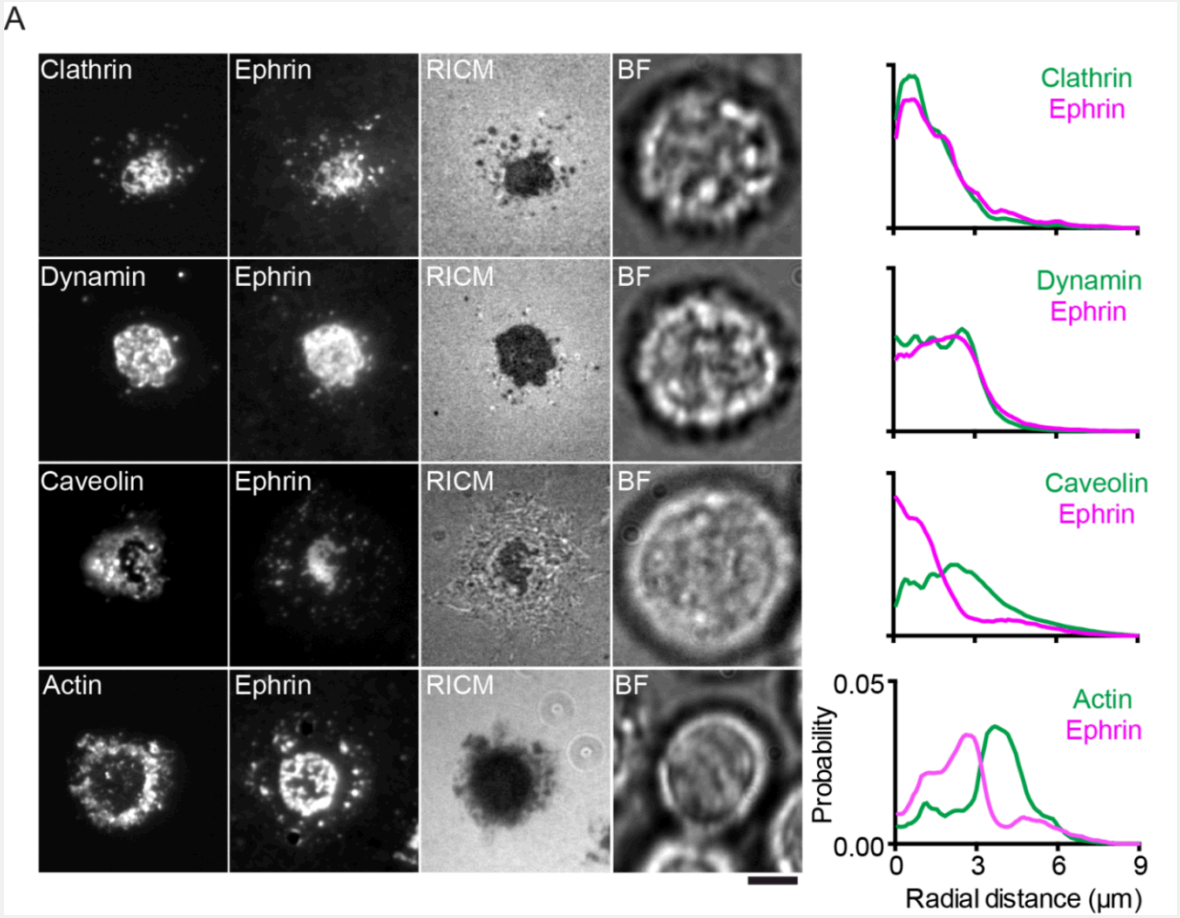


- Ephrin ligands on a supported membrane activate Eph receptors
  - Causes Eph receptors to be phosphorylated
  - Activates an actomyosin-driven contractility

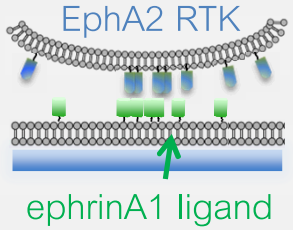


Salaita, K., *et al*. 2010. *Science*. 327(5971): 1380-5.
Xu, Q., *et al*. 2011. *Biophys J*. 101(11): 2731-9.

Molecular Physiology of EphA2-ephrinA1 Clusters

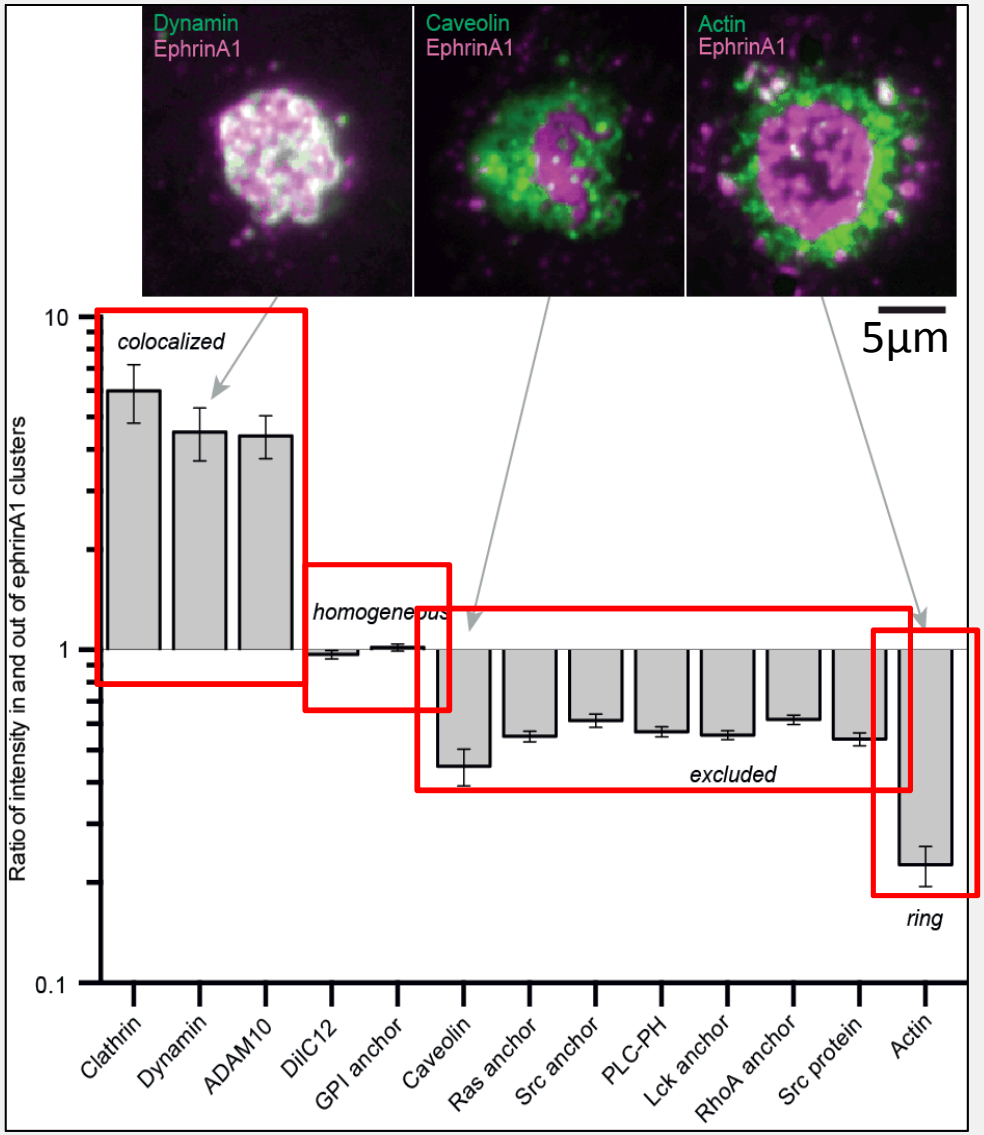


- Candidate fluorescent signaling molecules were transiently expressed in MDAMB231 cells

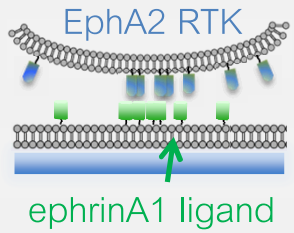


Greene, AC. and Lord, S. *et al.* 2014. *Biophysical Journal* 106: 2196-205.

EphA2-ephrinA1 Clusters Contain Endocytosis Molecules

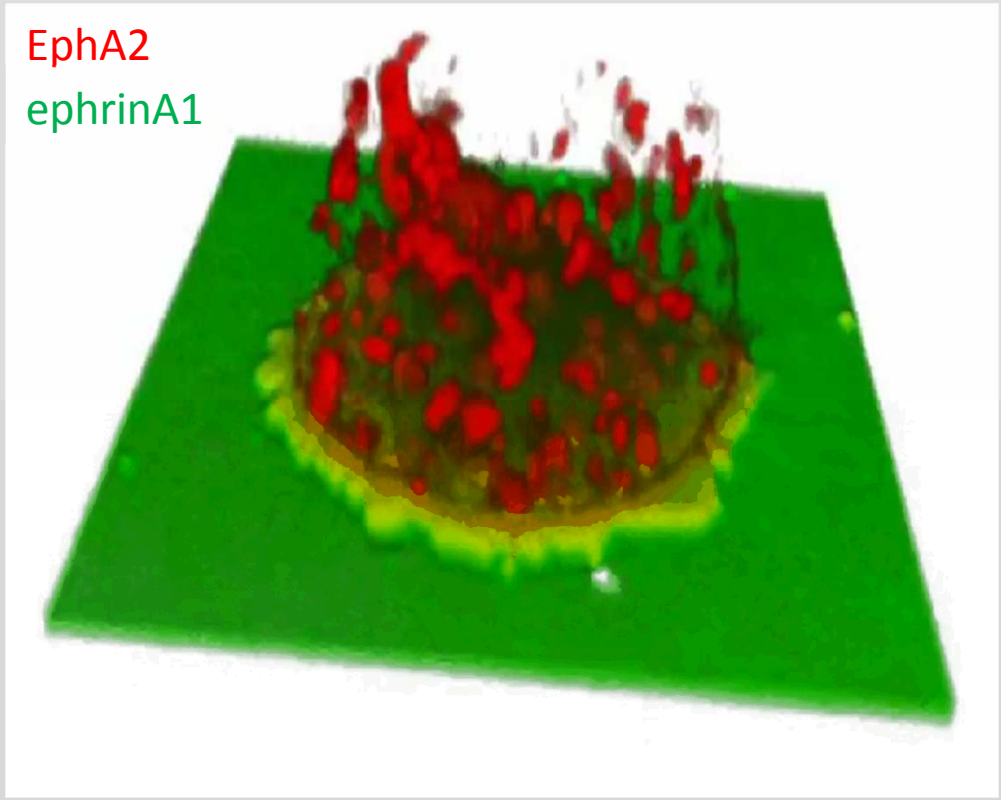


- Localization of signaling molecules to ephrinA1 was measured
- Four types of localization were characterized
  - Colocalization
  - Homogenous distribution
  - Anti-localization
  - Ring formation
- Maybe these clusters are important sites of endocytosis?



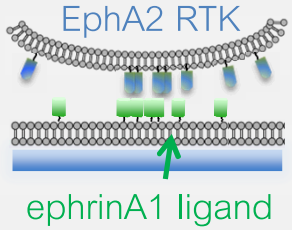
Greene, AC. and Lord, S. *et al.* 2014. *Biophysical Journal* 106: 2196-205.

Detecting Changes in EphA2 Endocytosis is Challenging



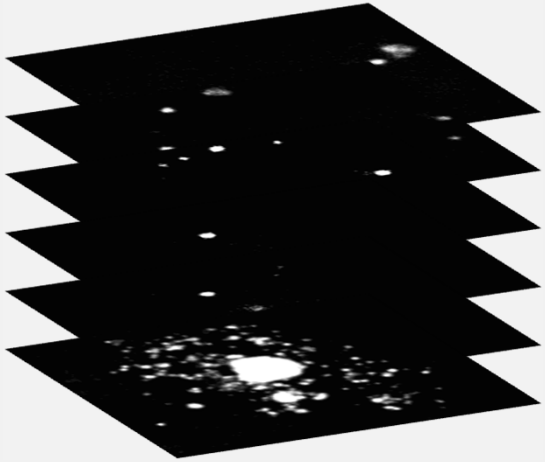
Problems:

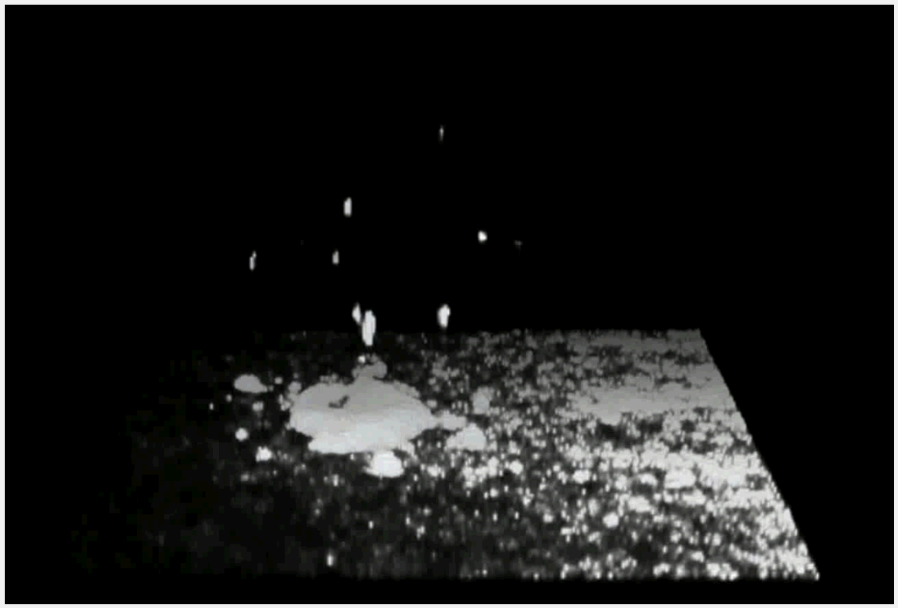
- Non-specific labeling
- Lots of background fluorescence
- Consistency with permeabilization and/or labeling
- Always a high background level of internal EphA2



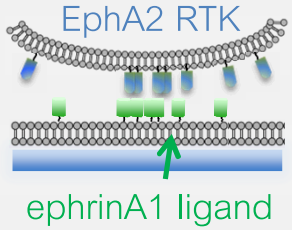
Developing a Live-Cell Endocytosis Assay

0.5µm Z-slices



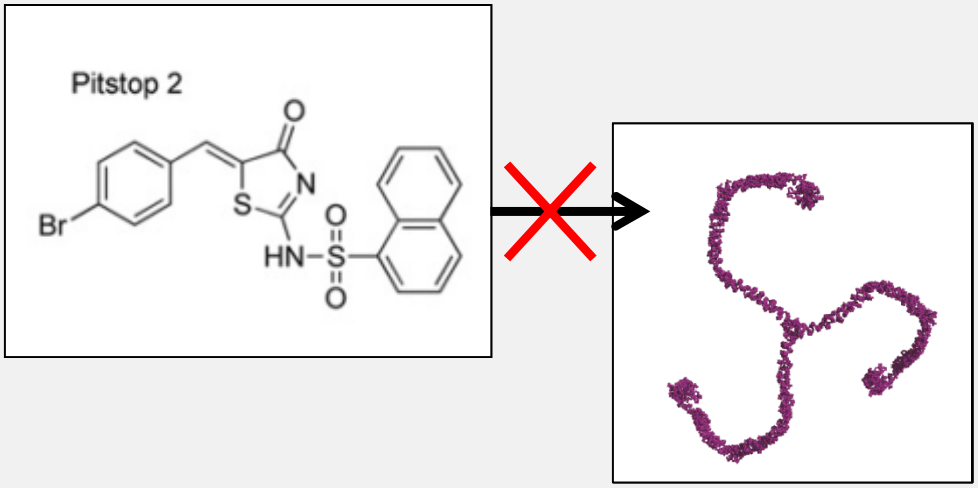


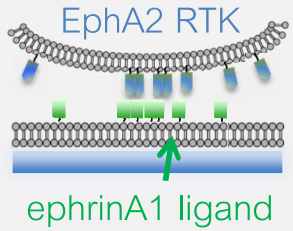
- Detect internal ephrinA1 using spinning disc confocal microscopy
- All signal must have come from the bilayer, so high signal to noise!
- Detection of small changes, very quantitative, cleaner/more reproducible assay than antibody staining
- Easy background threshold
- Selects for specific punctate spots using a size threshold

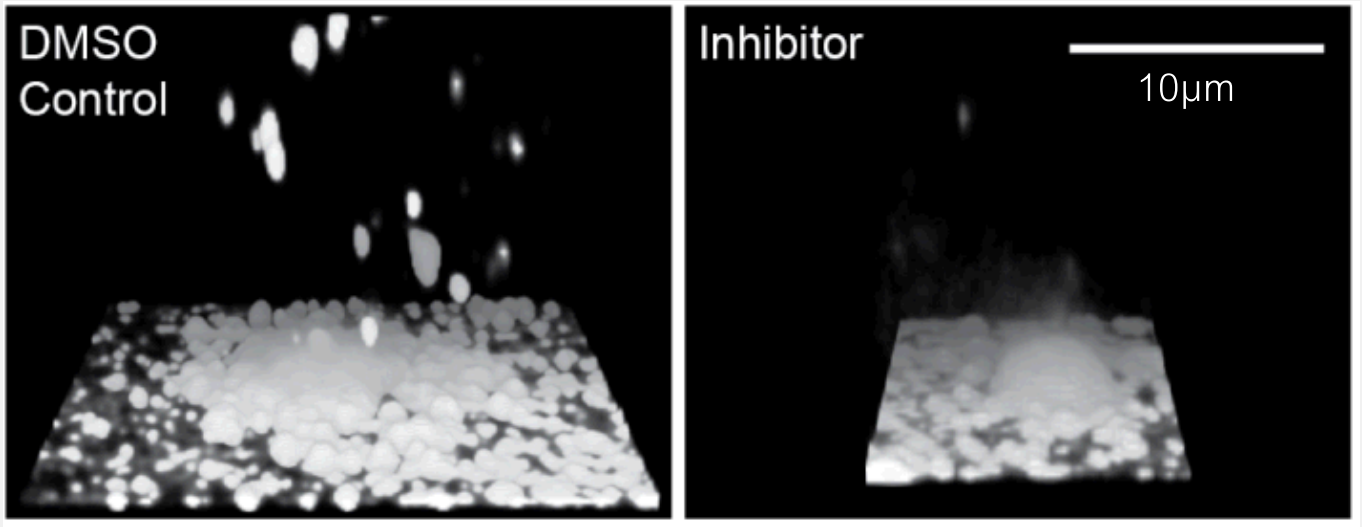


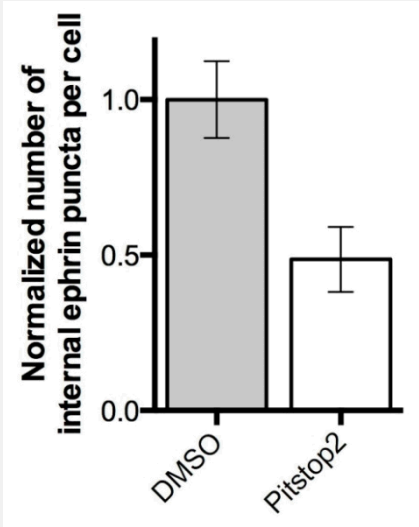
Greene, AC. and Lord, S. *et al.* 2014. *Biophysical Journal* 106: 2196-205.

Trans-Endocytosis of ephrinA1 Requires Clathrin





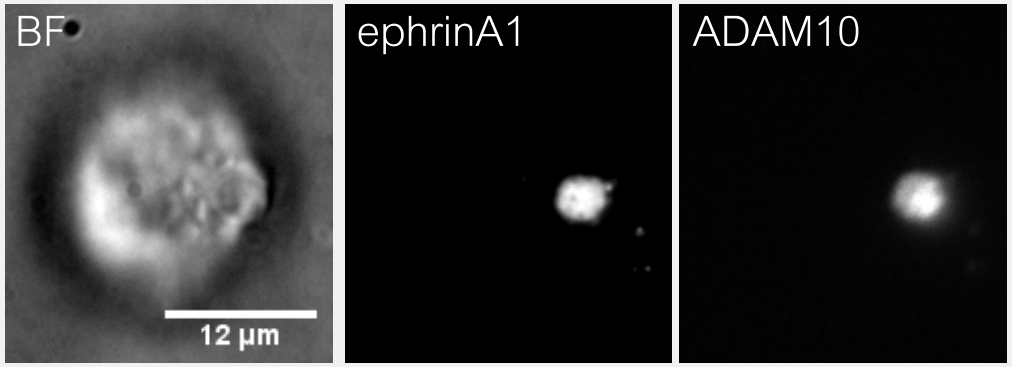


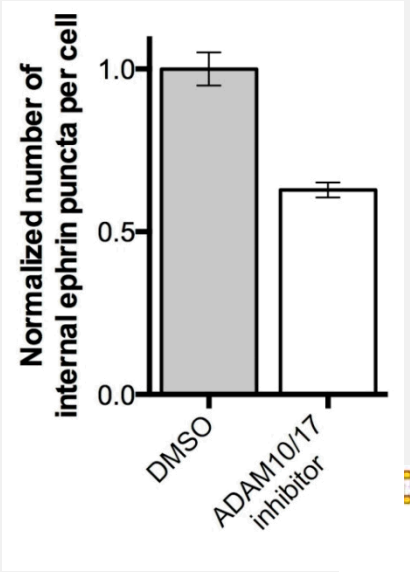


von Kleist, L. *et al.* 2011. Cell, 146(3): 471-84.

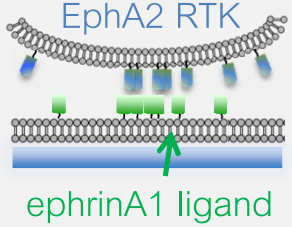
Greene, AC. and Lord, S. *et al.* 2014. *Biophysical Journal* 106: 2196-205.

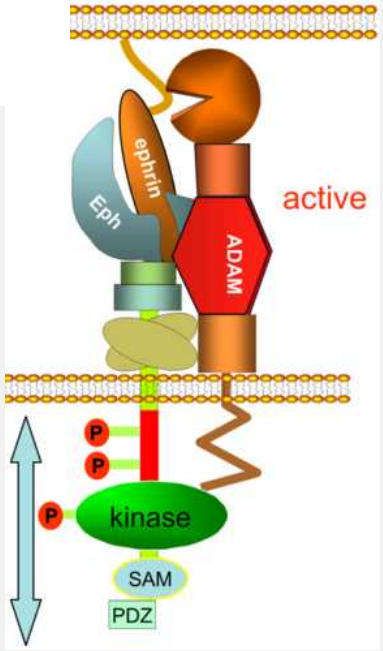
Trans-Endocytosis of ephrinA1 Requires ADAM10





- ADAM10 is a disintegrin and metalloprotease thought to be responsible for the trans-cleavage of ephrinA1
- ADAM10 is necessary for efficient ephrinA1 endocytosis
  - ADAM10/17 was inhibited with a small molecule

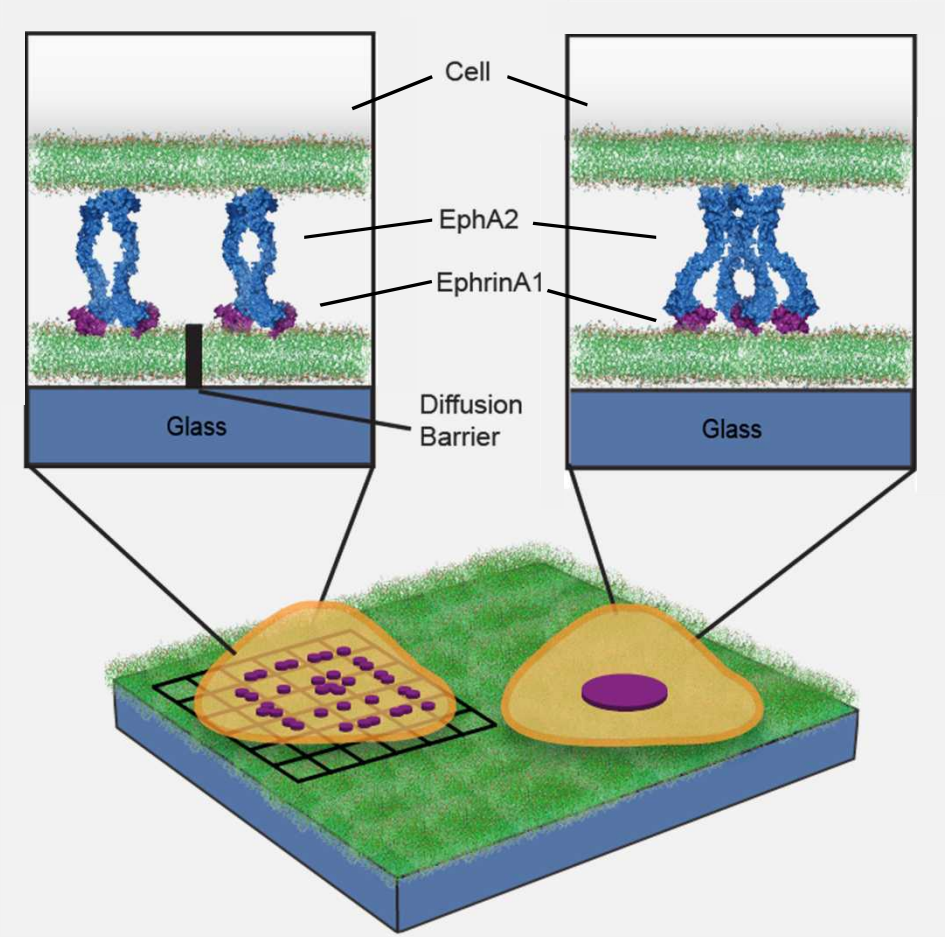




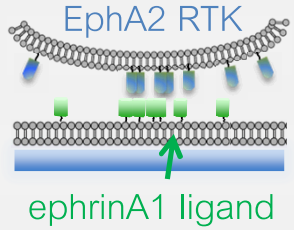
Janes, P. *et al.*. 2009. PLoS Biology 7(10): e1000215.

Greene, AC. and Lord, S. *et al.* 2014. *Biophysical Journal* 106: 2196-205.

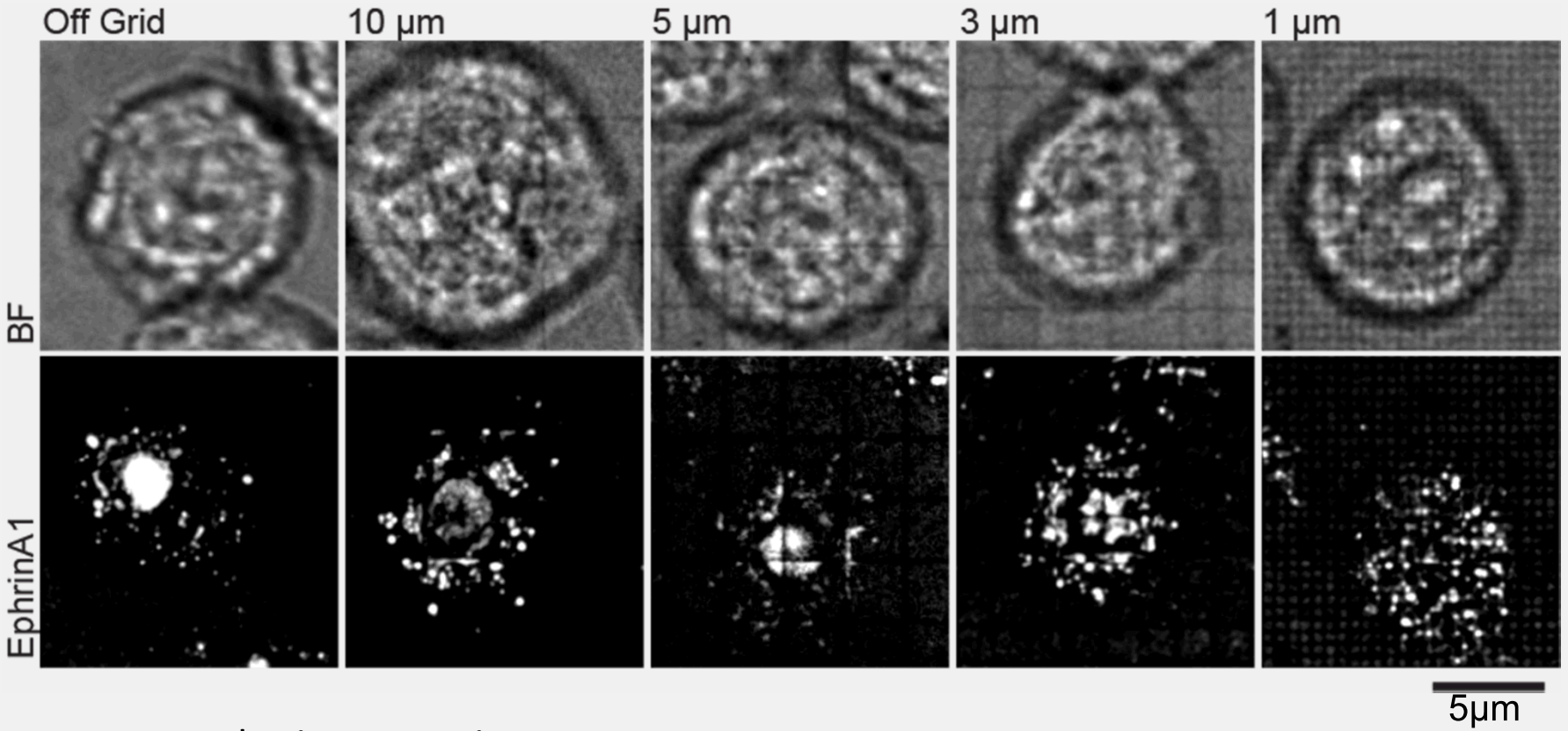
Using Diffusion Barriers to Alter Clustering: Creating a "Spatial Mutation"



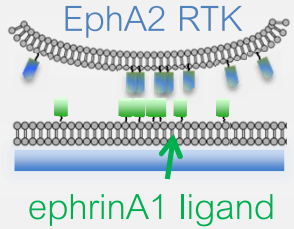
- Using electron beam lithography, diffusion barriers can be created to restrict receptor-ligand mobility
- This assay allows us to probe the importance of EphA2-ephrinA1 reorganization in the context of downstream signaling



Diffusion Barriers Alter EphA2-ephrinA1 Endocytosis

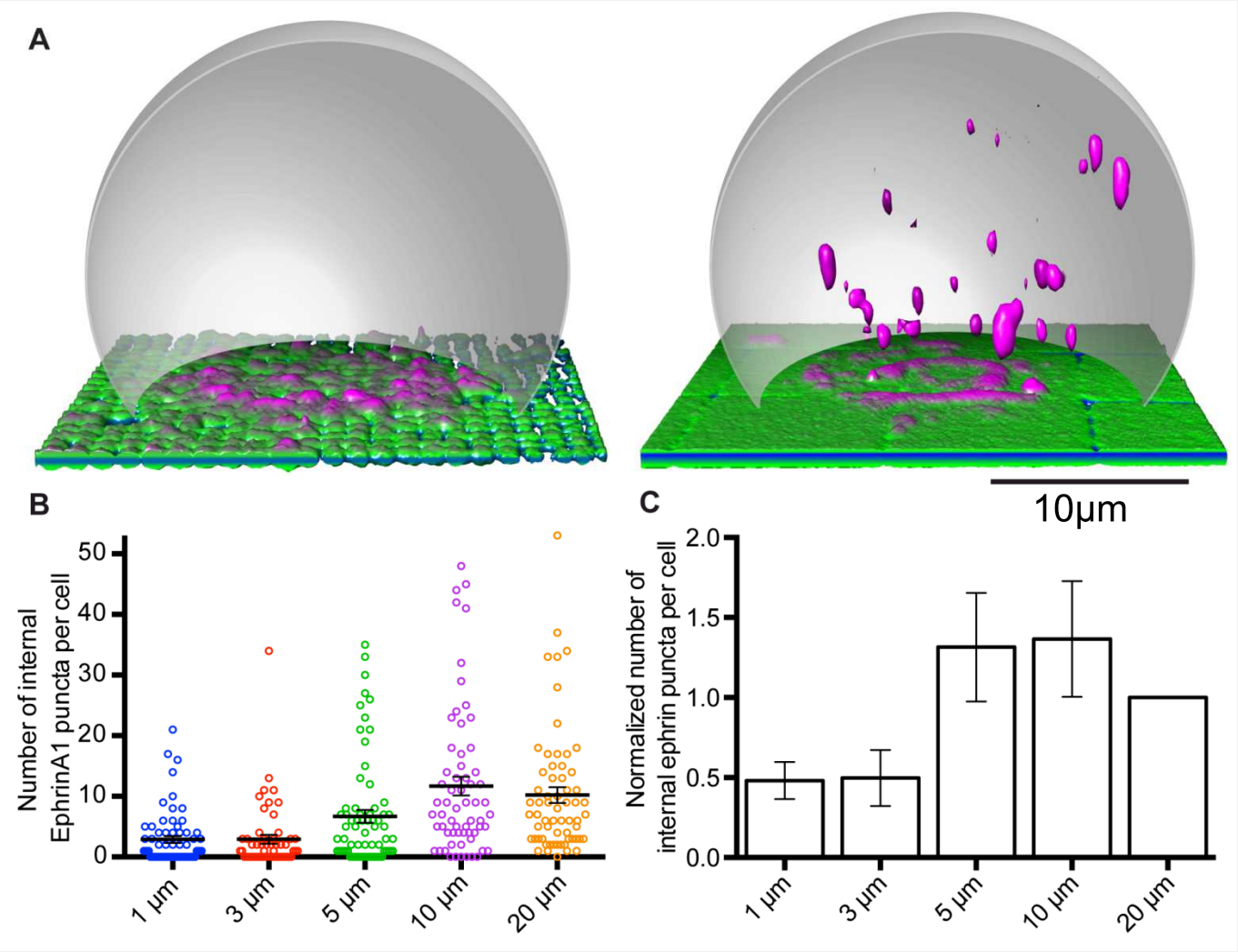


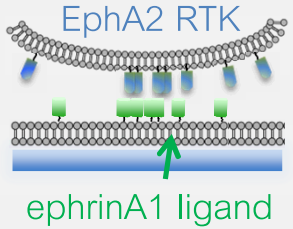
- Imaged using TIRF microscopy
- Only clustering and reorganization is altered
- Chemical composition remains the same



Greene, AC. and Lord, S. *et al.* 2014. *Biophsyical Journal* 106: 2196-205.

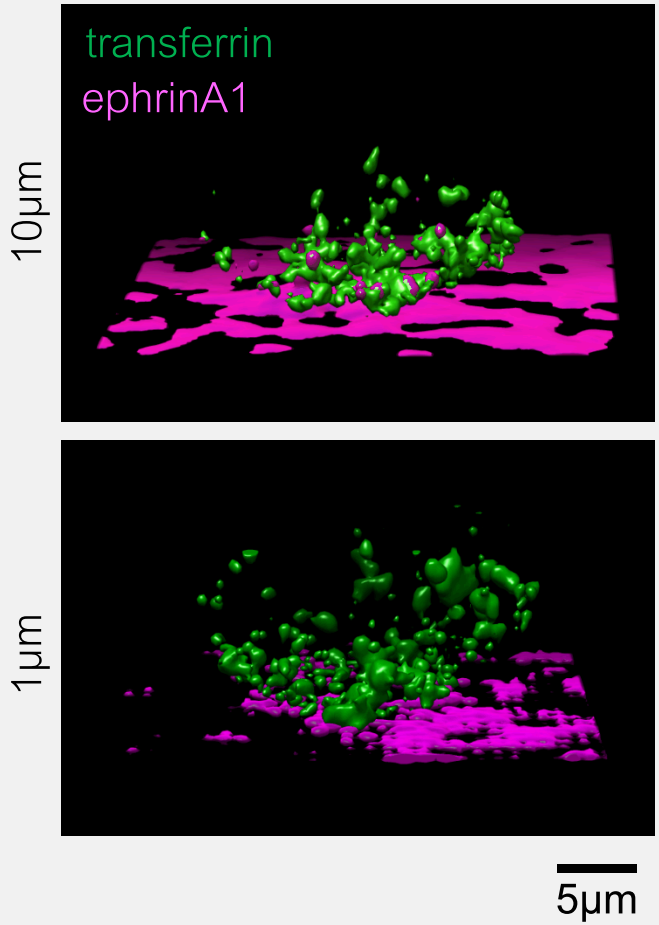
Trans-Endocytosis of ephrinA1 is Altered on Grids

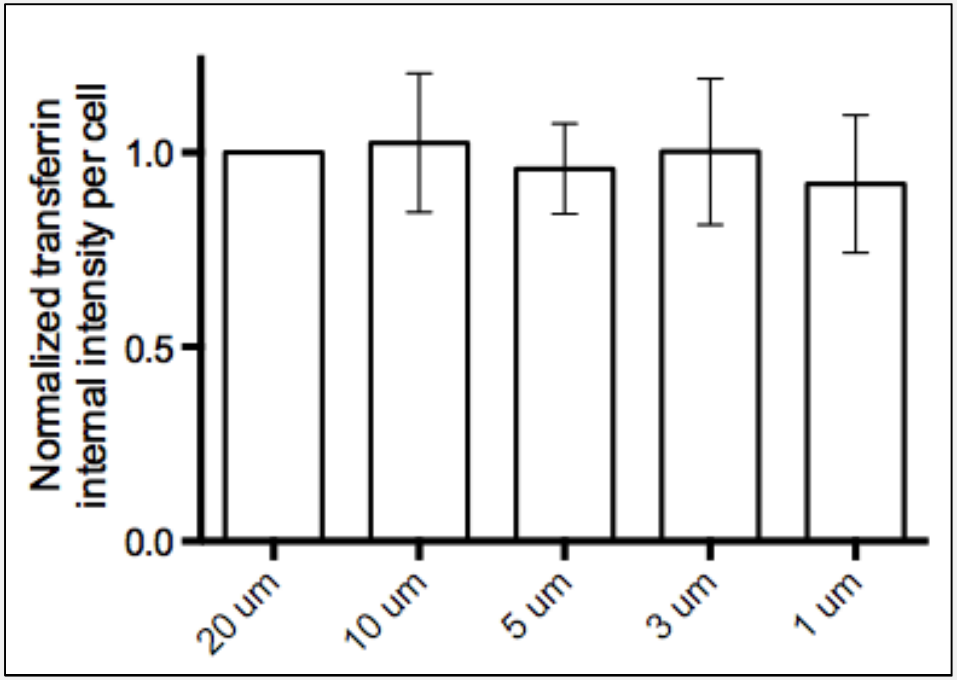




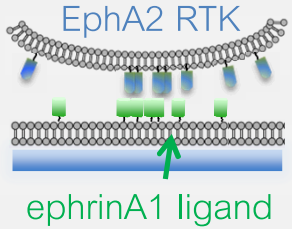
Greene, AC. and Lord, S. *et al.* 2014. *Biophysical Journal* 106: 2196-205.

Grids Do Not Alter All Clathrin-Mediated Endocytosis



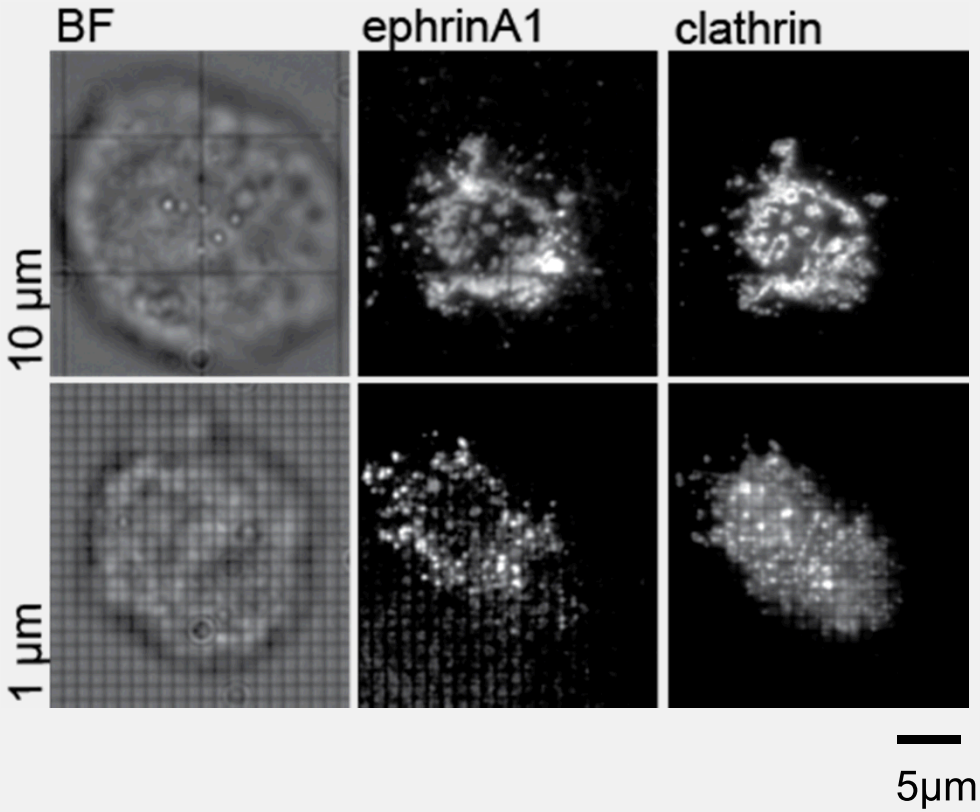


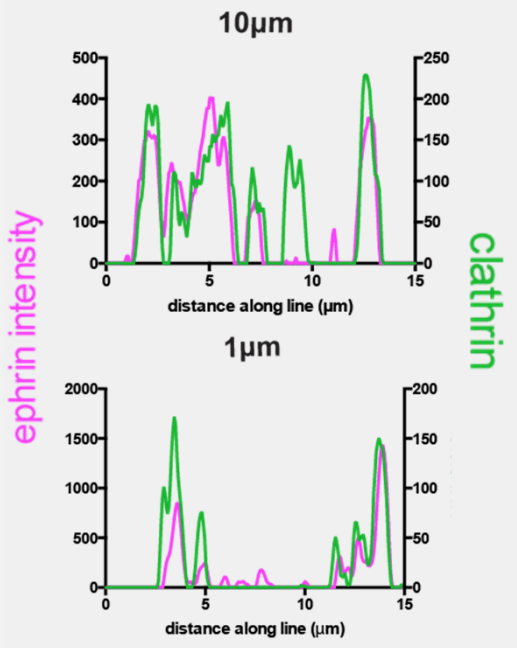
- Transferrin uptake on grids is not altered as a function of grid pitch
- There is some colocalization of transferrin and ephrinA1



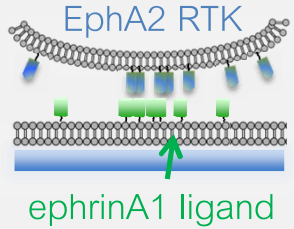
Greene, AC. and Lord, S. *et al.* 2014. *Biophysical Journal* 106: 2196-205.

Signaling is not Altered on Gridded Substrates



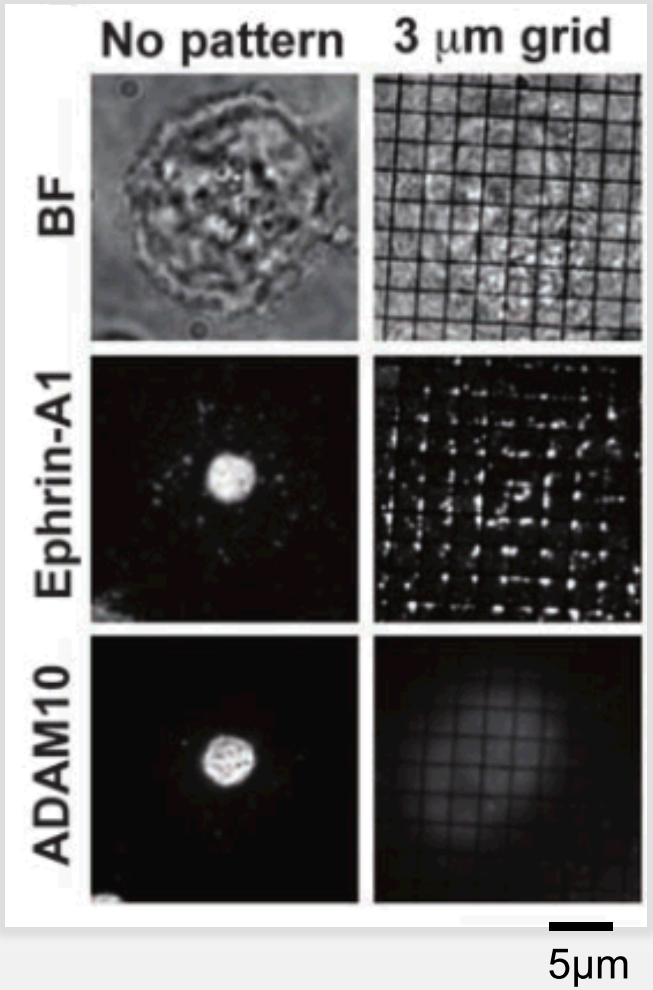


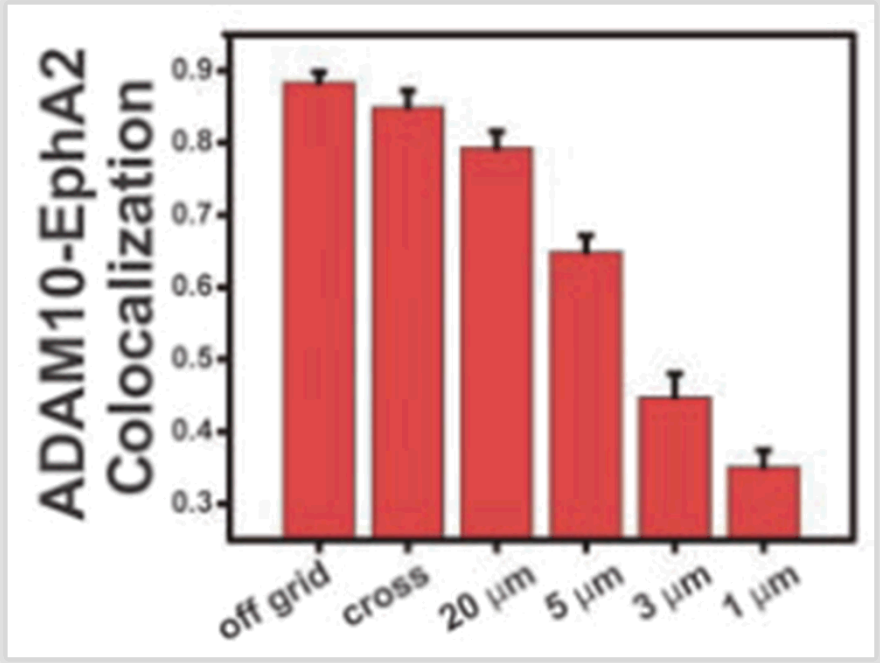
- Phosphorylation does not change
- Recruitment of most signaling molecules is not altered
  - Dynamin and clathrin are still colocalized
  - Caveolin is still anti-localized



Greene, AC. and Lord, S. *et al.* 2014. *Biophysical Journal* 106: 2196-205.

Recruitment of ADAM10 is Decreased on Grids

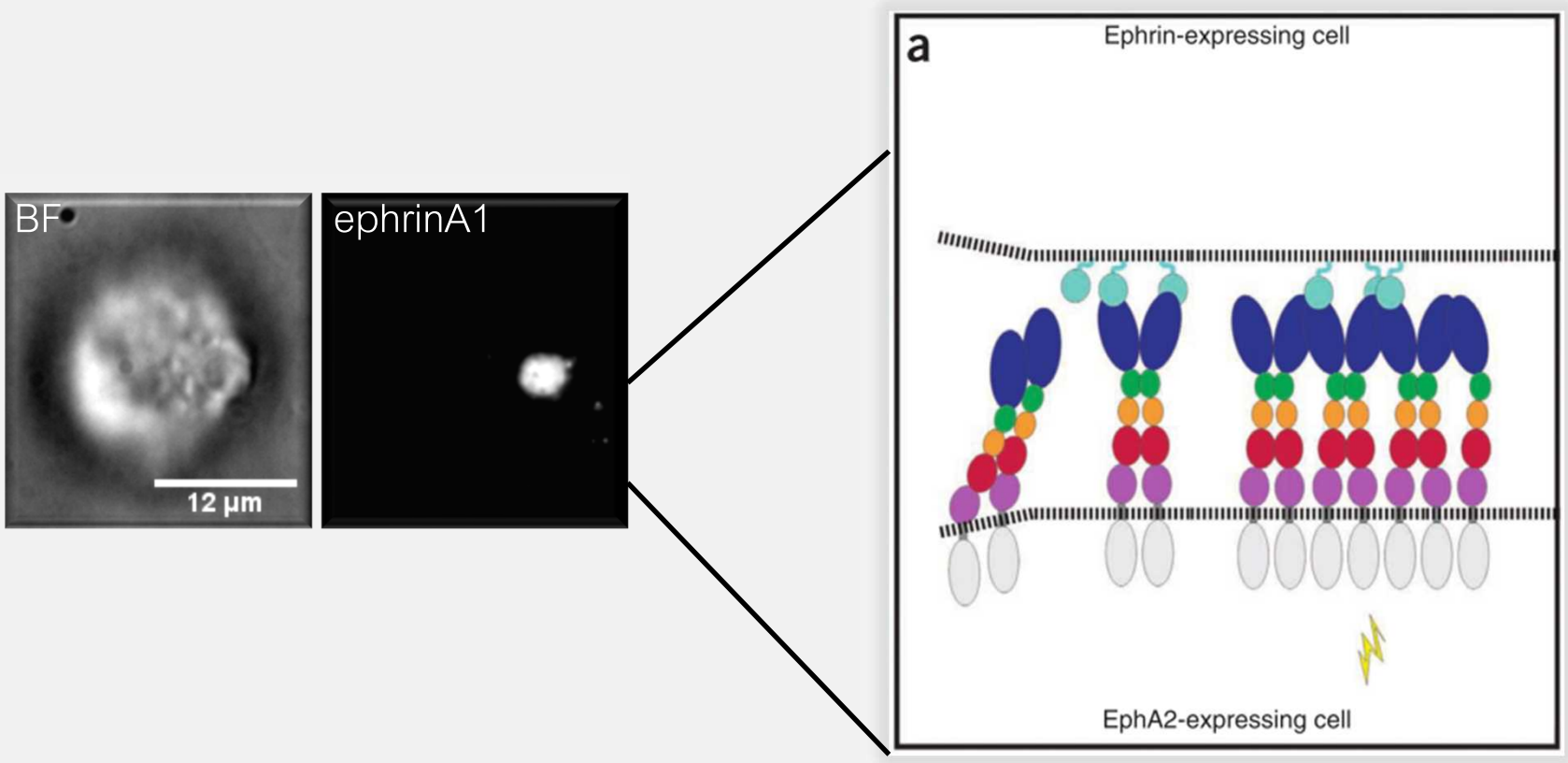




Salaita, K. and Nair P. *et al.* 2010. *Science* 327: 1380-5.

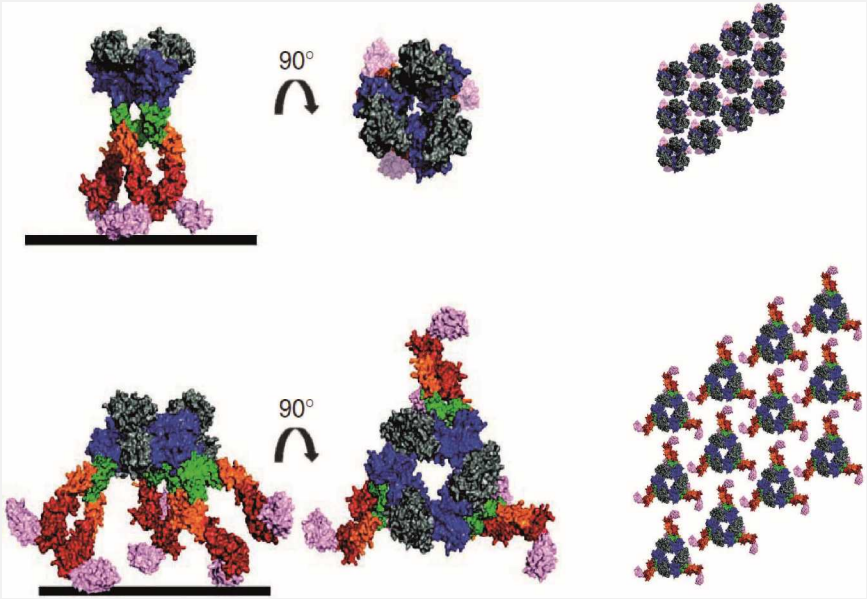
Structure and Function of EphA2 Clustering

How does clustering from the micron down to the nanoscale regulate signaling?



Seiradake, E., *et al*. 2010. *Nat Struct Mol Biol* 17(4): 398-402.

Molecular Architecture of EphA2 Clustering

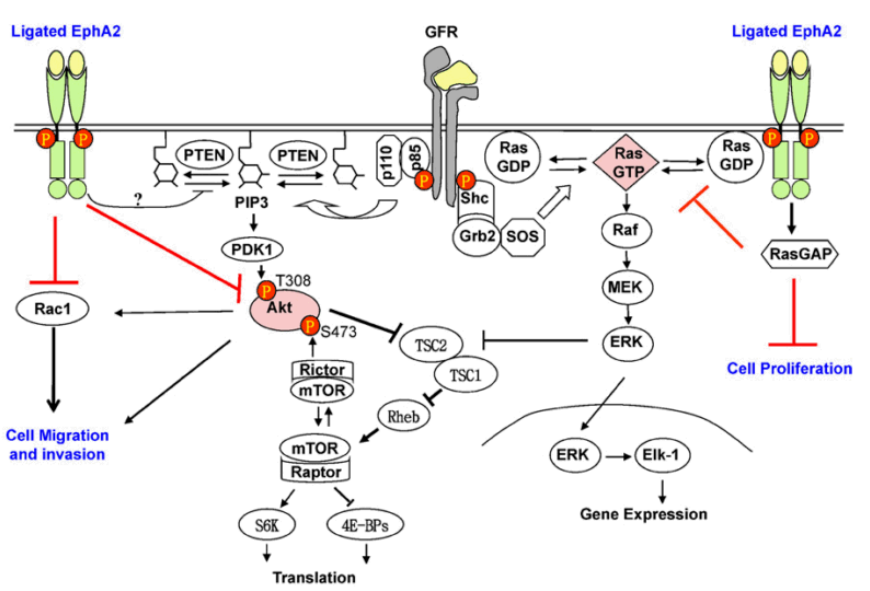


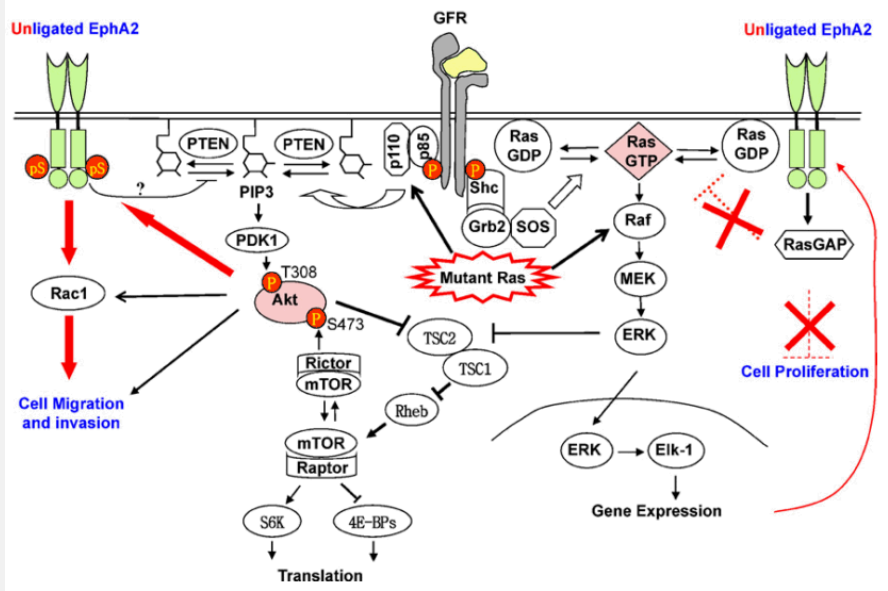
- EphA2 forms large-scale oligomers
  - Both in *cis* with other EphA2 receptors and in *trans* with ephrinA1 ligands

- We need to understand more about Eph molecular structure and clustering and how that alters signaling

Modified from Seiradake, E., *et al*. 2012. *Nat Struct Mol Biol* 20(8): 958-64.

EphA2 Signals in a Ligand-Dependent *and* Independent Manner



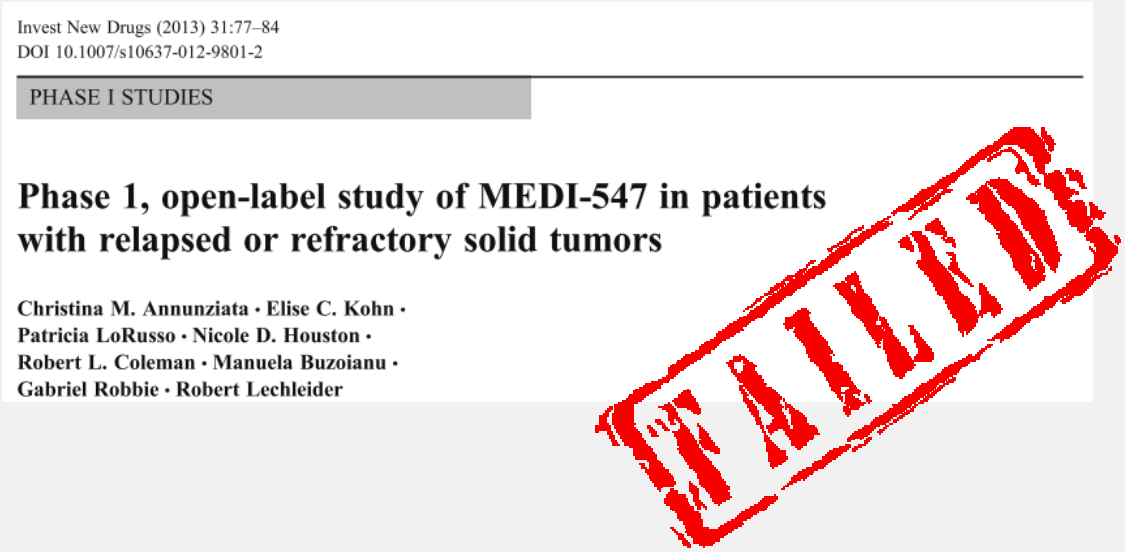


- Requires fine-tuning the balance of signaling based upon ligand-dependent and ligand-independent signaling; also context and cell type specific

- How Eph clusters (i.e. the stoichiometry of receptor/ligand binding) also changes this signaling map

Miao, H., and Wang, B. 2012. *Semin Cell Dev Biol* 23(1): 16-25.

Why Do We Care About EphA2 Clustering?



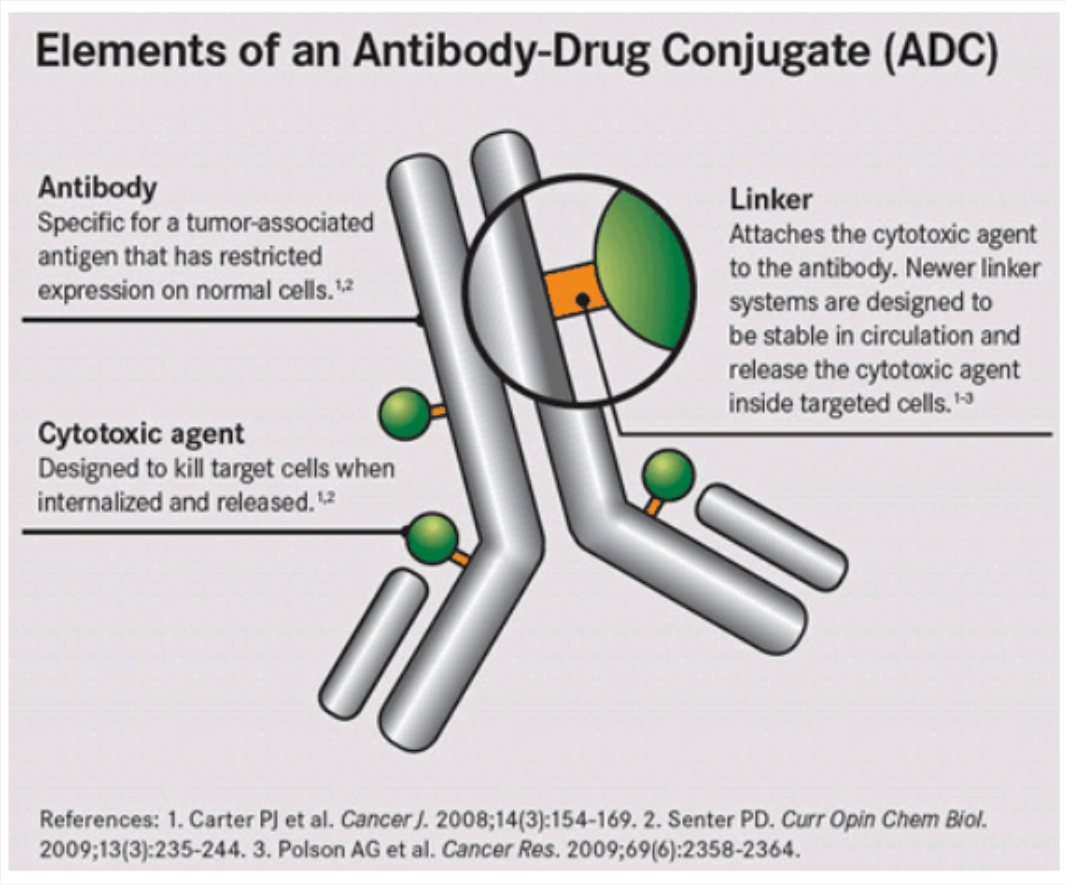
- Drug trial for targeting EphA2 expressing cells (e.g. triple negative breast cancers) failed in the Phase I Trial

- 6 women entered (breast, ovarian, endometrial and colon cancer patients)
  - Trial had disastrous effects; all women withdrew due to adverse affects (hemorrhage, liver disorder, etc.)

Annunziata, CM, *et al*. 2013. *Invest New Drugs* 31(1): 77-84.

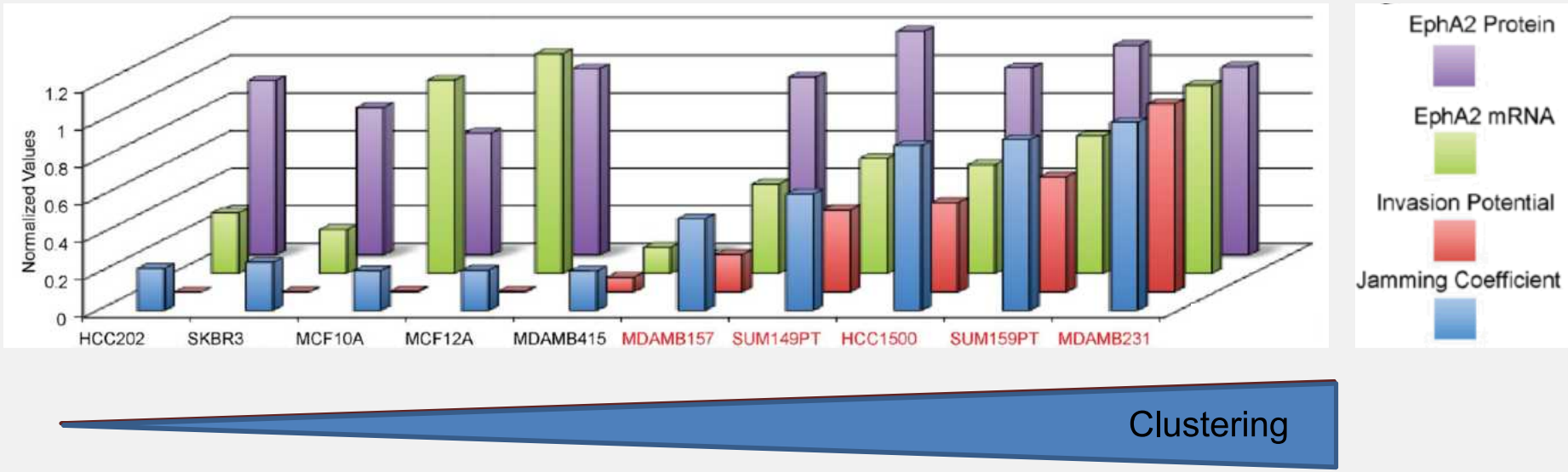
What Went Wrong with the Trial?

- Monoclonal anti-EphA2 antibody covalently attached to auristatin (microtubule inhibitor)
  - Likely NOT due to non-specific toxicity of auristatin
- Likely due to the antibody components of the antibody-drug conjugate
- In one patient, it caused pulmonary metastases
- **Maybe the artificial clustering induced by the antibody caused toxicity?**



Clustering Correlates to Disease State

- *The most aggressive cancers have the most tightly clustered EphA2 receptors*

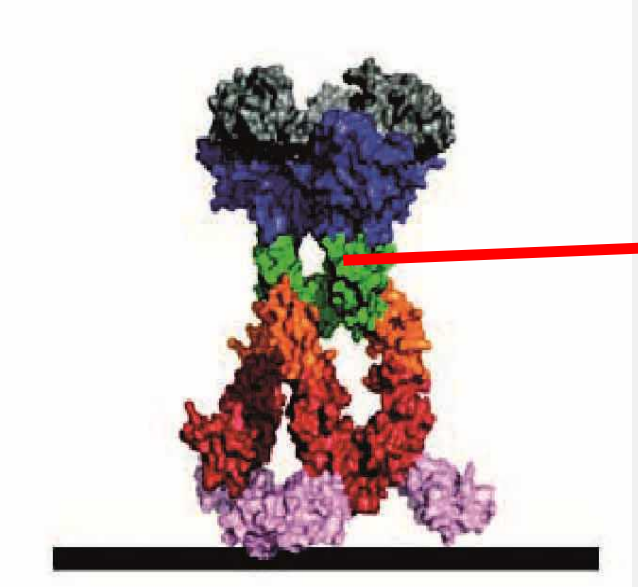


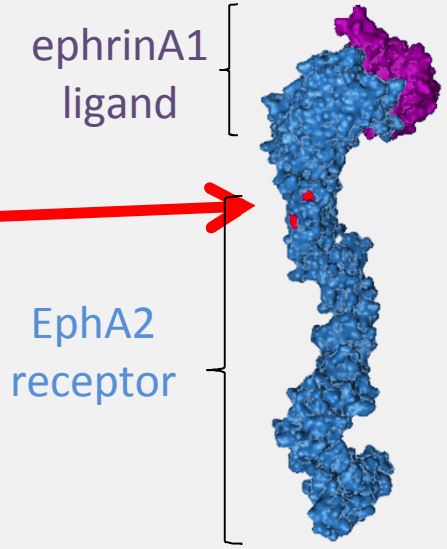
- Invasion potential of these cells only correlates to the EphA2 clustering phenotype

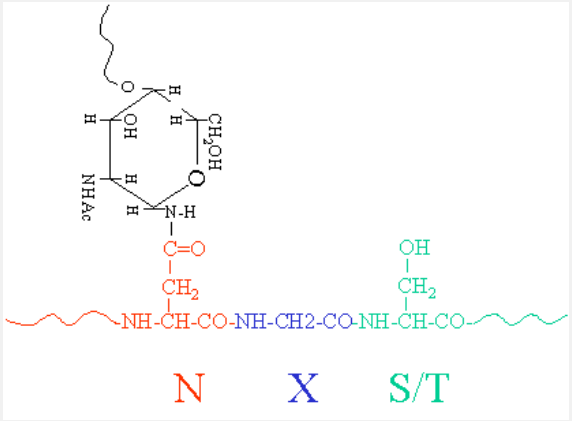
- Tightly clustered EphA2 indicative of more dangerous cancers?
  - Could the antibody-drug target be inducing more EphA2 clustering and causing an increased disease state of the cell?

Lohmuller, T., *et al*. 2013. *Nano Letters,* 13(7): 3059-64.

Genetic Mutation of EphA2 *cis* Clustering



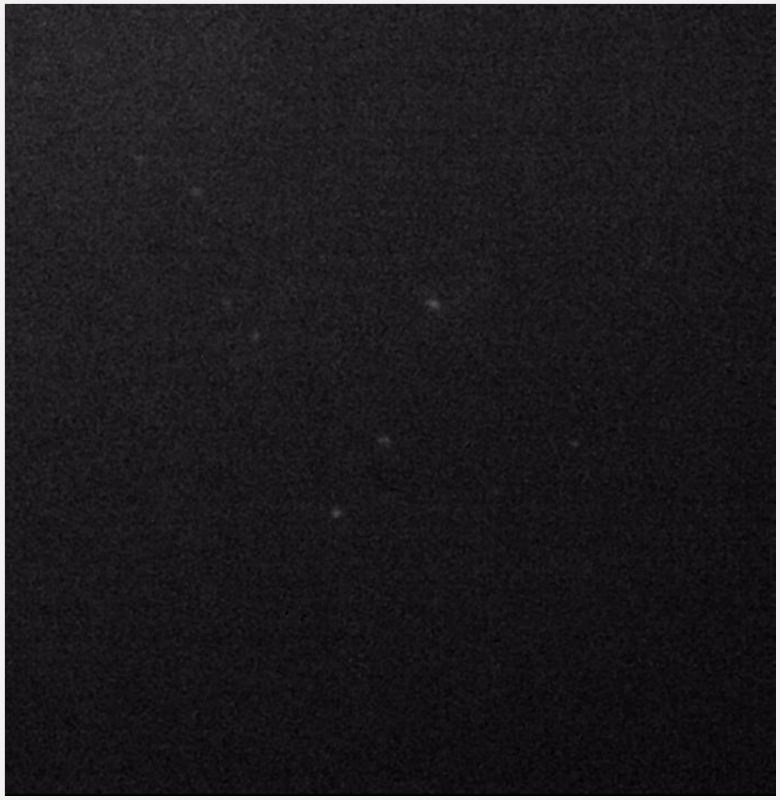




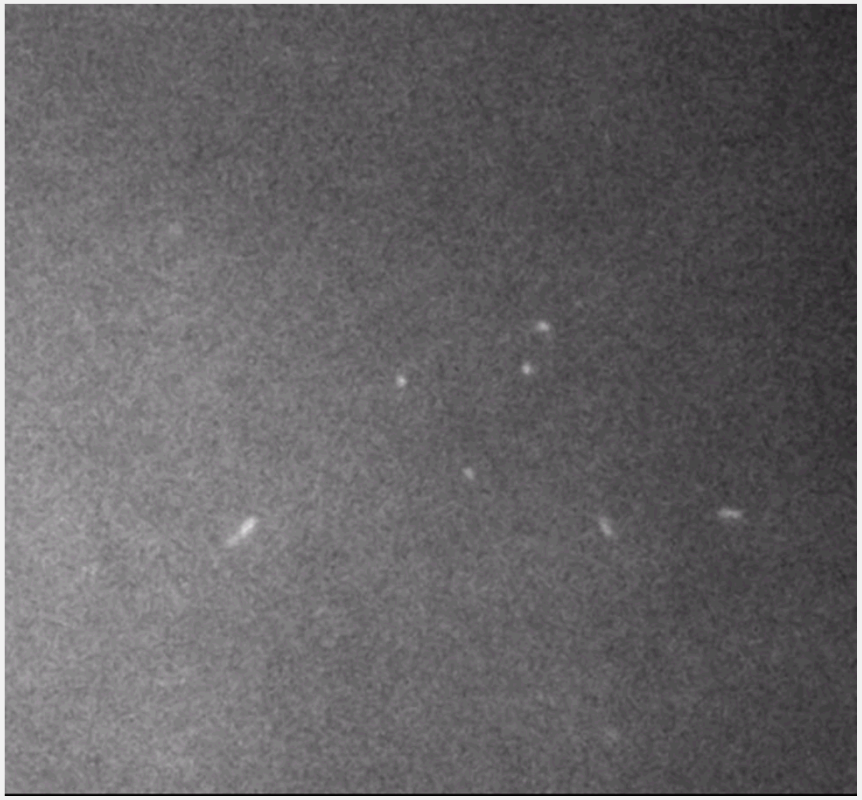
- Disrupting Eph clustering at the nanometer/angstrom scale requires genetic mutations
  - Target the sushi domain of the receptor to disrupt EphA2 *cis* clustering
- To do this in an endogenous context requires genome-editing
- We used CRISPR/Cas9 to permanently introduce two point mutations into the sushi domain resulting in an N-linked glycan in the domain

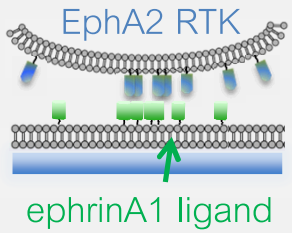
Mutants Cluster Faster than the Wildtype

Wildtype



Mutant



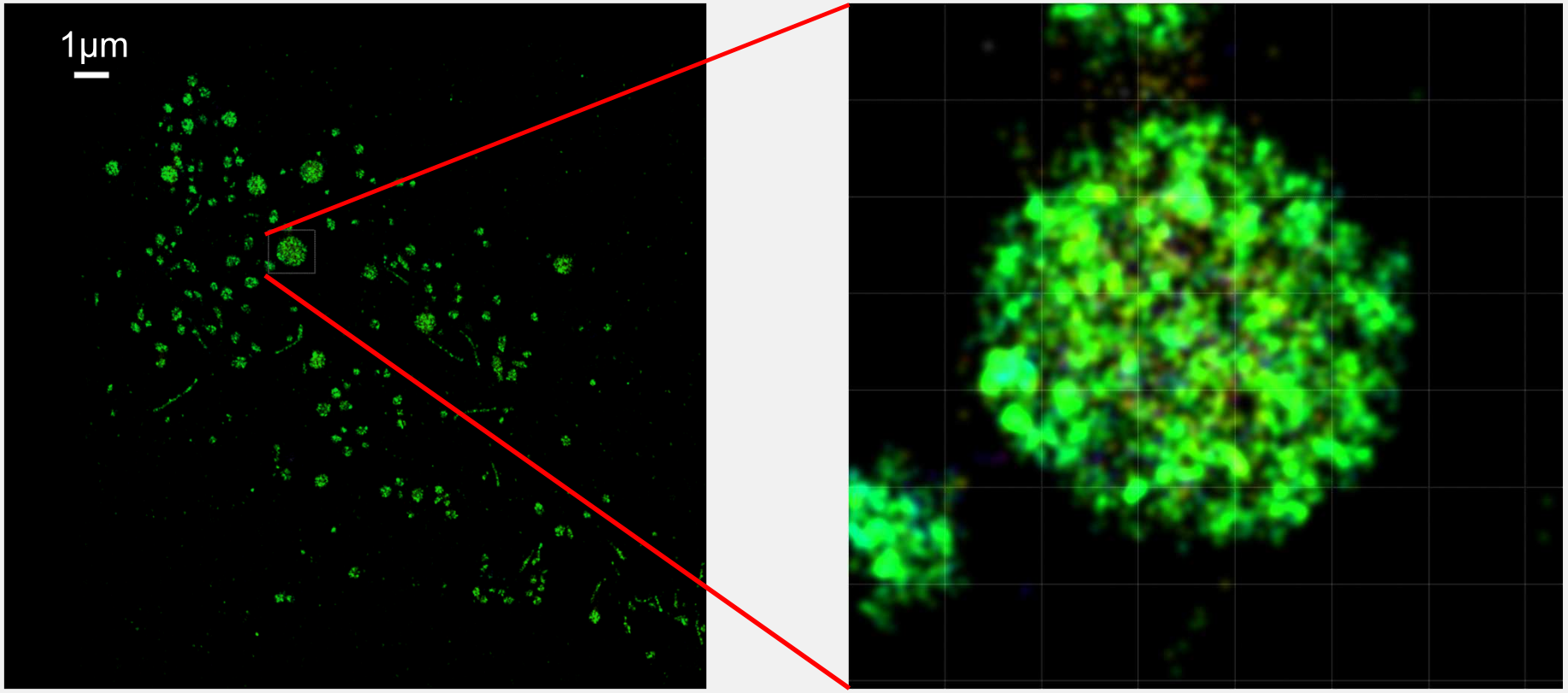


- Fluorescent signal is ephrinA1 on the supported membrane

Imaged every 1 second for 5 minutes
Movies are 5 frames per second

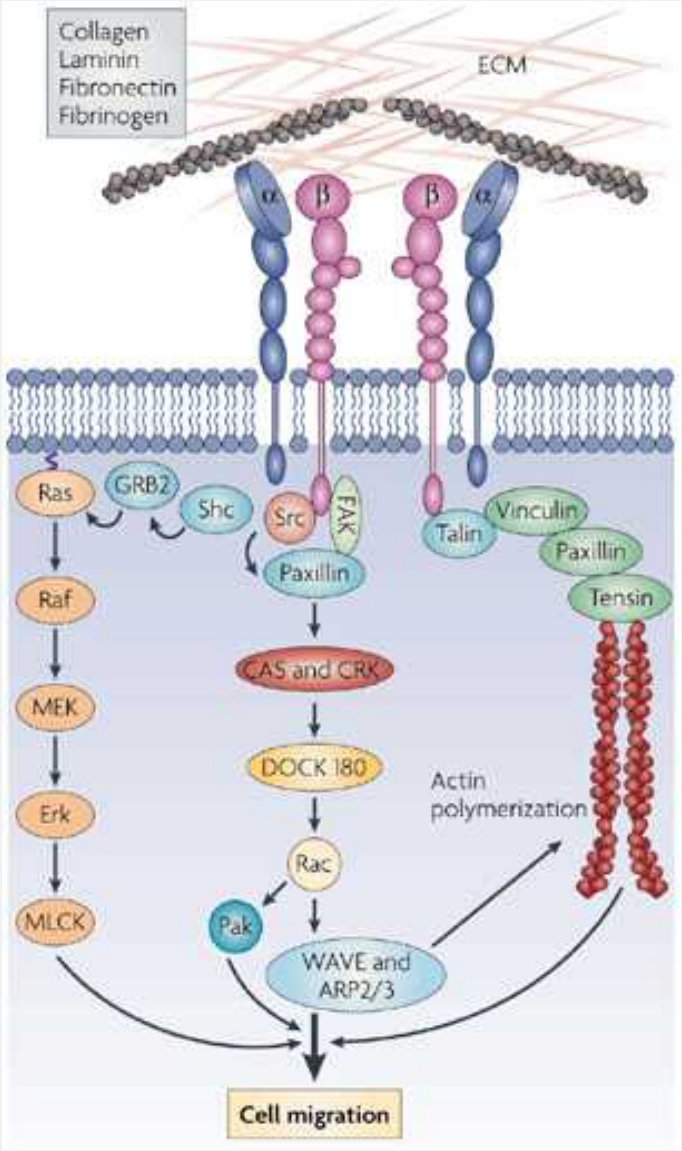
Determining Structural Differences at the Nanometer

- Use higher resolution STORM microscopy to define differences in Eph-ephrin structure at the nanometer scale
- Fluid bilayers allow for “too much” clustering; structures cannot be defined



Need to Flatten Cells and Confine Ligand Density

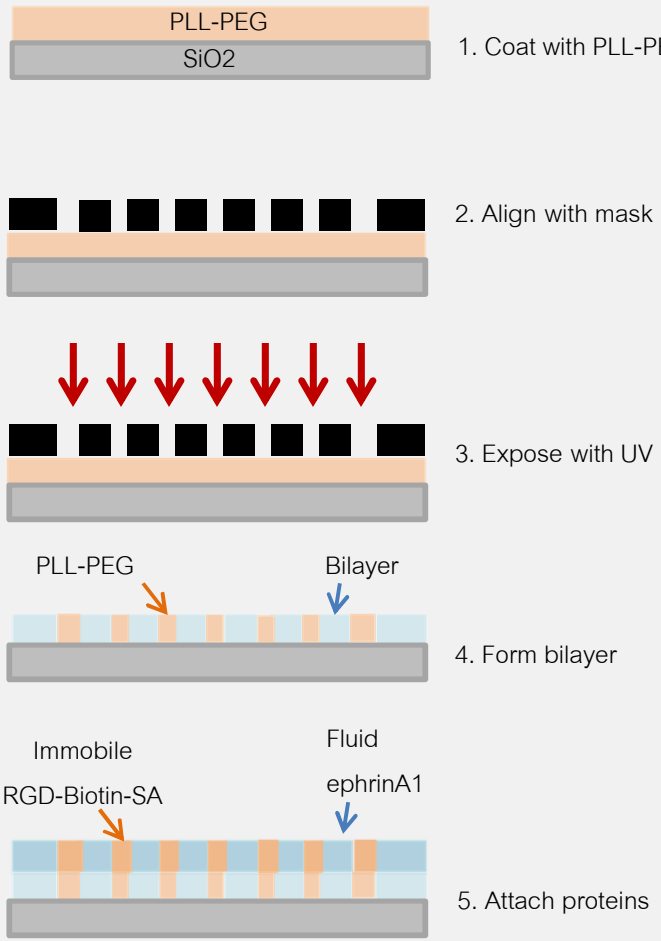
- Need a mechanism to flatten cells and simultaneously limit the ligand density available to the cells
- Simultaneously activate an adhesion pathway in addition to EphA2 RTK signaling
- Create a substrate with both integrin and EphA2 signaling



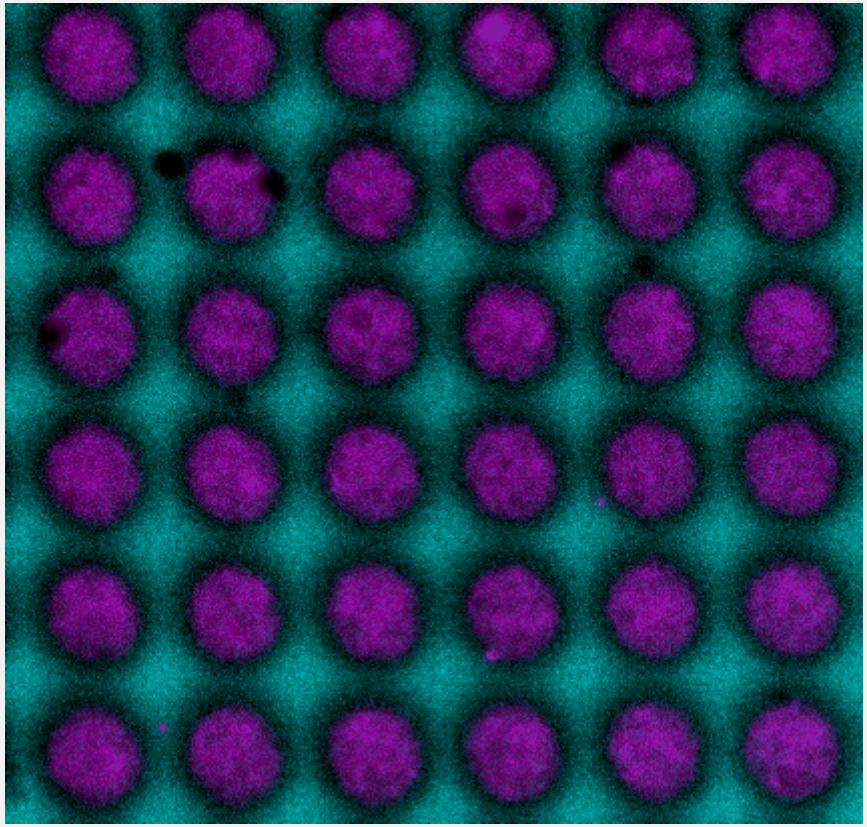
Christie, J. *et al*. 2008. *Nat Rev Cancer* 8: 604-17.

Confining Clustering to Determine Cluster Structure

- Confine clusters to defined regions and limit ligand density
- Flatten cells using integrin adhesions

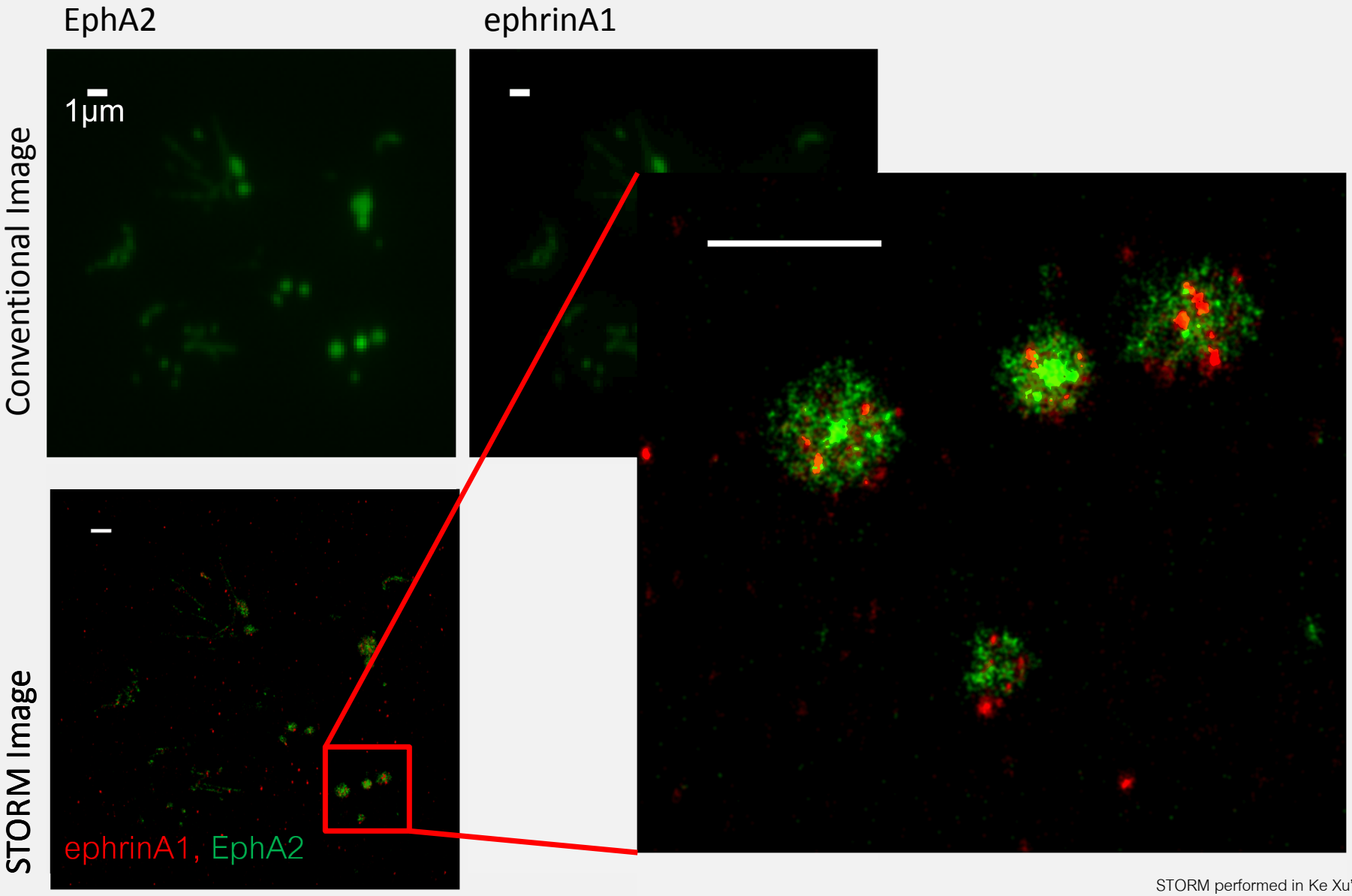


Immobile RGD, fluid ephrinA1



Mask designed by Zhongwen Chen

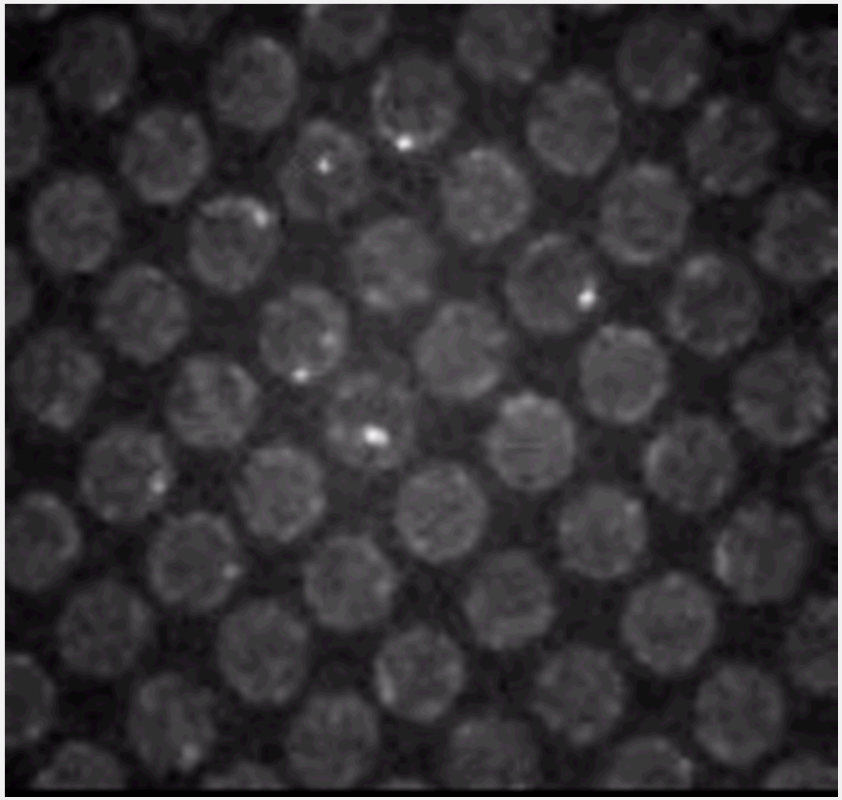
2-Color STORM Reveals Structural Information of Clusters



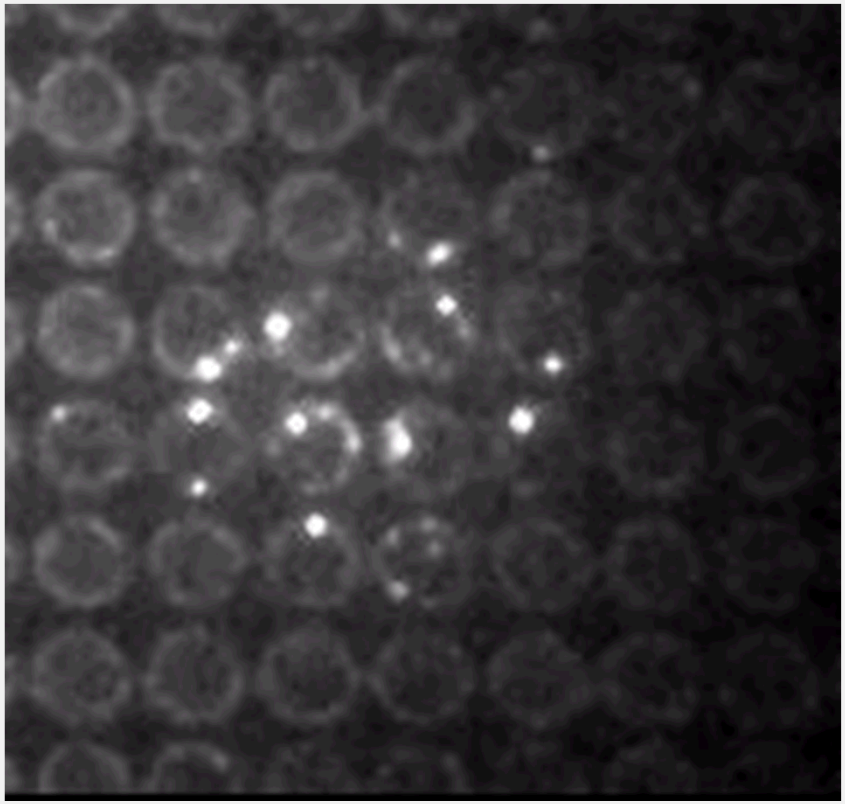
STORM performed in Ke Xu's lab

Mutant Clusters are More Dynamic and Transient

Wildtype



Mutant



5μm

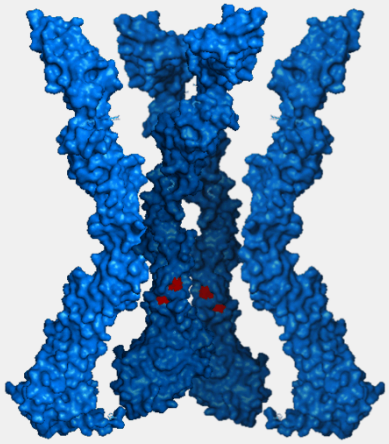
- Ligand density can be confined while adhering the cells using photolithography
- Mutants cluster faster and less definitively

Imaged every 10 seconds for 6 minutes

Movies are 5 frames per second

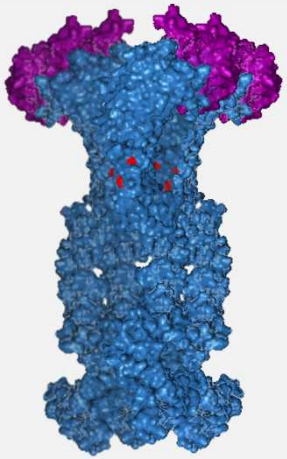
Disrupting EphA2 *cis* Interactions is Necessary for Clustering

Side view of unligated EphA2

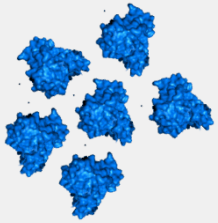


Rearrangement of Eph-Eph interactions

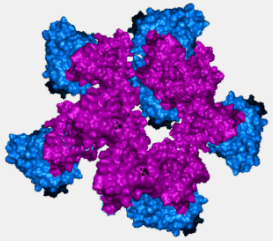
Side view of ligated EphA2



Top-down view of unligated EphA2



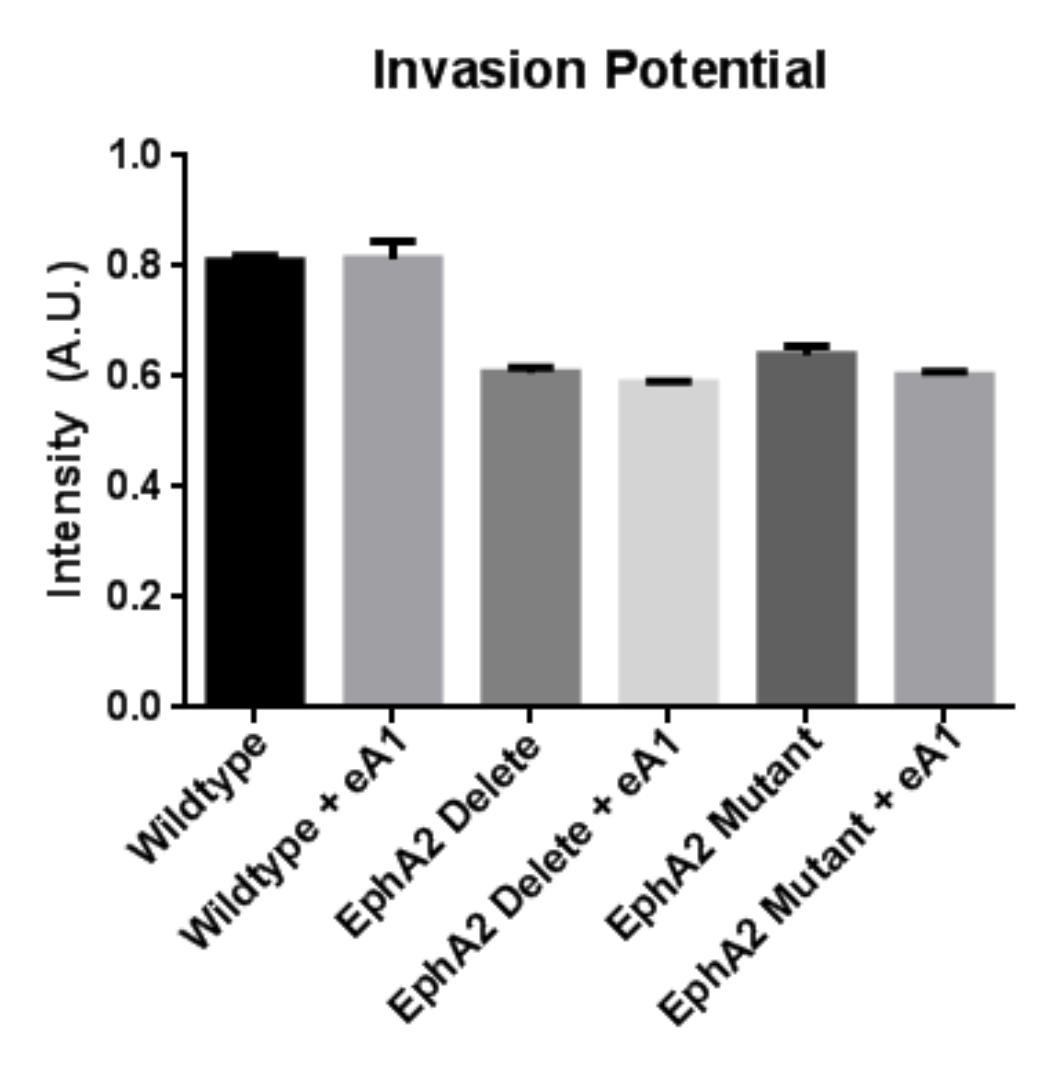
Top-down view of ligated EphA2



- Sushi domain mediates stable Eph-Eph interactions within the cell
- Micron-scale clustering requires binding to ephrinA1 and a disruption of Eph-Eph interactions

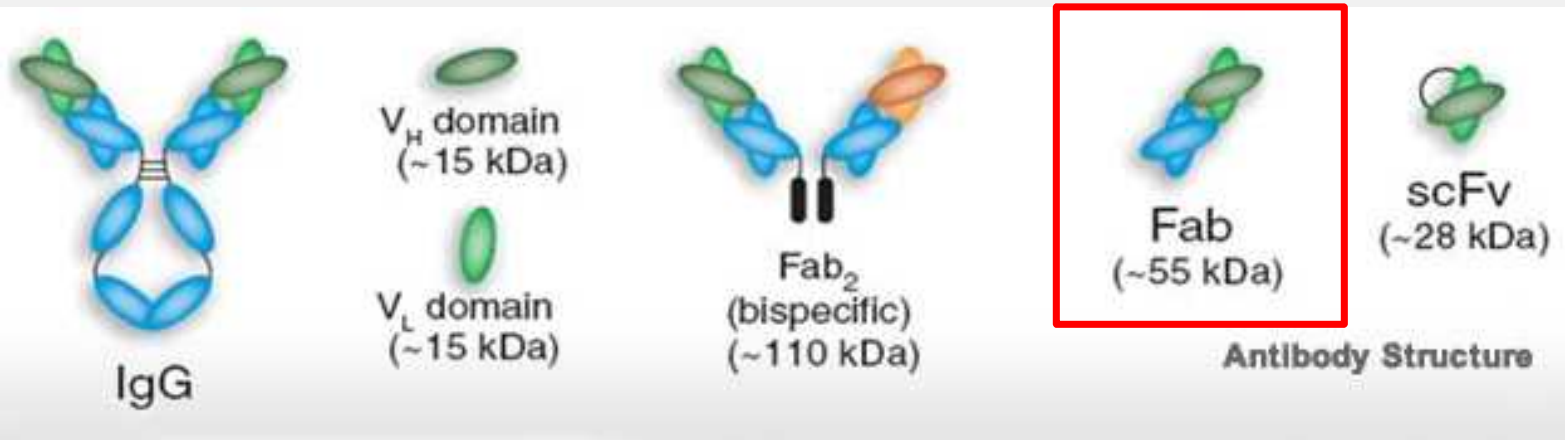
Changing Molecular Clustering Changes Invasion Potential

- Invasion potential measures the ability of a cell to leave a colony [tumor] and migrate through certain barriers; it is the hallmark of metastasis
- EphA2 with the sushi domain mutation is ~25% less invasive than the wildtype



Part 1 Conclusions

- The importance of spatio-mechanical regulation of signaling systems across length scales is becoming increasingly evident
- Understanding spatio-mechanical regulation of Eph receptors will provide insights into the misregulation of EphA2 in disease, particularly cancer
- Alternate strategies for targeting these kinds of receptors can be developed

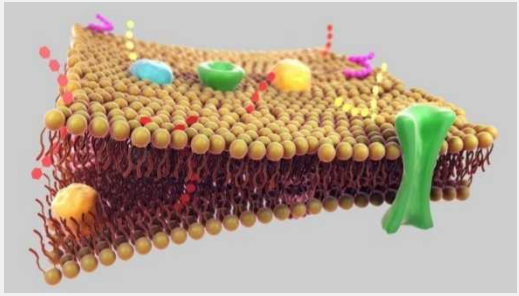


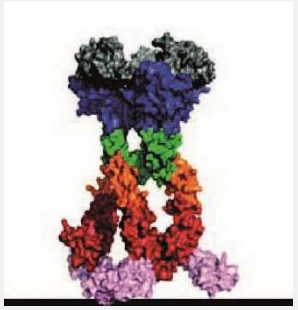
Understanding Regulatory Mechanisms of Biological Systems

Goal: Understand biological systems for applications-based technologies (therapeutic target design, diagnostic tools, biosensing and/or bioengineering technologies)

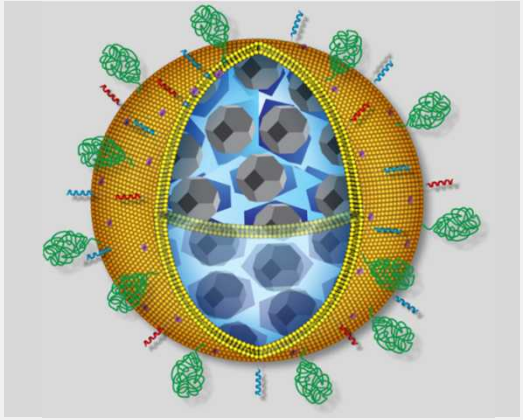
Novel and Effective Therapeutics

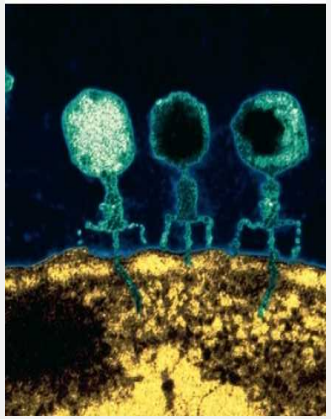
1. Receptor Signal Regulation





2. Targeted Therapeutic Delivery

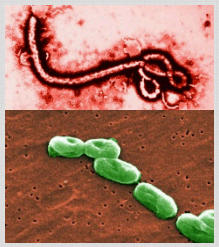




Targeted Delivery of CRISPR/Cas9 Therapeutics

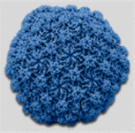
Experimental Approaches

CRISPR/Cas9 Toolbox



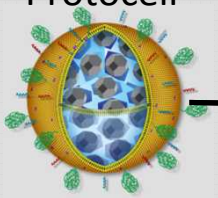
DNA

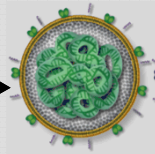
Phage

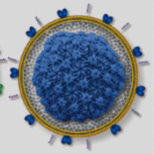


Packaging Therapeutics

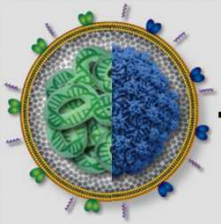
Protocell



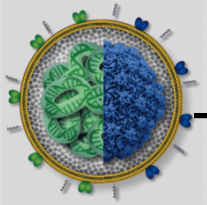




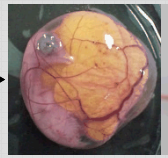
Testing *in vitro*



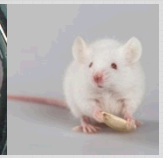
Model Organisms



CAM

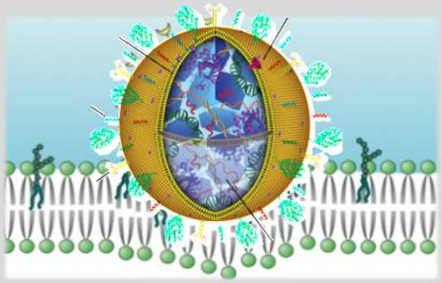


Mouse



Modeling Approaches

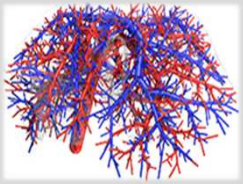
Molecular Level Modeling



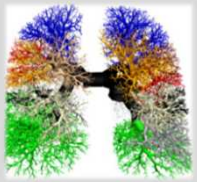
Vascular Level Modeling

Organ Level Modeling

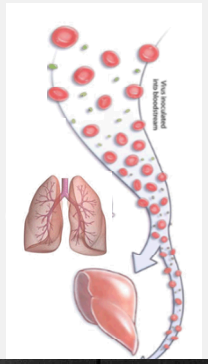
Liver

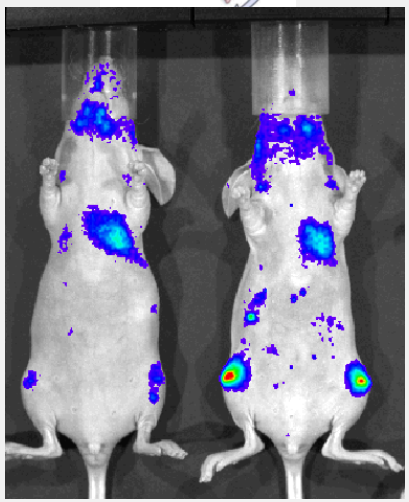


Lung



Optimization



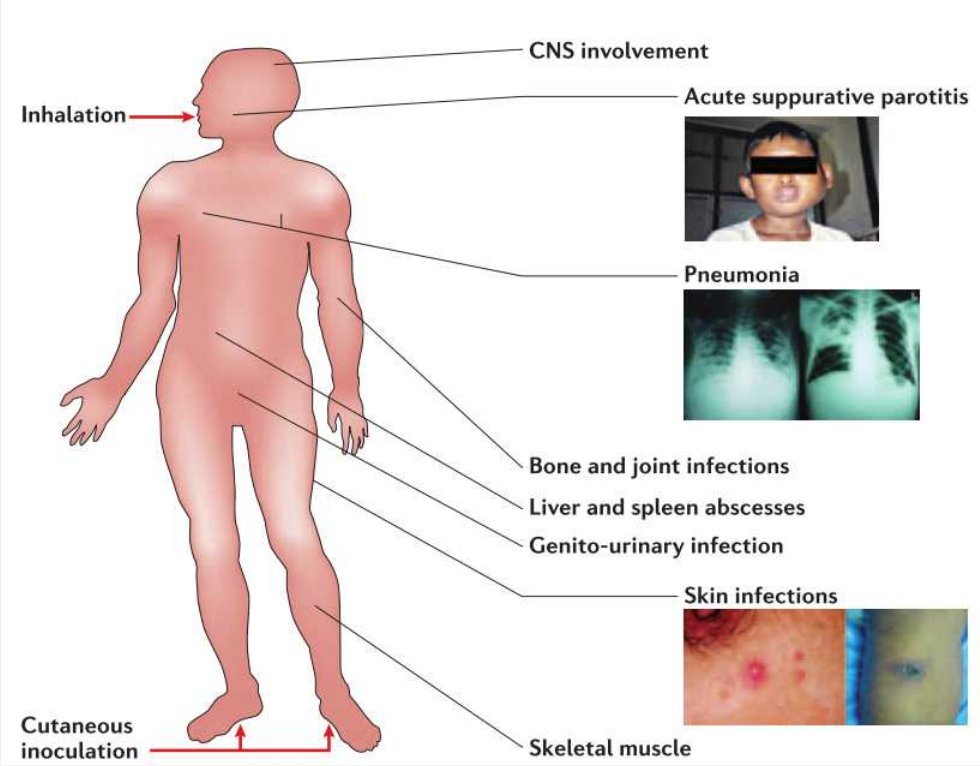


Goal: Targeted Therapeutic Delivery

Burkholderia pseudomallei

- *Burkholderia pseudomallei* is a highly drug resistant, intracellular gram-negative bacterium
- Common in Southeast Asia and Northern Australia
  - 20-50% mortality rate
- Infection is acquired by inoculation, inhalation, and aspiration
- Causes melioidosis
  - Pneumonia
  - Bone pain
  - Abscesses
  - Brainstem encephalitis

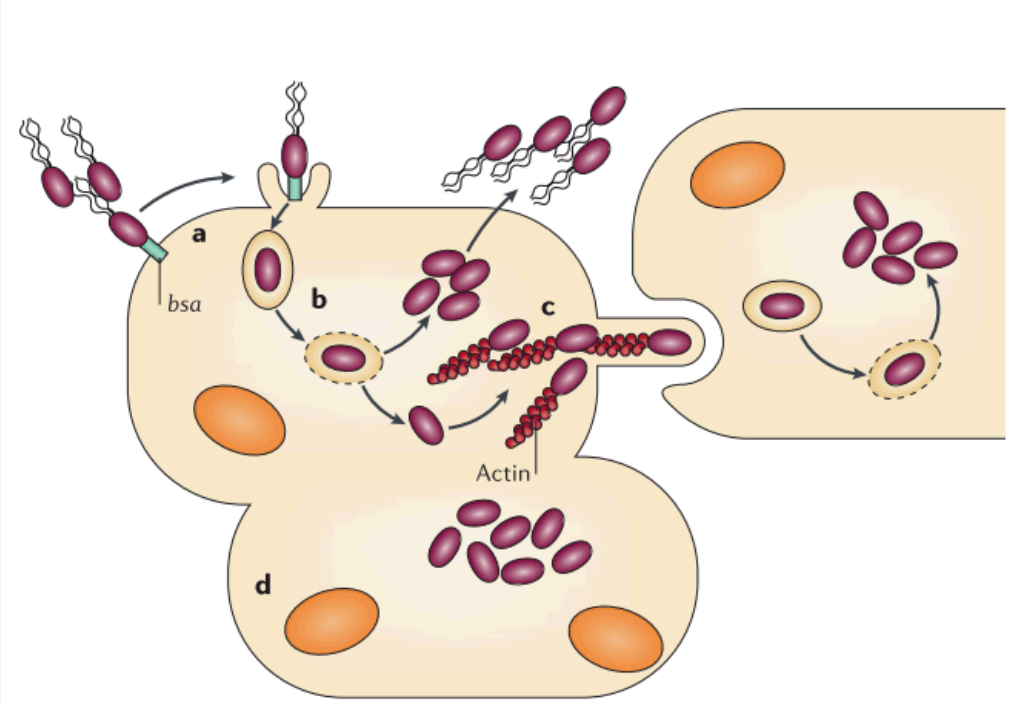
- ***Goal: Develop novel treatment strategies***



W. Wiersinga, *et al.*, Nature Reviews Microbiology, 2006, 4: 272-82.

The Life Cycle of *Burkholderia pseudomallei*

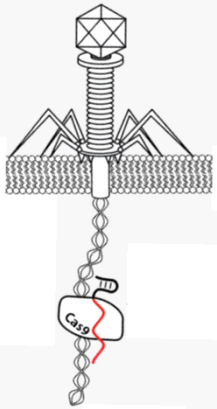
- Life cycle involves:
  - Adherence and entry into host cells
  - Phagosome escape
  - Cytosolic replication
  - Actin propelling within the cell
  - Spreading to neighboring cells



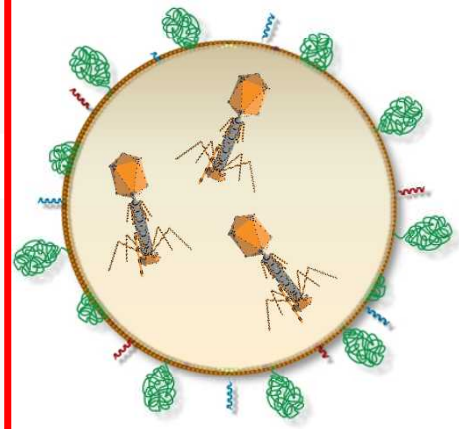
W. Wiersinga, *et al.*, Nature Reviews Microbiology, 2006, 4: 272-82.

How to Target Resistant Bacteria

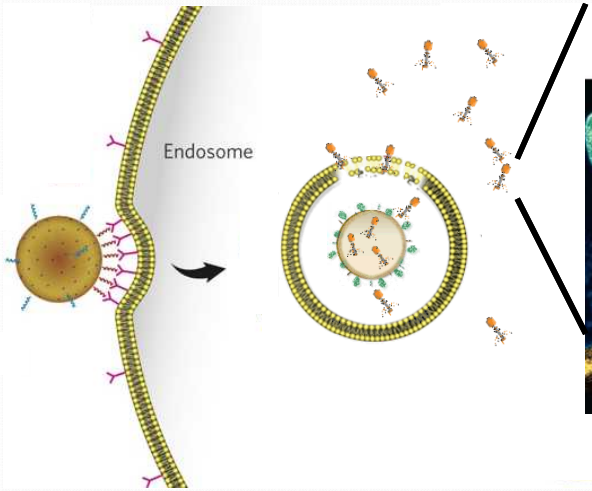
1. Genetically Encode Bacteriophage with CRISPR/Cas9



2. Encapsulate Bacteriophage into Nanoparticles



3. Deliver Bacteriophage to Bacterially-Infected Mammalian Cells

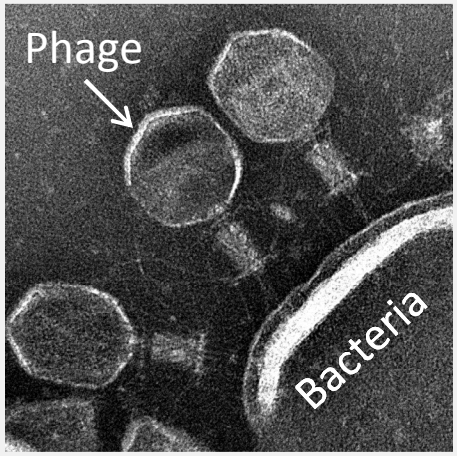


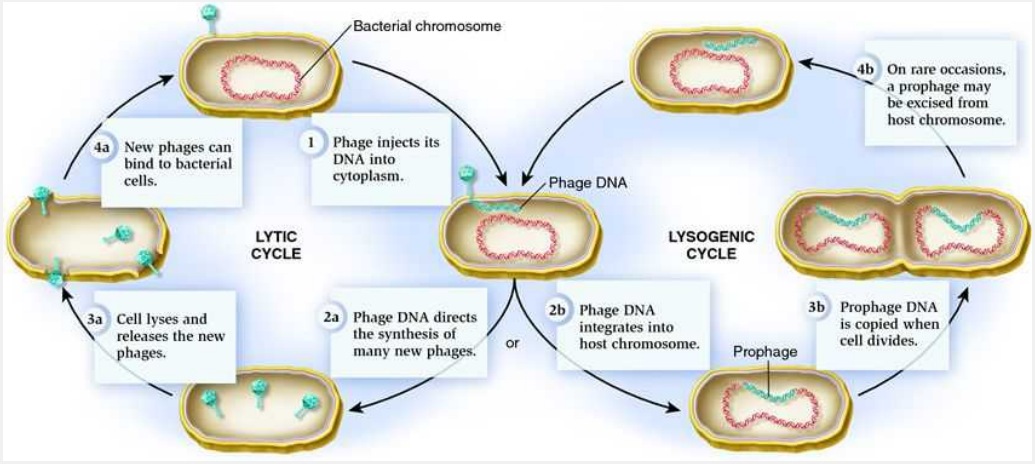
4. Bacteriophage Deliver CRISPR/Cas9 to Intracellular Bacteria

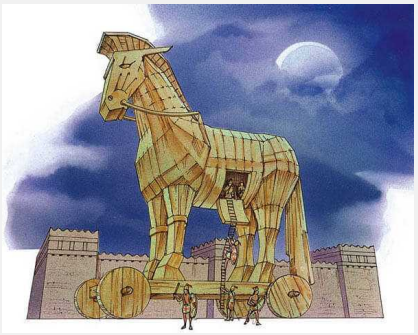
Protocell masks bacteriophage from initiating an immune response

Inhibit Antibiotic Resistant Bacteria Using Bacteriophages

Bacteriophages: Bacteria-Specific Viruses







Lytic Phage

- Fast bacterial killing on own
- Challenging to genetically modify

Lysogenic Phage

- Can exist without killing the bacteria
- Easier to genetically modify to contain CRISPR/Cas9; these phage will carry CRISPR as a “Trojan horse” for resistant pathogens

Goal: *Use Bacteriophage as a Trojan Horse for Delivering CRISPR/Cas9 Therapeutics*

https://phagehuntnz.wordpress.com/2014/05/13/phage-hunt-nz-post-fighting-with-phages/

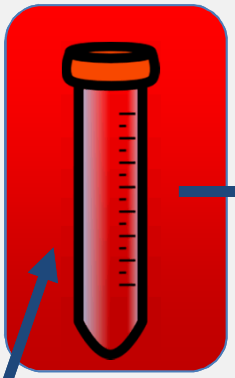
Environmental Isolation of Bacteriophage

Identify regions of interest to isolate phage based on epidemiology

Incubation

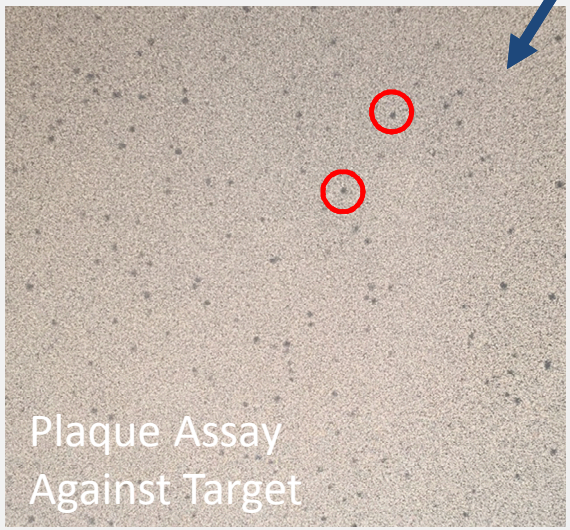
Filtration

Concentration



Sample types:

- Water
- Soil
- Sediment

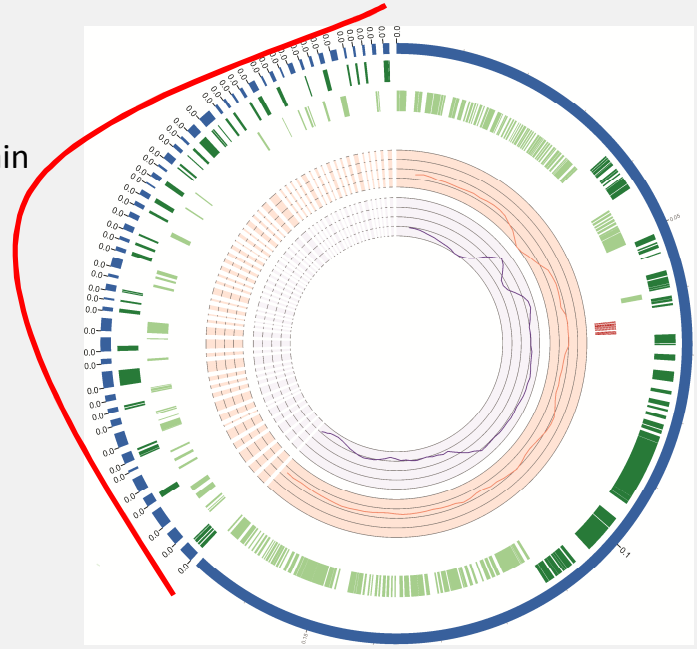


Samples Collected by Dr. Josh Santarpia

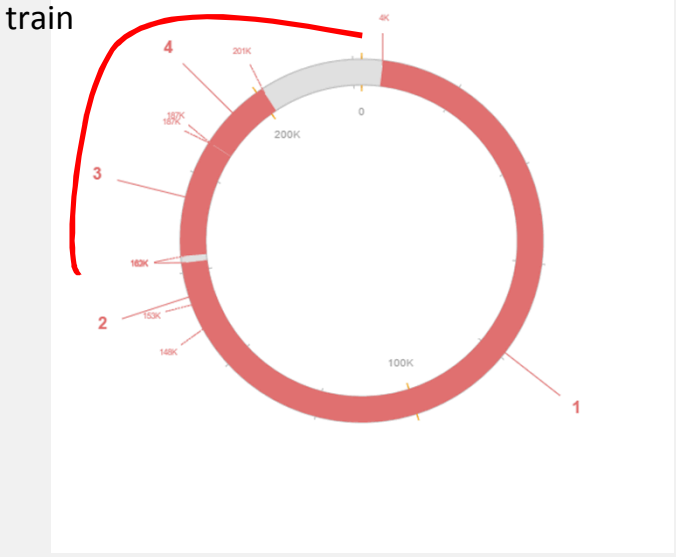
Environmental Isolation of Bacteriophage

- Isolated 2 new bacteriophage against *Burkholderia*
  - Isolated from samples collected in Louisiana (ME) and Arizona (AE)
  - Tested stability; currently purifying high titer stocks
  - Sequencing in progress
  - One is lytic and one appears to be lysogenic

CE-7 + train PATRIC annotation



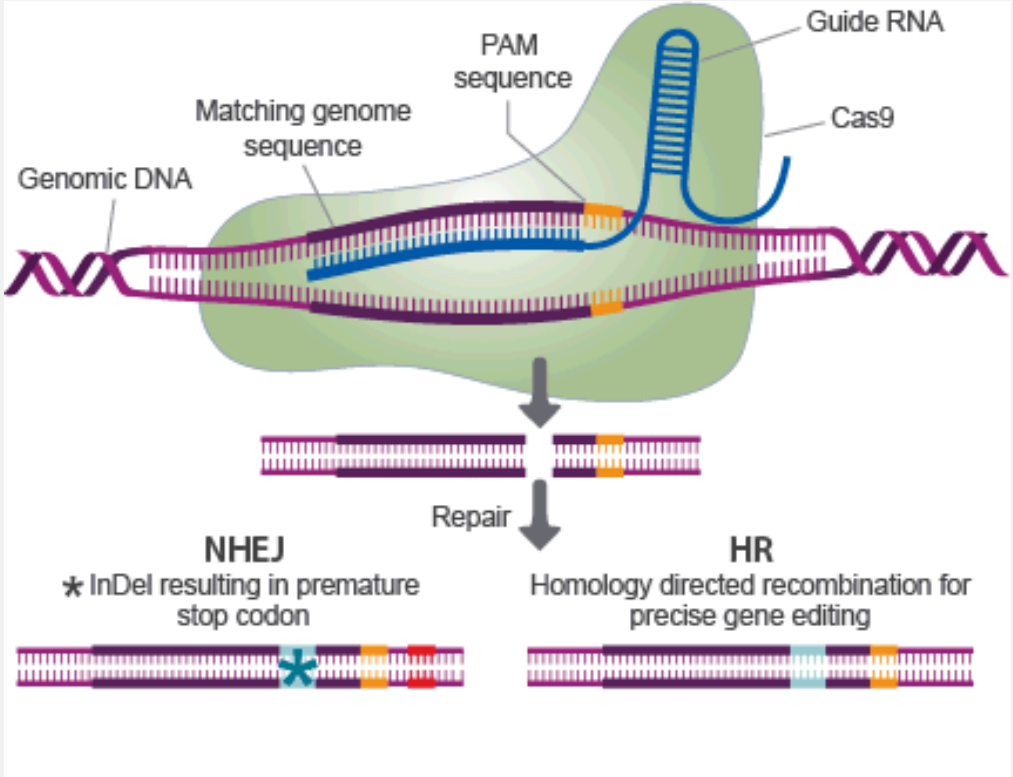
CE-6 + train PHASTER annotation



Sequencing Done by Dr. Yuriy Fofanov

CRISPR/Cas9 Gene Editing Technology

- CRISPR is a novel targeted gene-editing technology
  - Can introduce point mutations, delete genes, and add genes in specific genomic loci
- The technology uses an RNA-guided endonuclease to introduce a DNA break and relies on cellular DNA repair mechanisms to selectively edit genes



http://www.transomic.com/Products/CRISPR-Genome-Editing.aspx

Building a CRISPR/Cas9 Antimicrobial Library

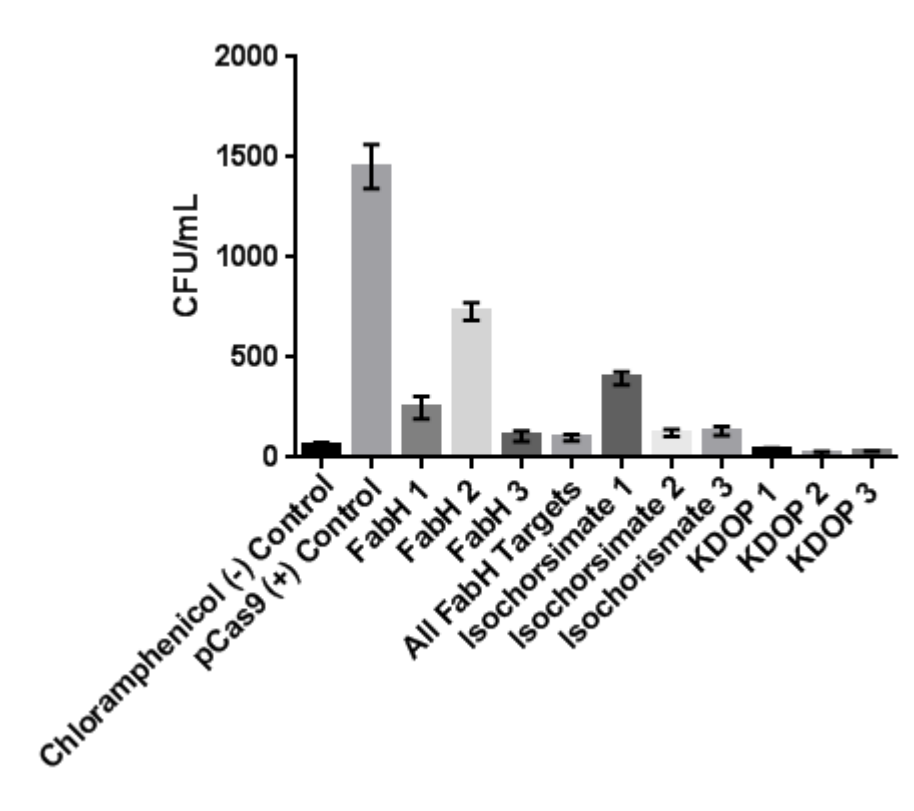
- Overall Goal: Use CRISPR/Cas as an antimicrobial to target intracellular bacteria using bacteriophage delivery
  - *Disrupt gene expression permanently*

- Target essential (genes required for viability) genes in *Burkholderia*, with no off target effects in humans or other organisms
  - FabH (fatty acid and phospholipid metabolism)
  - Isochorismate (iron uptake)
  - KDOP Synthase (synthesis/degradation of lipo- and polysaccharides)

- *Hypothesis: Using CRISPR/Cas9 to induce a double strand break in essential genes would result in cell death, thus acting as an effective "antibiotic"*

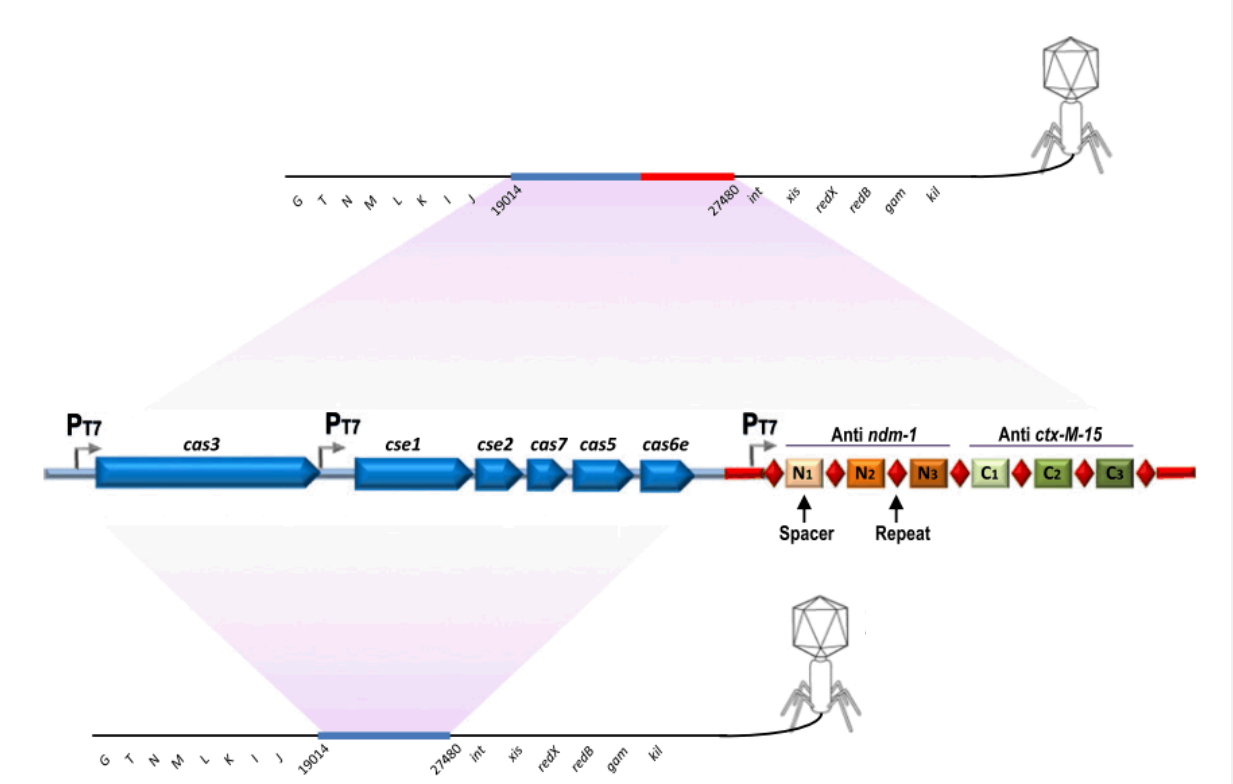
CRISPR Targets Kill *Burkholderia*

- Tested gene targets by directly delivering CRISPR/Cas9 components
  - Cloned sgRNA targets into vector expressing both Cas9 and sgRNA
  - Delivered plasmid encoding CRISPR and Cas9 to bacteria and monitored changes in viability
  - All CRISPR/Cas9 targets result in significant cell death



Putting it All Together: Encoding CRISPR/Cas9 Targets into Bacteriophage

- Next steps:
  - Genetically incorporate CRISPR/Cas system into bacteriophage genomes using molecular biology techniques

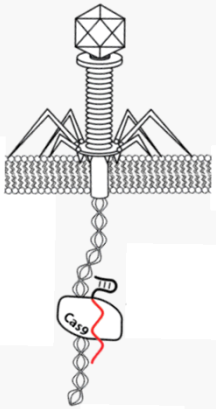


I. Yosef, *et al.*, PNAS, 2015, 112(23): 7267-72.

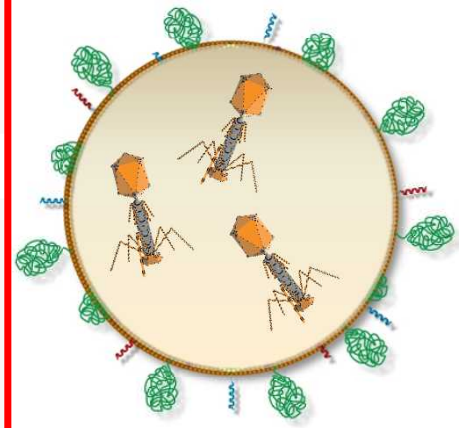
How to Target Resistant Bacteria

- Using protocells, we can package and deliver a variety of therapeutic cargos, including bacteriophage, to specific cell types

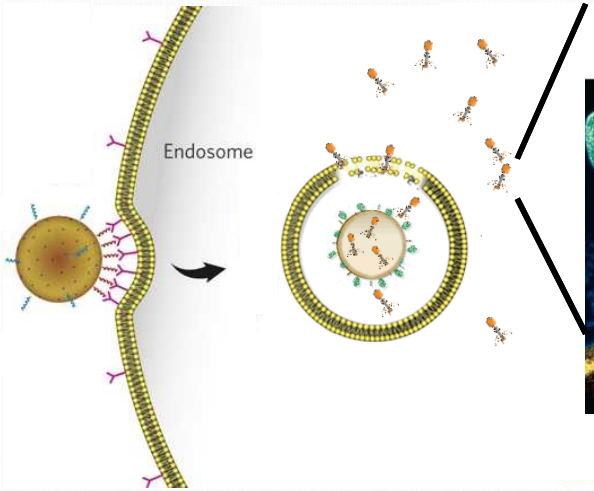
1. Genetically Encode Bacteriophage with CRISPR/Cas9



2. Encapsulate Bacteriophage into Nanoparticles



3. Deliver Bacteriophage to Bacterially-Infected Mammalian Cells



4. Bacteriophage Deliver CRISPR/Cas9 to Intracellular Bacteria

Protocell masks bacteriophage from initiating an immune response

Using Protocells to Deliver Therapeutic Cargoes

- Protocells are mesoporous silica nanoparticles (MSNP) that can be *easily* loaded with complex mixtures of cargo molecules (DNA, RNA, small molecules, proteins)
- Protocells have a 100-1000 fold higher capacity than other nanoparticles
- Properties of both the MSNP core and the bilayer shell can be precisely modulated to effect behavior

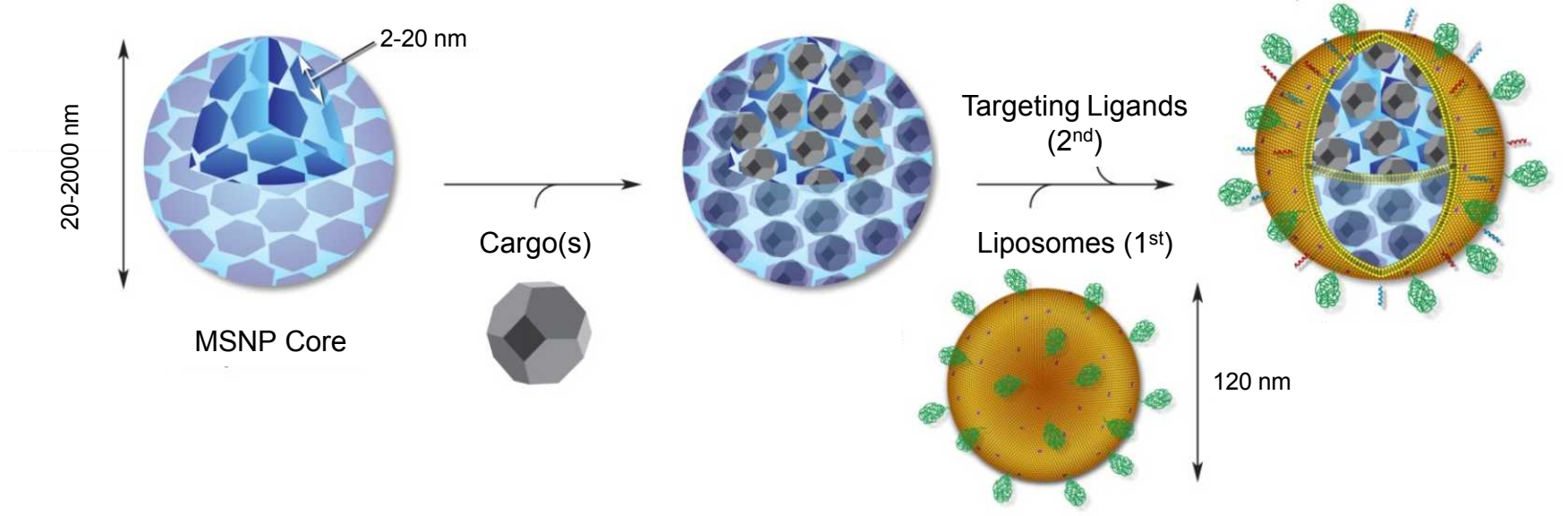


Image Courtesy of Dr. Eric Carnes

Using Protocells to Deliver Therapeutic Cargoes

- Functionalizing the supported lipid membrane with targeting, self and endolysmotic peptides, the protocells can:
  - Be selectively delivered to specific cell types
  - Escape the lysosome
  - Deliver the therapeutic cargo

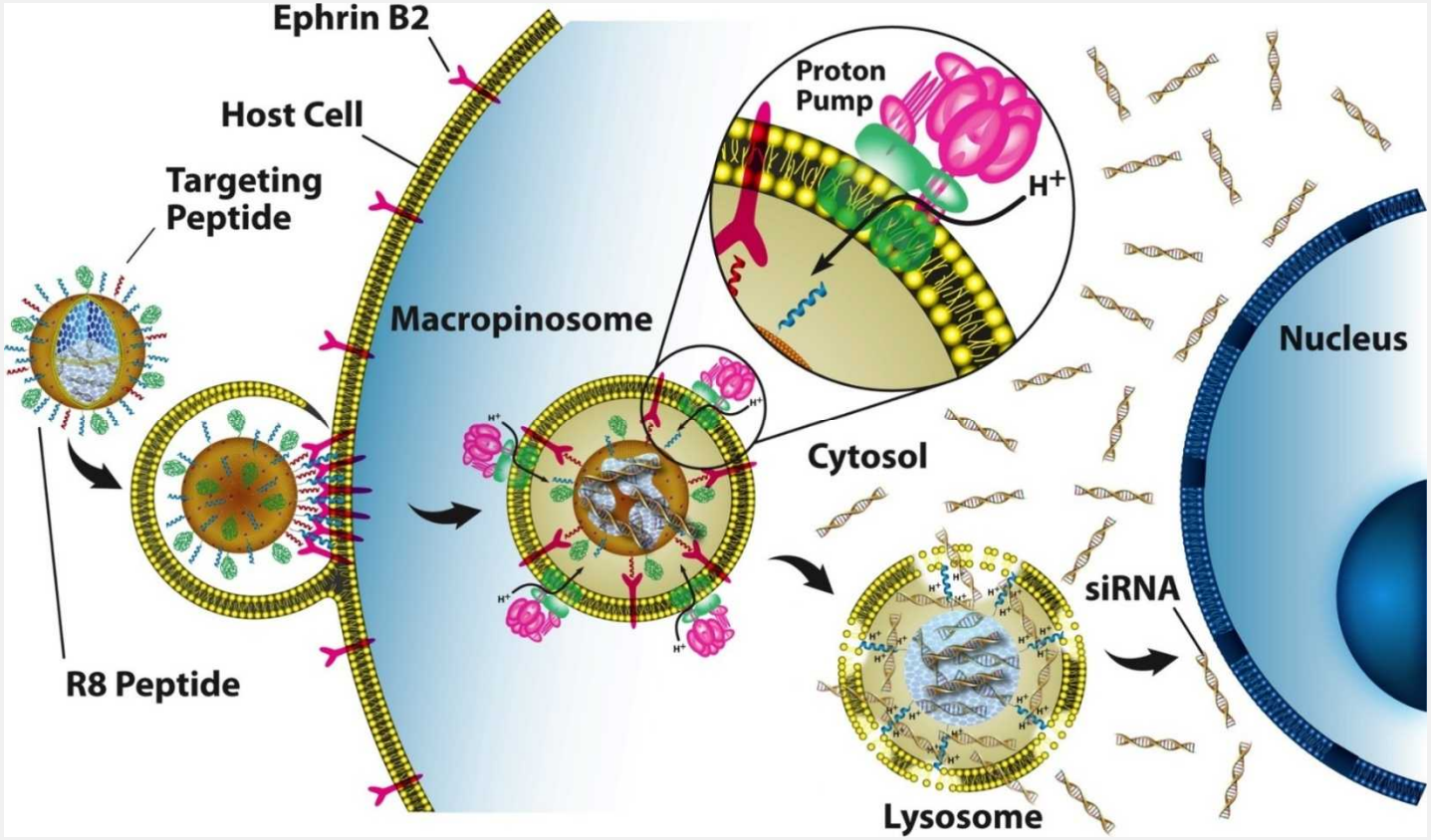
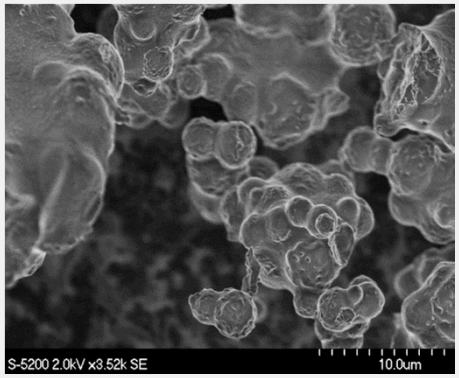


Image Courtesy of Dr. Eric Carnes and Dr. Carlee Ashley

Encapsulation of Bacteriophage into Protocells

- Tested encapsulation of bacteriophage
  - Bacteriophage were successfully encapsulated
  - Activity was maintained after release from the nanoparticle!



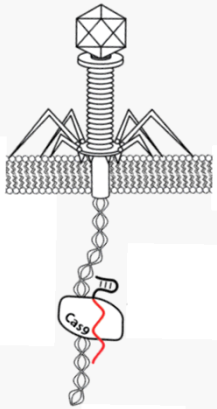
Phage	Initial Activity (pfu/mL)	Encapsulated Activity (pfu/mg)	Loss of Activity (Log pfu)
PL-4A	5.50E+10	1.05E+08	0.5
PL-4B	1.50E+10	4.00E+07	0.6
CE-6	2.38E+10	1.93E+06	2.0
CE-7	1.50E+10	6.50E+06	1.4

SEM Images: Dr. Eric Carnes

Next Steps

- Using protocells, we can package and deliver a variety of therapeutic cargos, including bacteriophage, to specific cell types

1. Genetically Encode Bacteriophage with CRISPR/Cas9



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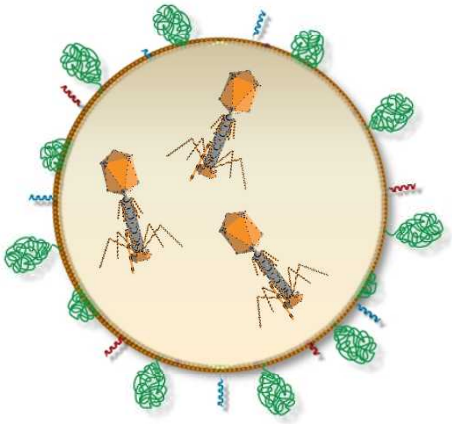

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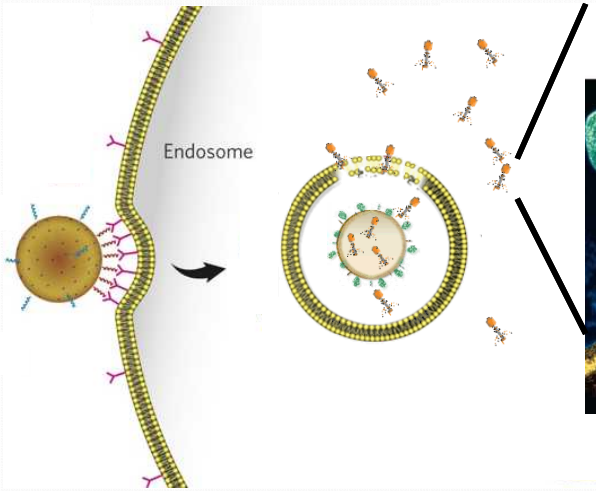
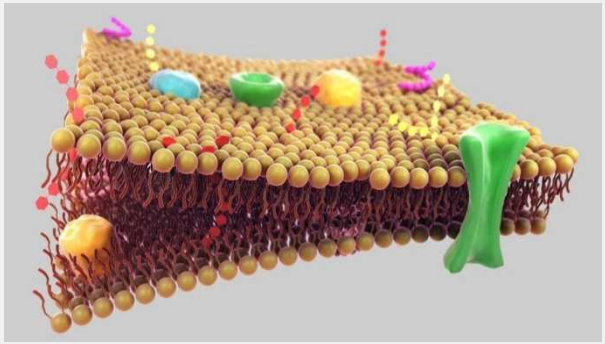
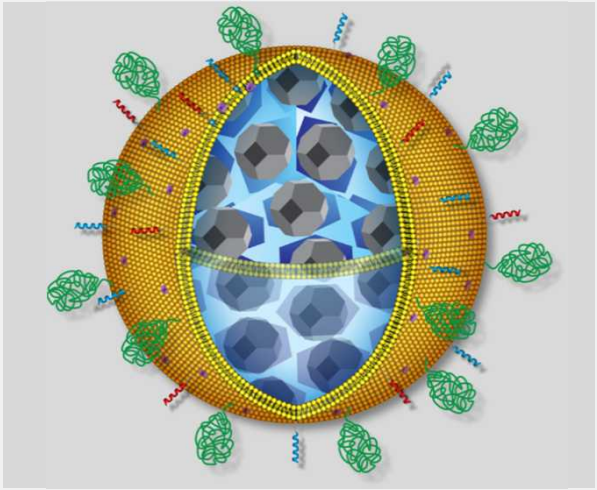


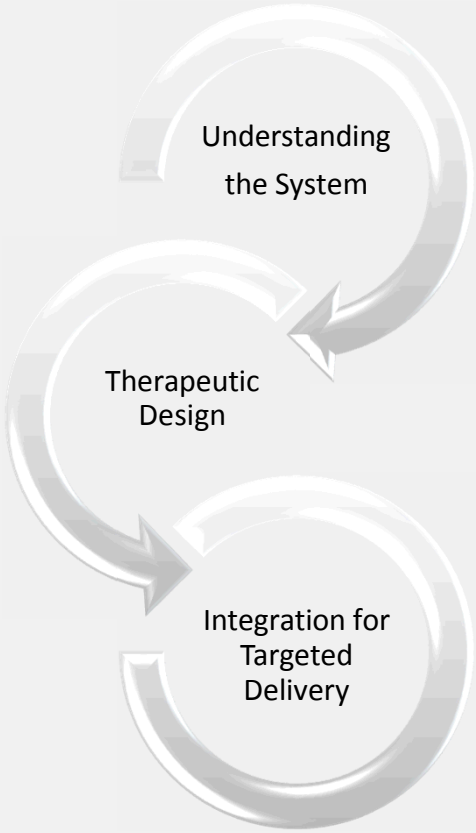
Protocell masks bacteriophage from initiating an immune response

Understanding Biological Systems for Integrated Science Applications

Goal: Understand biological systems for designing targeted therapeutics. We can couple our expertise with protocells and cancer cell biology to design and selectively target/deliver novel, more effective chemotherapies







Mouse Image: Courtesy of Dr. Carlee Ashley

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