High Resolution Fluorescence Imaging of *E. coli* Chemotactic Receptors

Original proposal

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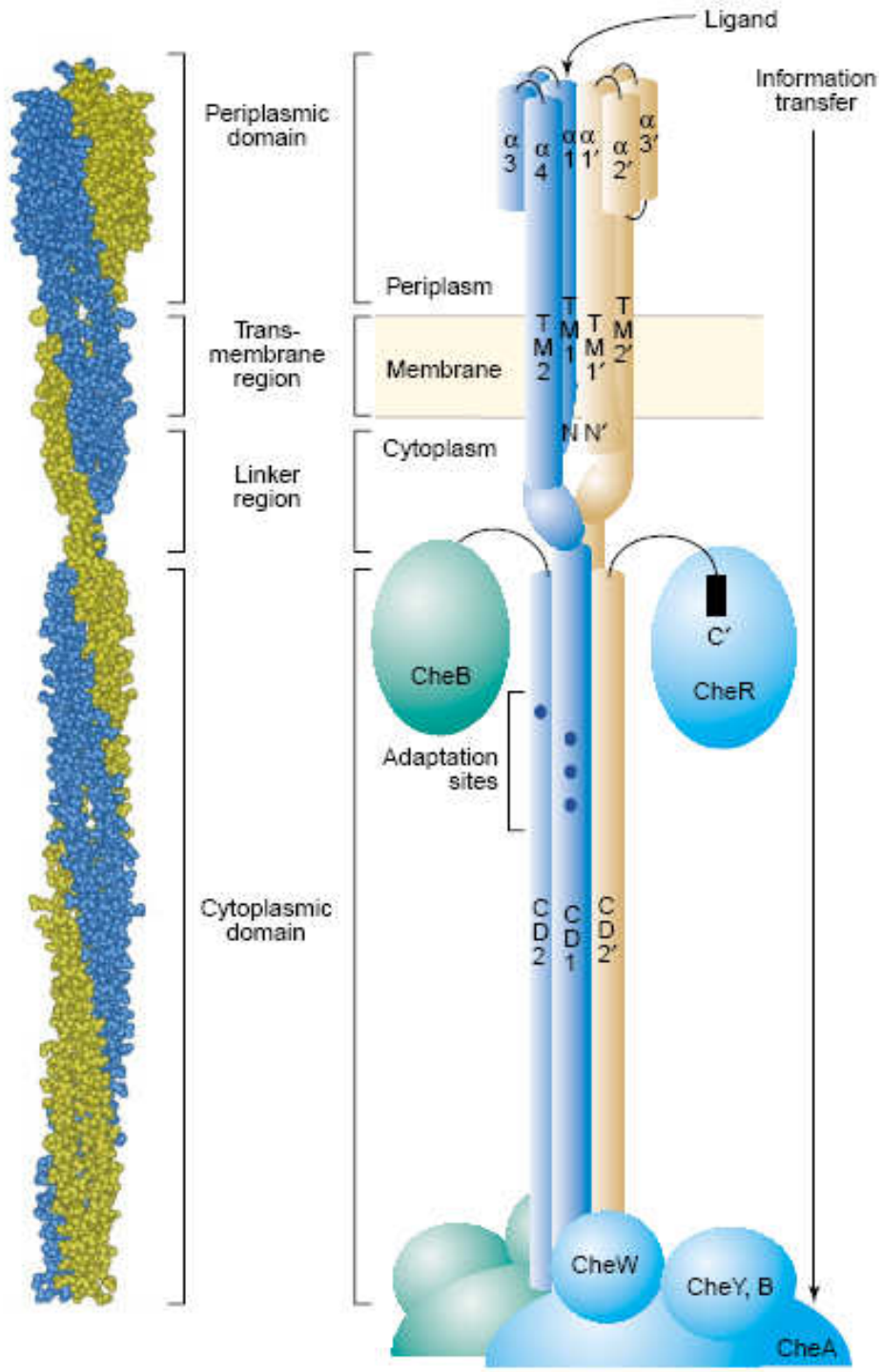
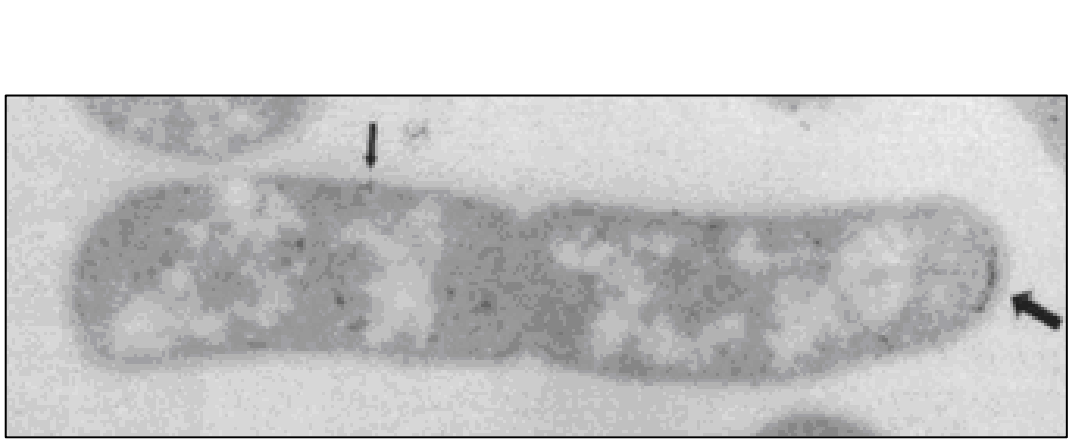
Chemotaxis Receptors (MCPs)

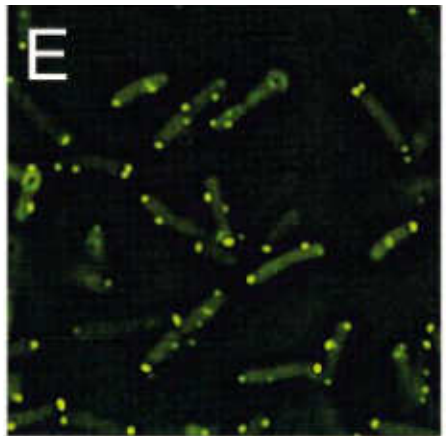
- **Chemotaxis:** directed movement of cells in response to external environment
- Transmembrane receptor proteins (MCPs) amplify the signal
- Five types of receptors: Tsr, Tar, Aer, Tap and Trg
- Proteins CheW and CheA form complexes with receptors, pass signal to CheY
- Receptors have dimeric structures



Chemotaxis Receptor Clusters

- Receptors form large clusters (~200 nm) in all bacteria
- Interactions between receptors likely plays a role in large (~100x) signal amplification
- Many experiments have been performed, giving several models of receptor interactions and organization
- Actual cluster organization hasn't been visualized
- High resolution (~10 nm) fluorescence experiments should be able to visualize receptor organization





Objectives

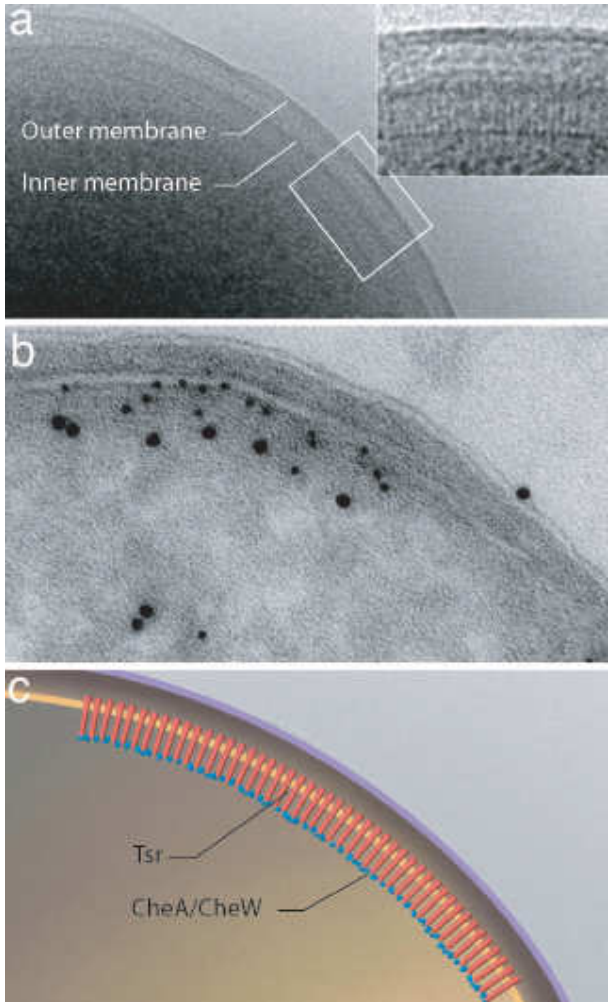
- *Visualize the lateral MCP receptor clusters* that are formed in *E. coli* cells with a PALM microscope
- Compare the obtained data to the models of receptor clustering that have been developed (e.g. trimer of dimers, hexagonal lattice, close packed lattice, hedgerow, etc.)
- Extend this method to other systems that demonstrate receptor clustering

Novelty

- Both receptor clustering and the PALM methodology have been well-established
- However, there has been *no known mention* of high resolution fluorescence possibilities in the literature
- Perhaps due to the relatively short lifetimes of these techniques or complexity of instruments
- However, in a recent email contact with Victor Sourjik he disclosed that his lab has considered starting to perform PALM based imaging in a collaboration with their physics department

Competing Technologies

- Other high resolution fluorescence techniques (e.g. NSOM, STED, SSIM, STORM, etc.)
- Cryoelectron microscopy

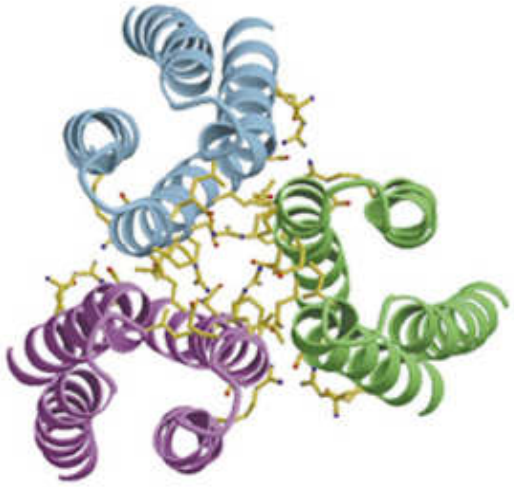


PNAS, 2007, 104, 3777

Receptor Cluster Models and Calculations

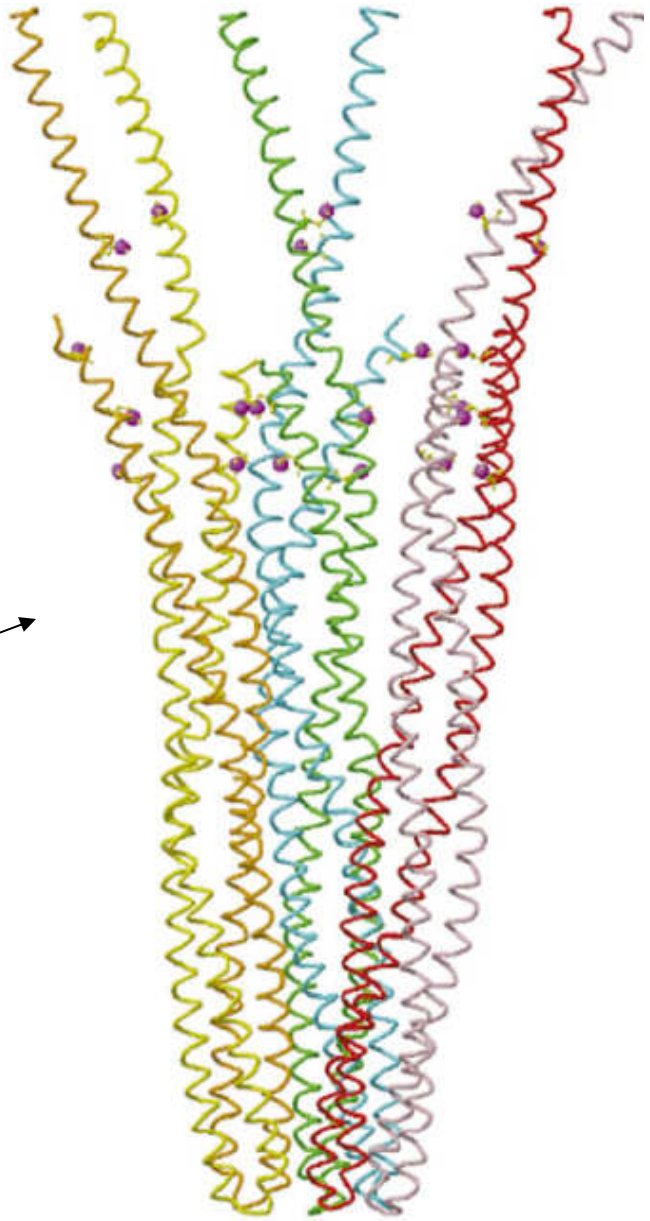
Trimer of Dimers, 1999

- X-ray crystal structures of the Tsr cytoplasmic domain showed that a *trimer of dimers* forms
- Trimers result from interactions of highly conserved signaling domains



Each dimer is different color

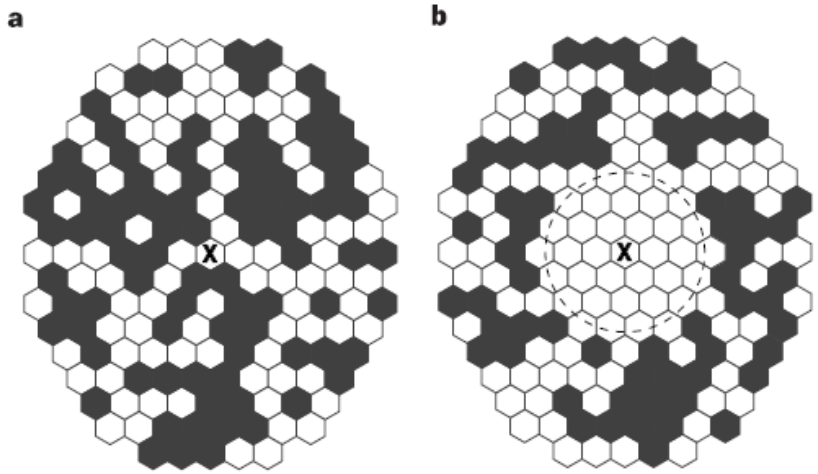
Each monomer is different color



Nature, 1999, 400, 787

Bray, 1998 & 1999

- Receptors are “active” or “inactive”
- “Activity” is likelihood of existing in active state
- Binding of ligand may affect receptor and surrounding receptors
- This would result in lower threshold of detection but narrower range of detection
- For a wide range, must be able to change aggregation or propagation with changes in land concentration

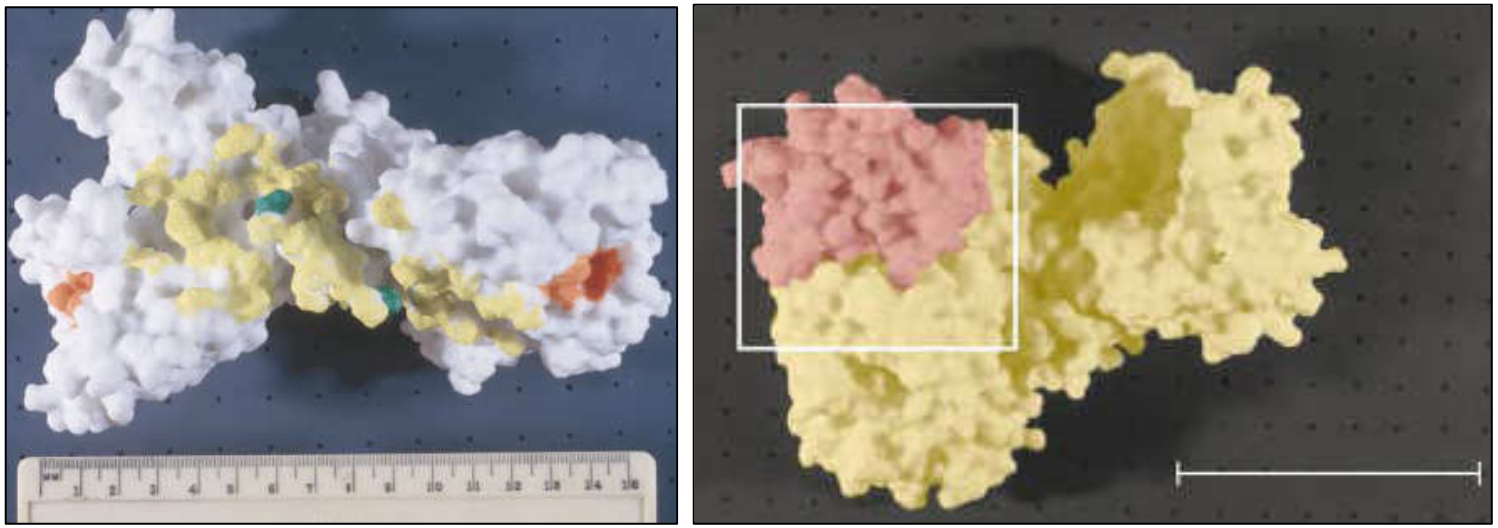


Bray, D., Levin, M.D., Morton-Firth, C.J., *Nature*, 1998, 393, 85

Duke and Bray, *PNAS*, 1999, 96, 10104

Bray, hexagonal lattice, 2000 (1)

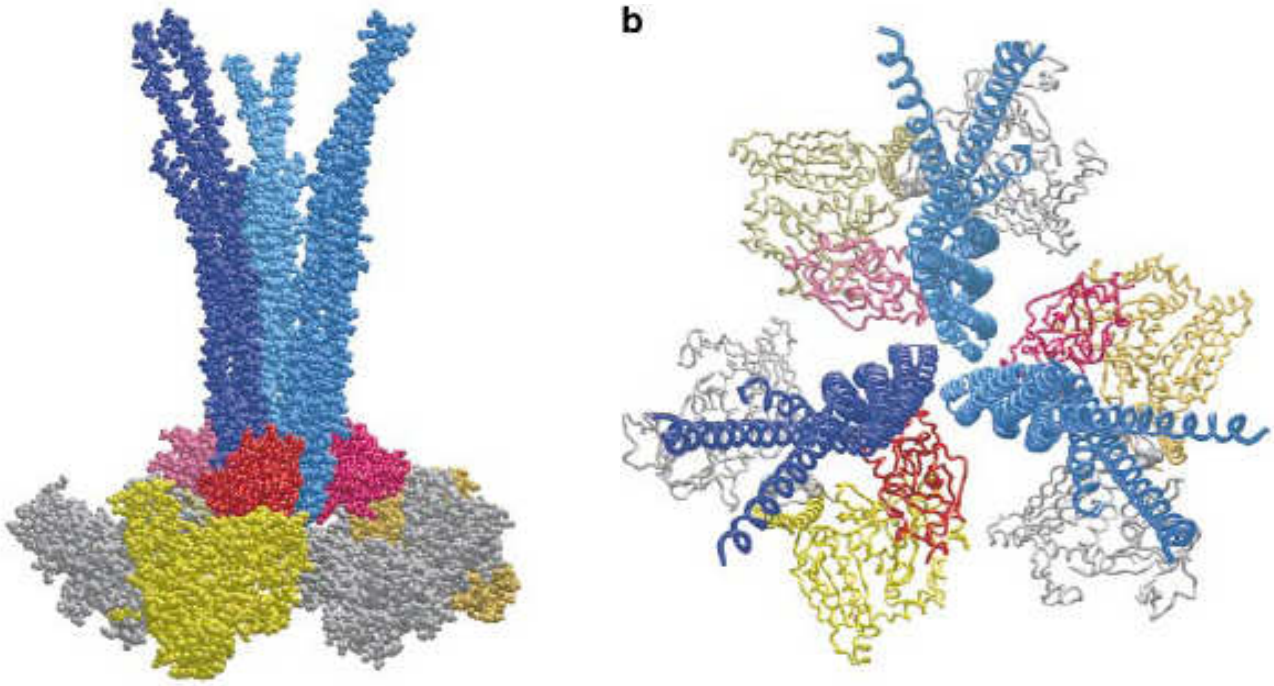
- Made solid models of CheA dimer, CheW and Tsr trimer of dimers structure
- First presumed that reactive sites of CheA would face into cytoplasm
- Used models to find likely positions of CheA-CheW binding and CheW-Tsr binding



Nature Cell Biol., 2000, 2, 792

Bray, hexagonal lattice, 2000 (2)

- Arranged three CheW-CheA complexes around a receptor “trimer of dimers” to form a “trigonal vault”

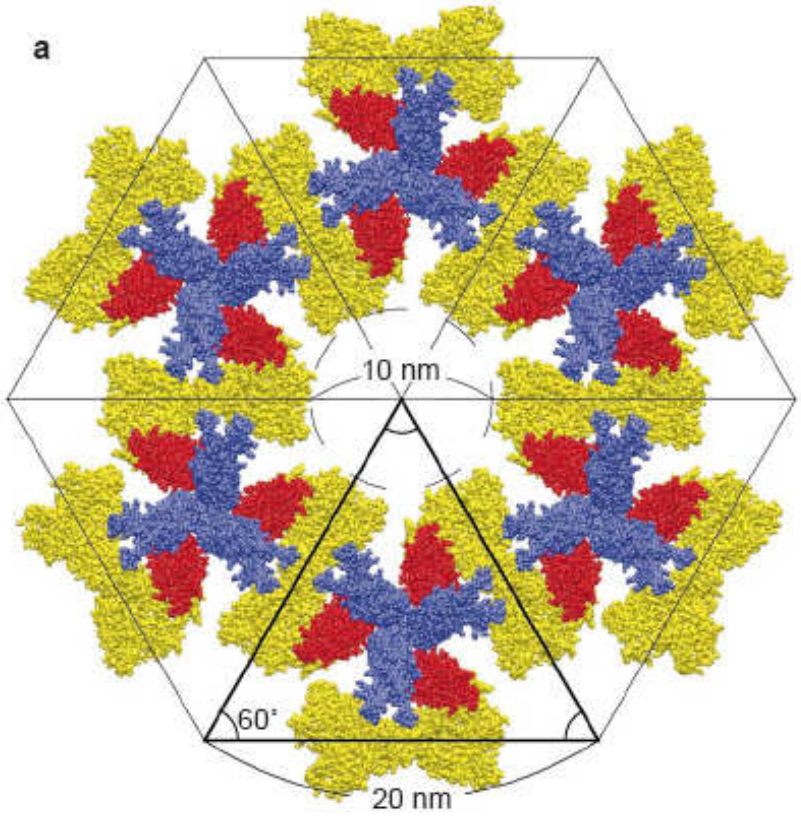


Receptors (blue)
CheW (red)
CheA monomer (yellow)
CheA from other subunits (gray)

Nature Cell Biol., 2000, 2, 792

Bray, hexagonal lattice, 2000 (3)

- The “vault” can be arranged in a repeating **hexagonal lattice** via CheA dimer formation between vaults
- CheA interactions may allow for communication between receptors

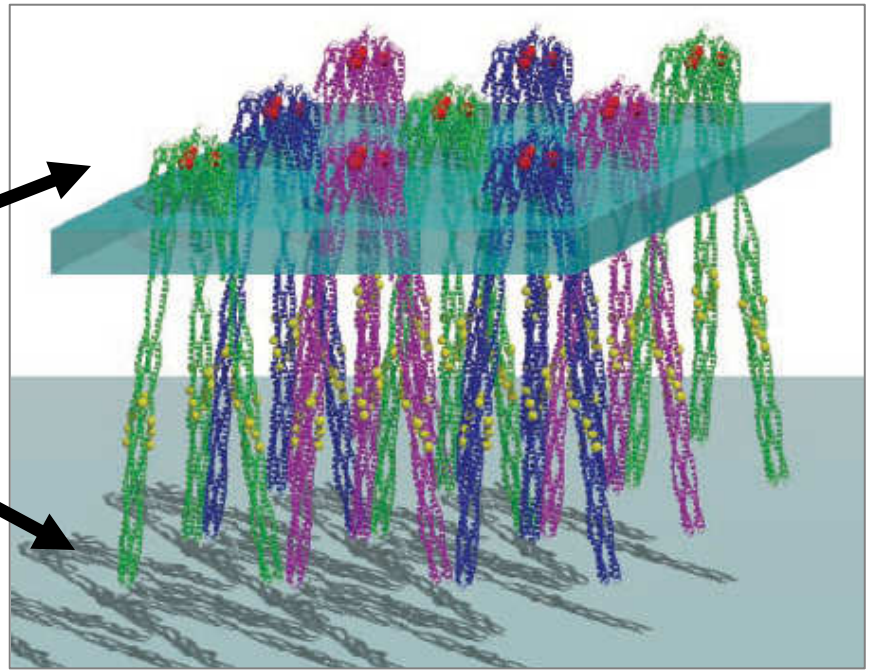


Receptors (blue)
CheW (red)
CheA (yellow)

Nature Cell Biol., 2000, 2, 792

Kim, trussed slab, 2002

- Propose 2-d “trussed slab” in which each receptor dimer interacts with two other dimers via the “trimer of dimers” arrangement, but also interacts with two more dimers via its ligand-binding domain
- Propose a **close-packed arrangement**

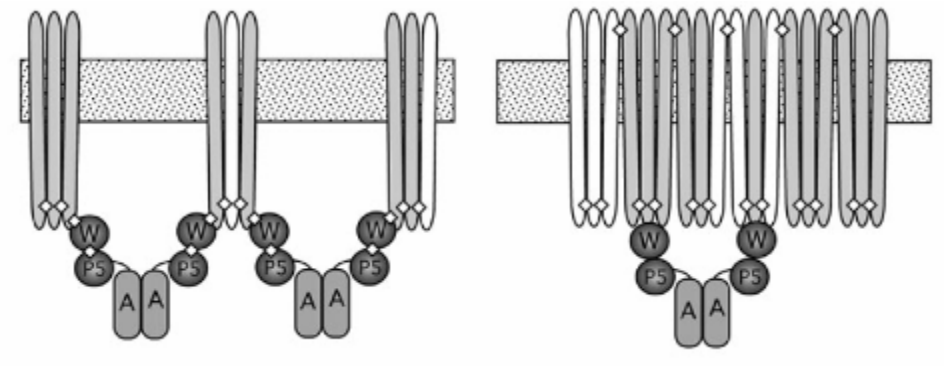


PNAS, 2002, 99, 11611

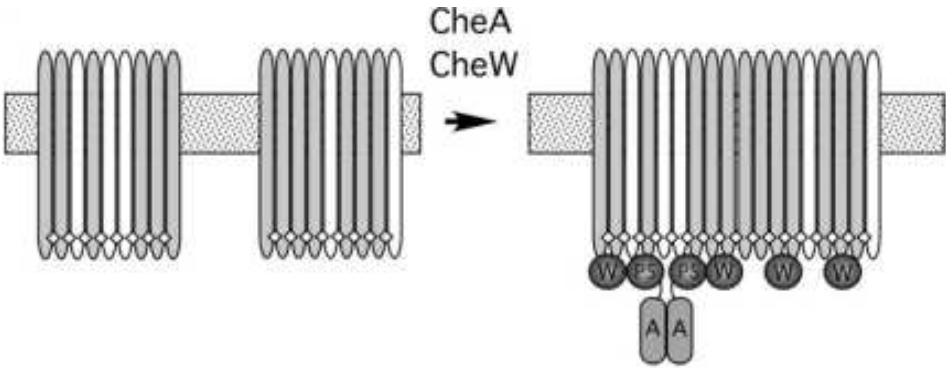
Kentner, signaling domains, 2006

- Results suggest that signaling domains of receptors are the vital components for receptor clustering
- CheA and CheW may just tighten these clusters
- Contrast with models that suggest CheA interactions or periplasmic receptor interactions as means of cluster organization

Previous models



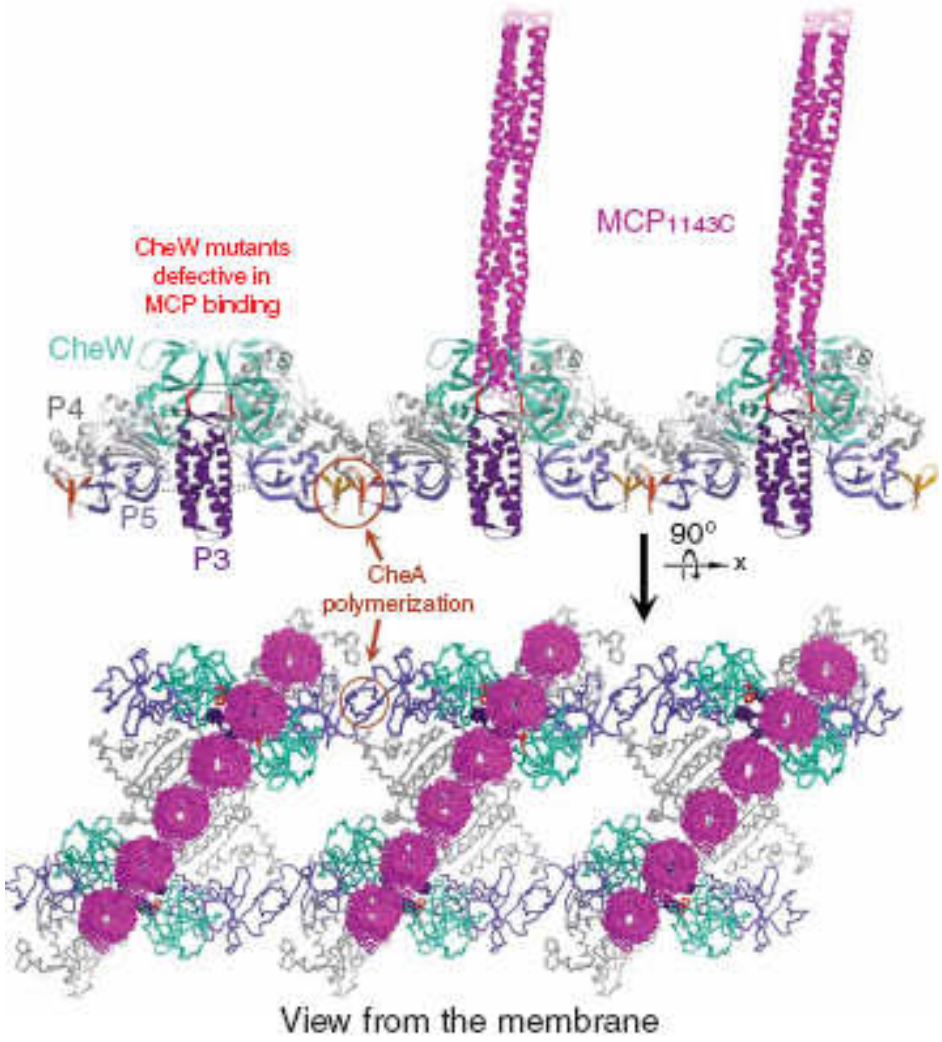
new model



Molec. Microbiol., 2006, 61, 407

Park, hedgerows, 2006

- Obtained crystal structure of MCP from *T. maritima* bacteria
- Suggested the MCPs may form “hedgerows” that combine with each other via CheA interactions



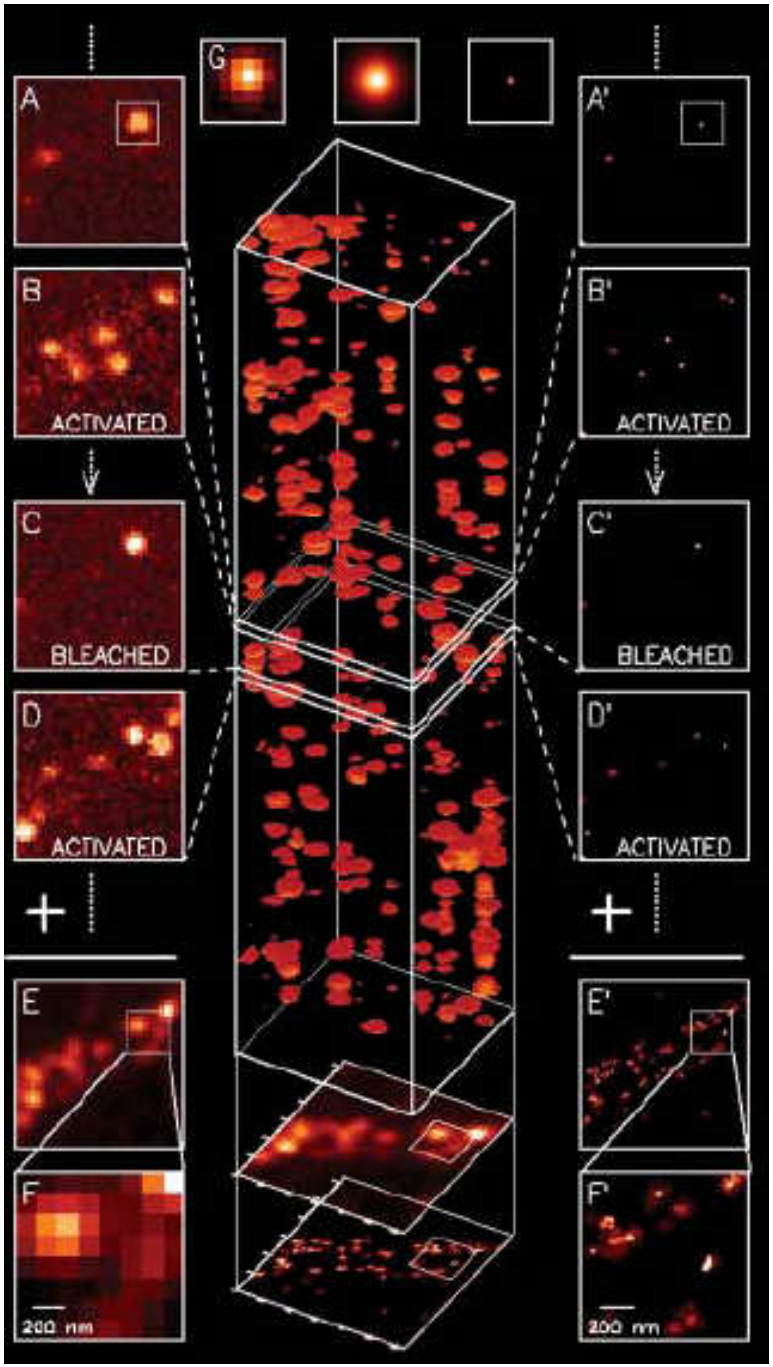
Green: CheW
Gray: CheA
Purple: receptors

Nature Struct. Molec. Biol., 2006, 13, 400

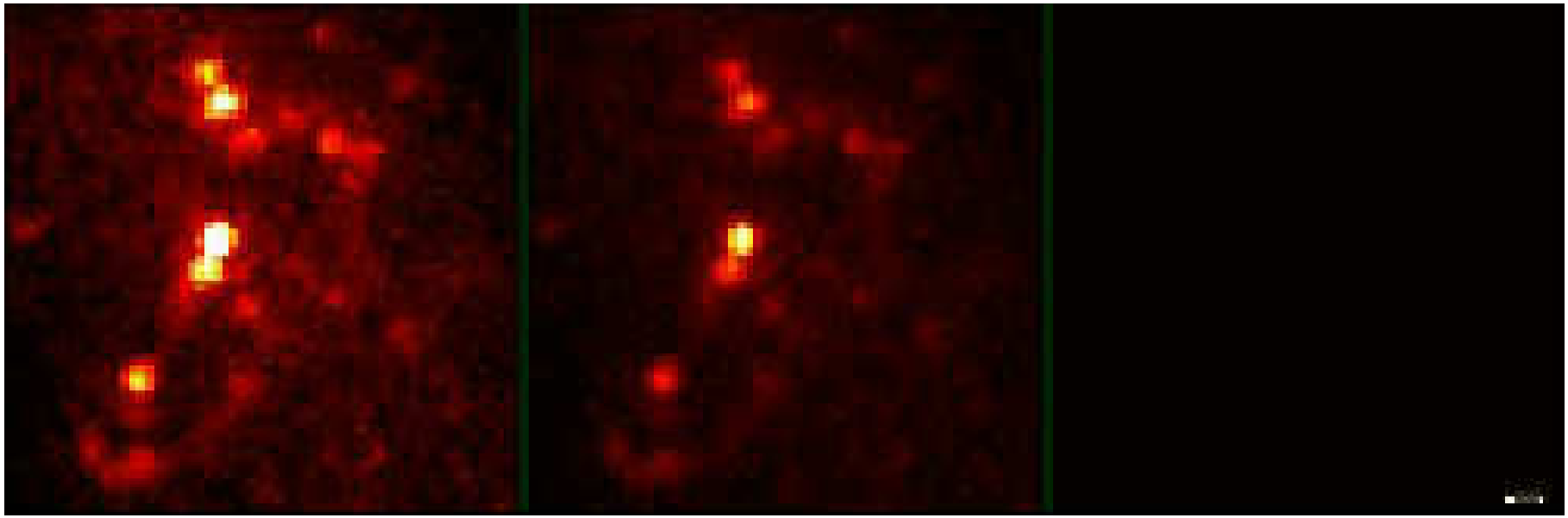
Construction of PALM microscope

PALM methodology

- Continually apply excitation light
- Activate small number of tags with short, low intensity laser pulse (~400 nm)
- Collect photons until photobleached and statistically pinpoint locations
- Apply another activation pulse and pinpoint more locations
- Repeat many times and add up the locations to form a high resolution image



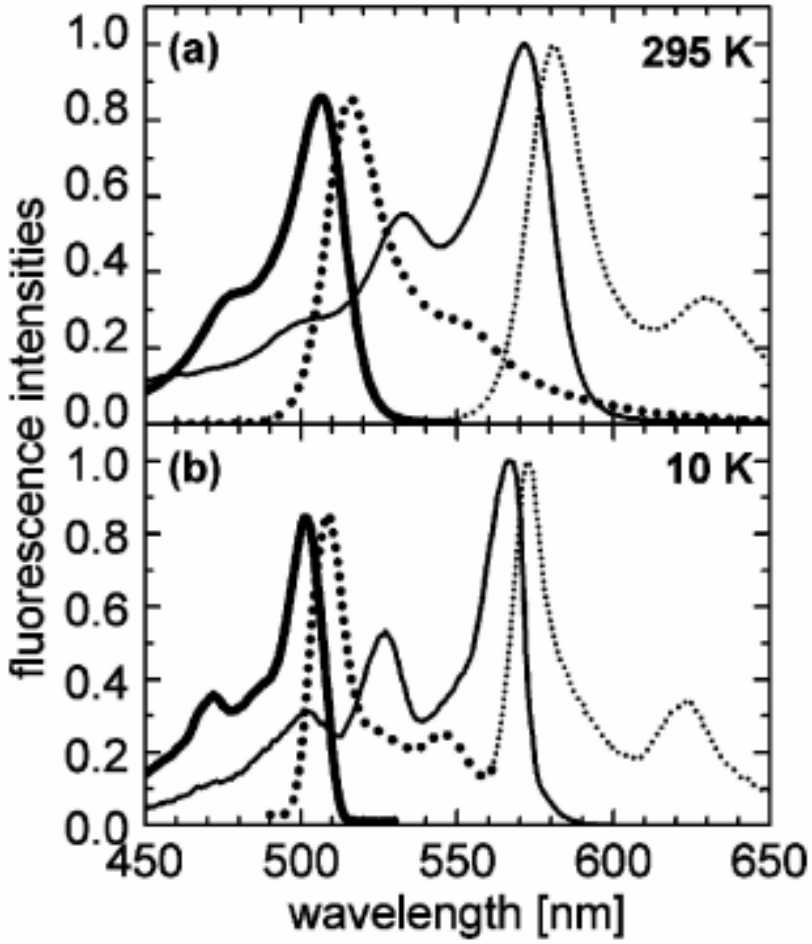
PALM methodology (2)



Capable of ~10 nm resolution

Photoactivatable Tag: EosFP (1)

- From stony coral *Lobophyllia hemprichii*
- UV activation (~400 nm) changes emission from 516 to 581 nm
- Wild-type exists in tetrameric form
- Formed dimer by changing V123 to threonine
- Formed monomer by changing T158 to histidine

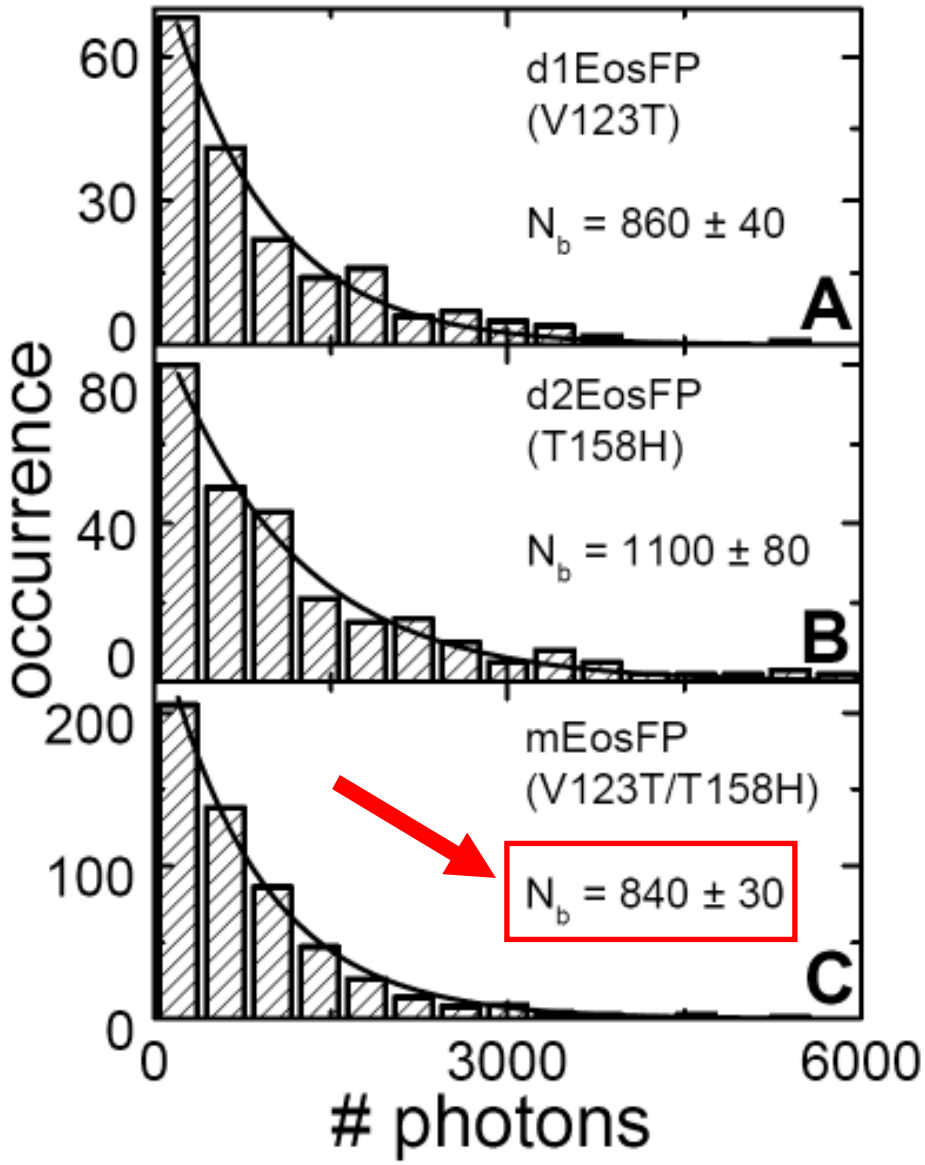


Solid: absorbance; dotted: emission
Thick: normal, thin: activated

PNAS, 2004, 101, 15905
J. Biol. Phys., 2005, 31, 249

Photoactivatable Tag: EosFP (2)

- Determined average number of detected photons from activated oligomeric forms of EosFP
- ~840 photons detected on average before bleaching (estimated typical 0.04 detection efficiency)



PNAS, 2004, 101, 15905
J. Biol. Phys., 2005, 31, 249

Design Considerations

- PALM pinpoints positions of individual activated fluorophores
- Mean squared position error is:

$$\left(\sigma_x^{\,2}\right) \approx \frac{r_0^{\,2} + q^2/12}{N} + \frac{8\pi r_0^{\,4} b^2}{q^2 N^2}$$

r_0 = diff. limited resolution

q = pixel size

N = # photons collected

b = # background photons collected

- For highest possible resolution, want to maximize number of photons collected and minimize background noise
- Also must minimize sample drift

Science, 2006, 313, 1642 | *Biophys. J*., 2006, 91, 4258

TIRF geometry

- Increases signal to noise ratio
- Light striking the coverslip-sample interface at an angle greater than the critical angle will create an evanescent field that extends a *few hundred nanometers* into the sample

Characteristic decay depth

$$d = \frac{\lambda}{4\pi n_2}\left(\frac{\sin^2\theta}{\sin^2\theta_c} - 1\right)^{-1/2}$$

Field strength

$$I = I_0 e^{-z/d}$$

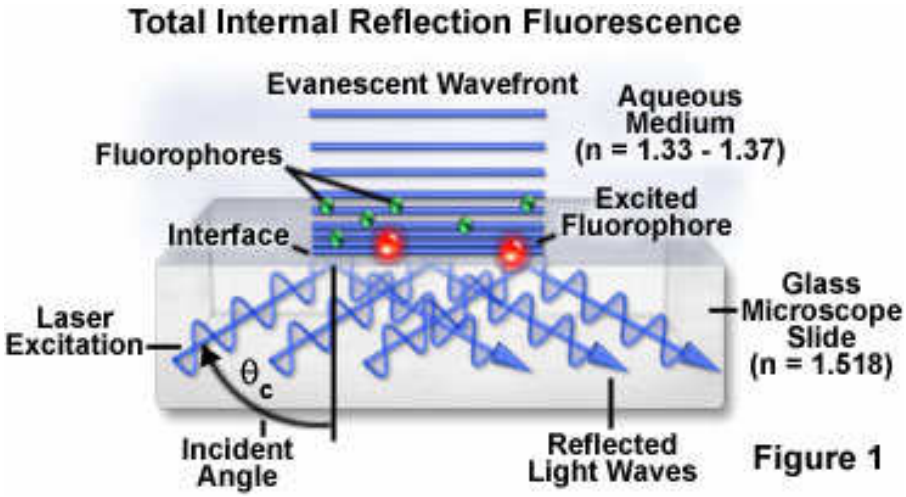
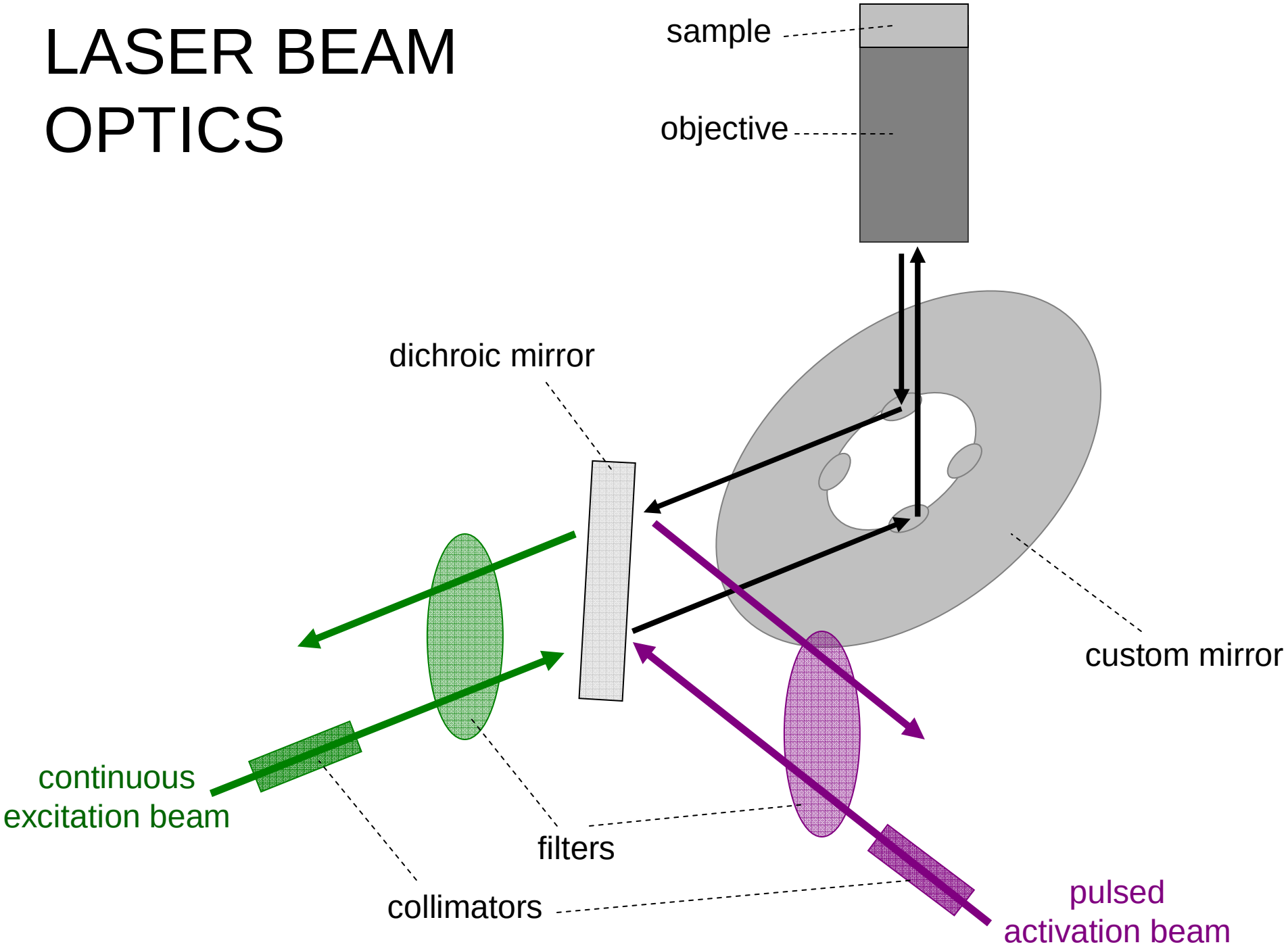


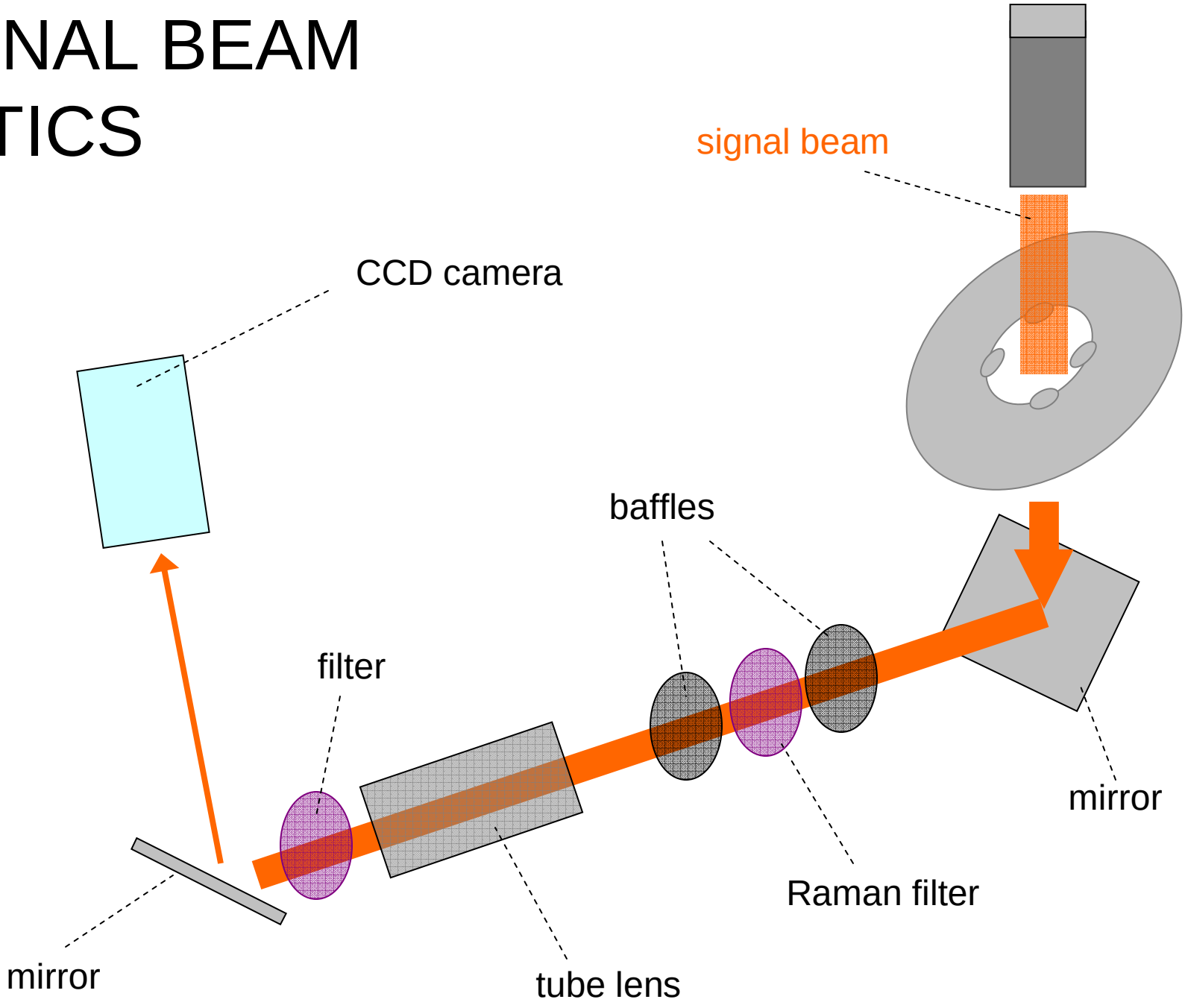
Figure 1

http://www.microscopyu.com/articles/fluorescence/tirf/tirfintro.html

LASER BEAM OPTICS



SIGNAL BEAM OPTICS



Appropriate Pixel Size

- Ideal pixel size is comparable to the standard deviation of the PSF (for best resolution in PALM)
- Use tube lens with ~400 nm focal length to achieve this.
- Such a lens gives overall magnification of:

$$f_{\text{tube lens}}/f_{\text{objective}} = [400\text{nm}/(180\text{nm}/60)] = 133$$

- With 16 micron pixels on detector, effective pixel size is 120 nm
- Since detector is 512x512 pixels, field of view is 61 microns

Resolution Estimation (1)

$$\left(\sigma_x^{\,2}\right) \approx \frac{r_0^{\,2} + q^2/12}{N} + \frac{8\pi r_0^{\,4} b^2}{q^2 N^2}$$

r_0 = diff. limited resolution
q = pixel size
N = # photons collected
b = # background photons collected

$$f_0 \approx \frac{0.55\lambda_{emission}}{NA} = \frac{0.55\left(580\cdot 10^{-9}\, m\right)}{1.4} = 2.28\cdot 10^{-7}\, m$$

$$r_0 \approx f_0\sqrt{2.2\ln 2} = 2.28\cdot 10^{-7}\, m\cdot 1.235 = 2.82\cdot 10^{-7}\, m$$

N= 8.4E2 (from EosFP papers)

q = 1.2E-7 m (previous slide)

b = ~1.02 photons/frame/pixel (from PALM papers)

Resolution Estimation (2)

$$\left(\sigma_x^{\,2}\right) \approx \frac{r_0^{\,2} + q^2/12}{N} + \frac{8\pi r_0^{\,4} b^2}{q^2 N^2}$$

r_0 = diff. limited resolution
q = pixel size
N = # photons collected
b = # background photons collected

$$\left(\sigma_x^{\,2}\right) \approx \frac{\left(2.82\cdot 10^{-7} m\right)^2 + \left(1.20\cdot 10^{-7} m\right)^2/12}{\left(8.4\cdot 10^2\right)} + \frac{8\pi\left(2.82\cdot 10^{-7} m\right)^4 (1.02)^2}{\left(1.20\cdot 10^{-7} m\right)^2 \left(8.4\cdot 10^2\right)^2}$$

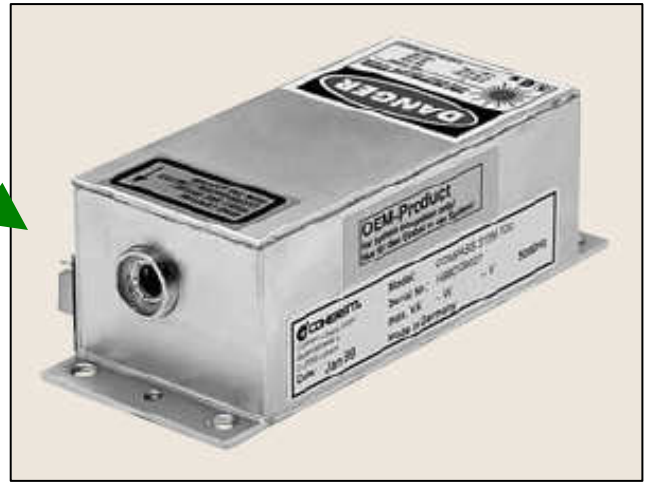
$$\left(\sigma_x\right) \approx 10nm$$

- On average, location can be pinpointed with 10 nm accuracy
- Should be sufficient for comparison of data to proposed models.

Instrument Components

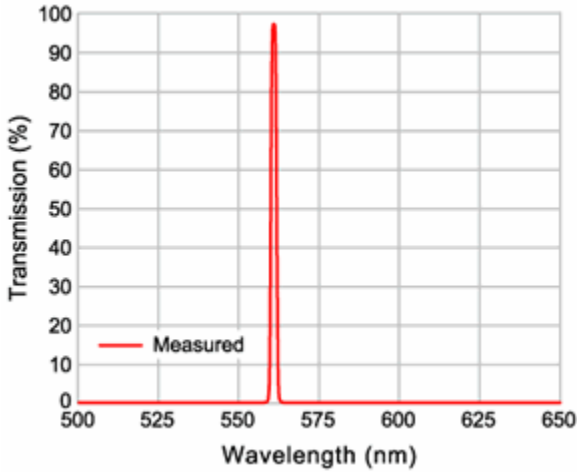
Lasers

- The monomeric form of EosFP will be utilized
- Lasers with appropriate wavelengths are necessary (405 nm and 561 nm)
- **Activation:** 50 mW, 405 nm CW diode laser from Coherent, Inc. (Compass 561)
- **Excitation:** 10 mW, 561 nm pulsed diode laser from Coherent, Inc. (Compass 405)
- Collimators from Coherent, Inc. are coupled to the lasers



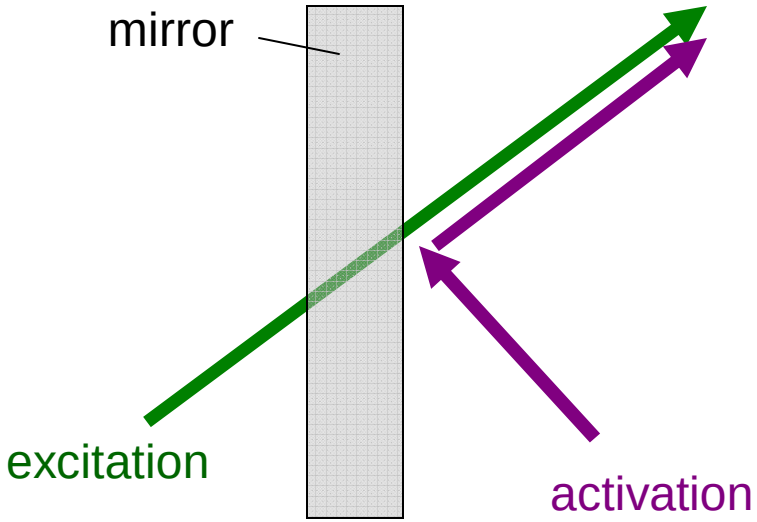
Line Filters

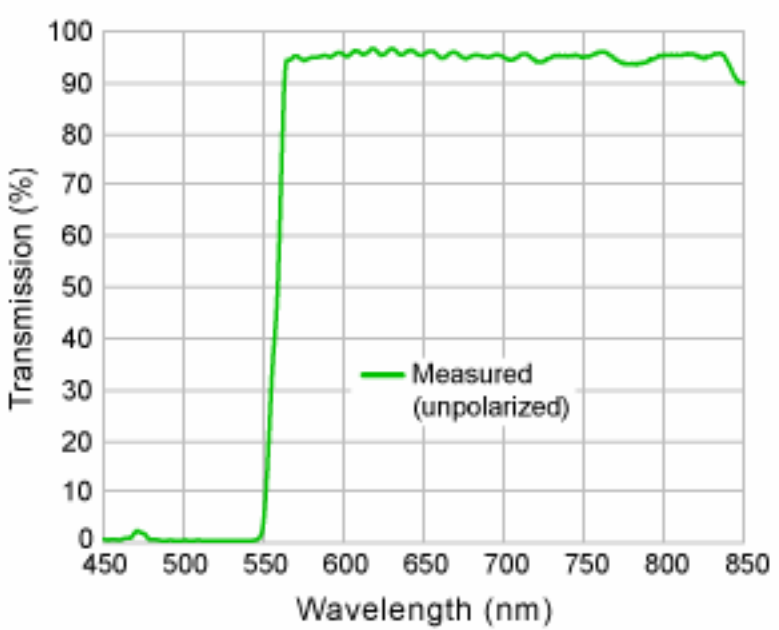
- 405 nm filter from CVI Laser (RNF-405.0) eliminates emission noise and autofluorescence from the activation laser
- 561 nm filter from Semrock performs the same tasks on the excitation beam



Dichroic Mirror

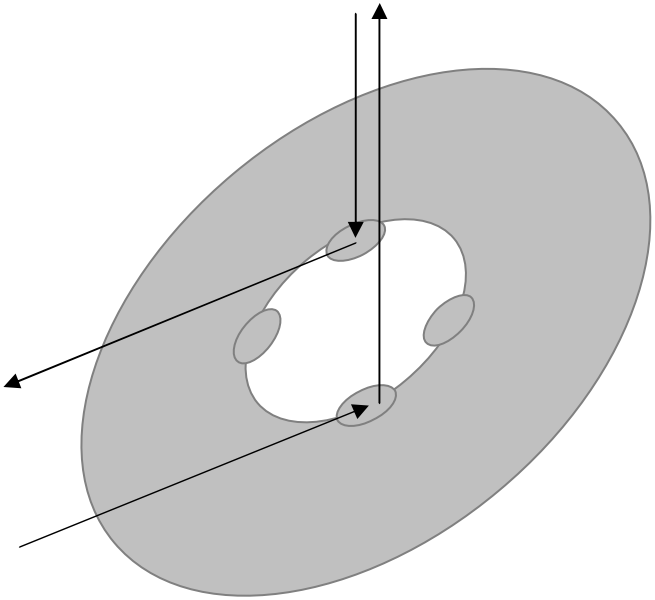
- FF560-Di01 from Semrock reflects the activation and passes the excitation
- This puts the beams on the same path



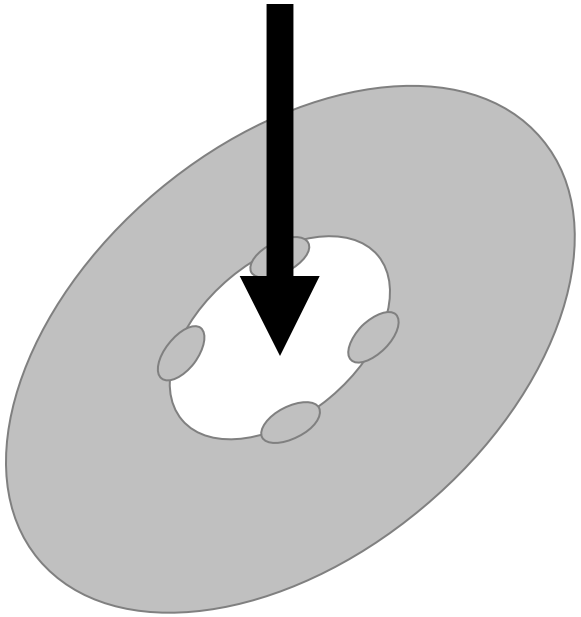


Custom Mirror

- Custom-made aluminized mirror by Reynard Corp.
- Reflects the input and output beams from elliptical spots
- Has a elliptical aperture which the signal beam passes through (projects as circle with same diameter as rear pupil)
- Transfers with high efficiency and is wavelength-independent



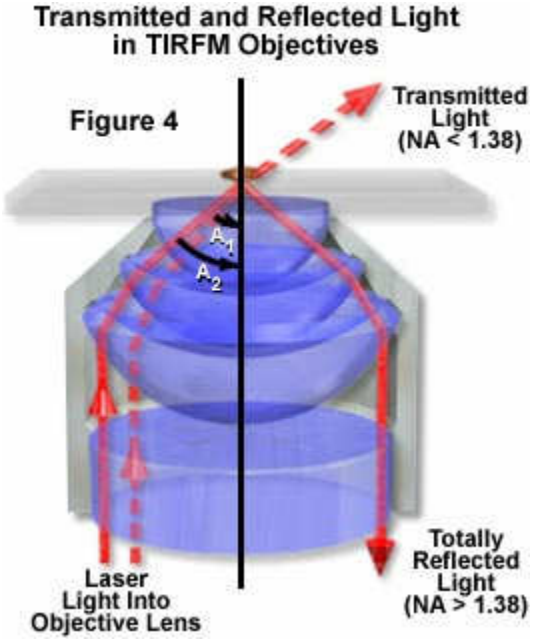
excitation/activation



signal

Total Internal Reflection Fluorescence (TIRF) Objective

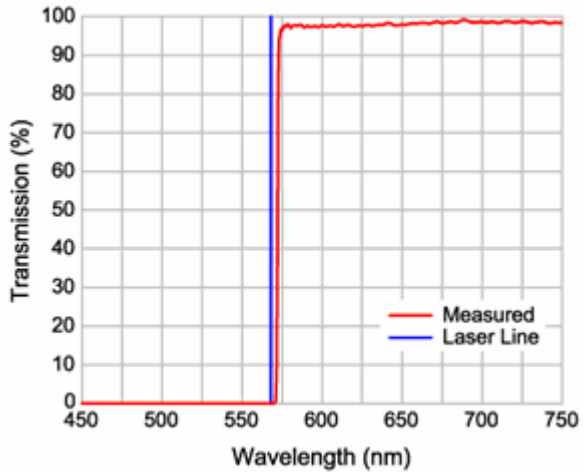
- Olympus 60x or 150x 1.4NA
- Brings input beam in at sufficient radius to strike the sample-cover slip interface at an angle greater than the critical angle
- Produces evanescent field in sample
- At 250 nm into the sample, field intensity drops to ~40%
- Use low autofluorescence immersion oil from Structure Probe Inc.



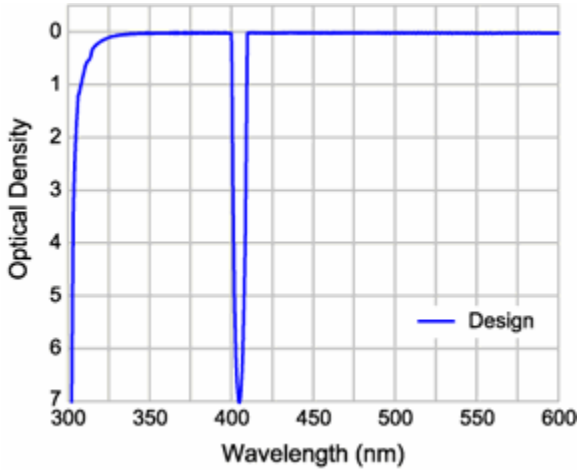
Signal Beam

- Emitted light from EosFP is at ~581 nm with higher wavelength shoulder
- Mirror redirects to detection optical axis
- Raman edge filter from Semrock filters out activation/excitation wavelengths
- Baffles remove high incident angles
- Achromatic tube lens from Edmund Optics focuses the beam
- Notch filter from Semrock further ensures that activation light is removed

edge filter



notch filter



Detector

- Signal finally impinges on electron-multiplying CCD camera from Andor Technology
- 512 x 512 pixels
- 16 micron pixel size
- Cooled to -50℃

Microscope Tube

- Sample holder, objective and optics are all mounted onto a cylindrically symmetric tube
- This minimizes sample drift during image acquisition
- XYZ stage will allow appropriate positioning of the sample relative to the objective

Sample Preparation

E. coli strains

- The RP437 strain has been utilized by the Sourjik lab as a wild-type for chemotaxis.
- RP437 samples with in-frame deletions of *tar* and/or *tsr* genes will be obtained from his laboratory (or from Molecular Biology Resources)
- The fusions of mEosFP to Tar and Tsr will be expressed in place of the deleted genes

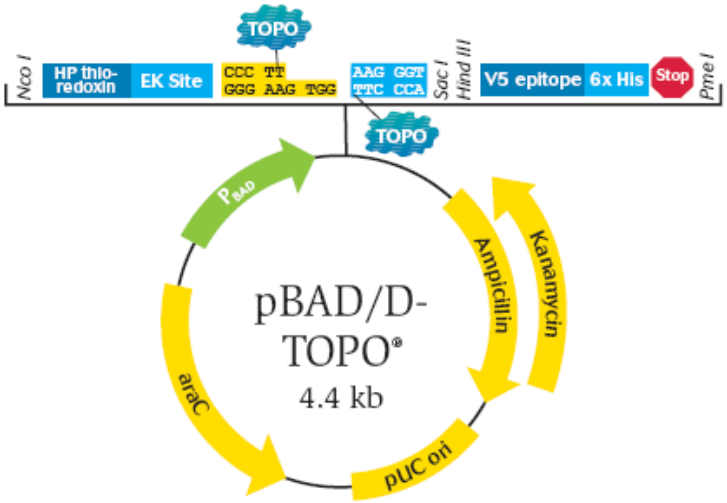
J. Bacteriol., 1982, 151, 106

Fusion Plasmid Construction

- The lab of Dr. Jorg Wiedenmann will provide the plasmid pcDNA3-Flag1 EosFP T158H/V123T
- Amplify cDNA from this plasmid with N-terminal primer that encodes for a 5 glycine overhang
- Amplify DNA of Tsr or Tar gene with C-terminal primer having complementary 5 glycine overhang
- Anneal the amplified DNAs together by their 5 glycine linker regions

Fusion Plasmid Construction (2)

- Amplify fusion gene with PCR.
- If using PBAD18K from Sourjik's lab, use primers with SacI or XbaI restriction sites
- If using pBAD TOPO vectors from Invitrogen, use CACC as 5' primer overhang
- Anneal the amplified gene with the plasmid



E. coli Transformation

- Used incorrect “transient transfection” term in the written proposal
- Cells are rather *transformed* by weakening walls with $CaCl_2$
- The fusion plasmids are then taken up by the cells

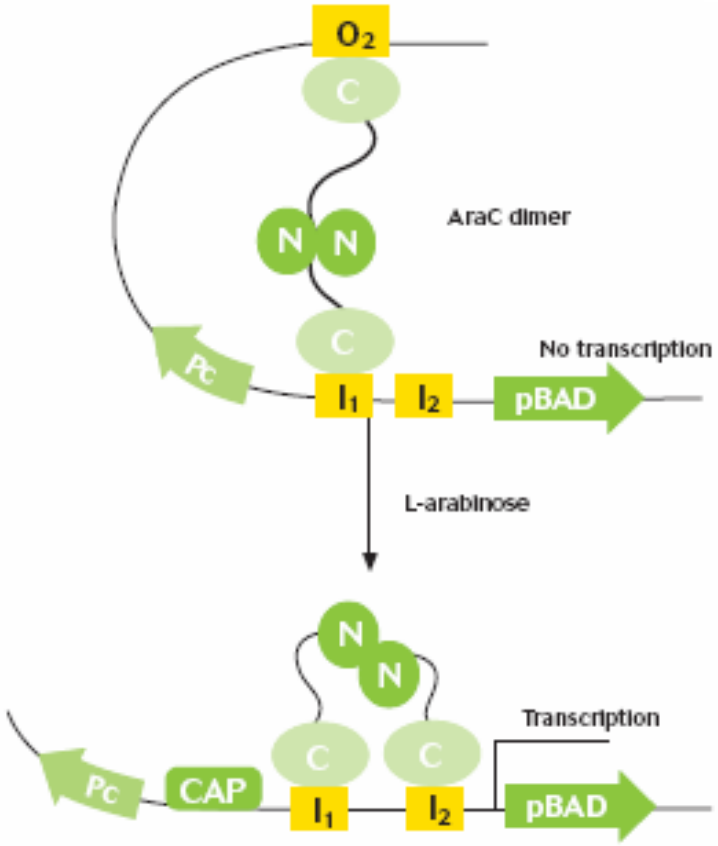
Cell Growth

- Grow the transformed *E. coli* cells in tryptone broth (1% tryptone, 0.5% NaCl) at 30℃ with ampicillin
- Grow directly on the cover slips
- The 30 degree temperature is required by the monomeric form of EosFP, which does not fold correctly at higher temperatrues

Fusion Plasmid Expression

- pBAD promoter on plasmid allows for tight control of expression (induction: expression level can be 1200)
- Arabinose is inducer; glucose is repressor
- ~0.01% arabinose has provided ideal expression in previous studies with MCP fusions

- Arabinose binds to AraC. The protein releases the O_2 site and binds the I_2 site, which is adjacent to the I_1 site. This releases the DNA loop and allows transcription to begin (5).
- The cAMP activator protein (CAP)-cAMP complex binds to the DNA and stimulates binding of AraC to I_1 and I_2.



Cell Fixation/Immobilization

- Cells can be fixed with paraformaldehyde or glutaraldehyde
- If these fixation chemicals are detrimental to receptor proteins, then cells can be immobilized with polylysine
- Immobilization should be acceptable since the lateral clusters are fixed in position

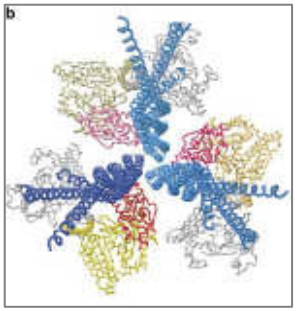
EXPERIMENTS

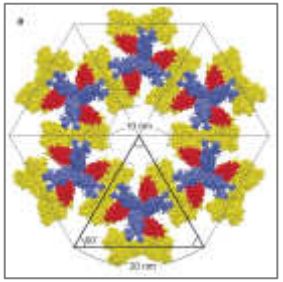
1. Demonstrate Clustering

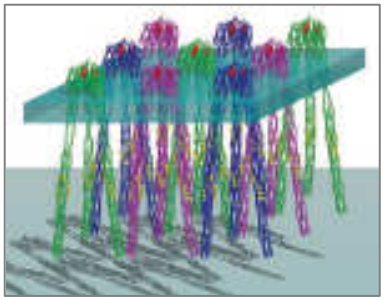
- Test the *tar/tsr* deletion cells on agar plates to show that they do not chemotax
- Express fusion of full length Tar and mEosFP in the cells and visualize receptors with a normal fluorescence microscope
- Perform truncations on Tar, if needed, until clustering occurs
- Determine whether or not cells can still chemotax
- Perform similar experiments with Tsr-mEosFP fusion

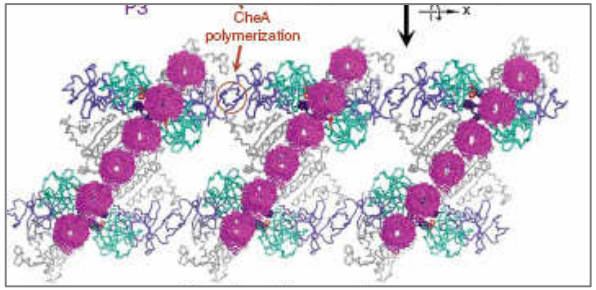
2. Visualize Tar and Tsr Fusions

- Express the Tar-mEosFP and Tsr-mEosFP fusions from plasmids in the *E. coli* cells
- Use PALM microscope to visualize the receptor organization in high resolution
- Compare this data to predictions made by various models (e.g. trimer of dimers, hexagonal lattice, close-packed lattice, and hedgerows)









3. Determine Effects of CheW and CheA on Clustering

- Obtain PALM images of Tsr and Tar fusions in cells that have in-frame deletions of the CheA and/or CheW strains
- Determine what effect these proteins have on the receptor organization

4. Visualize CheW and CheA Fusions

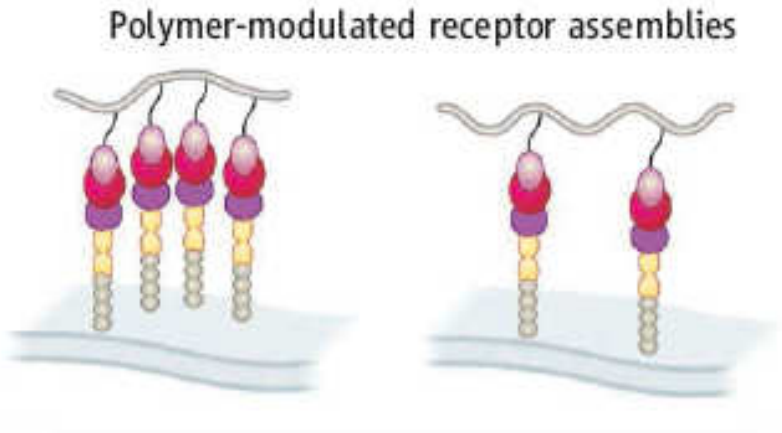
- Prepare fusion plasmids of CheA or CheW with mEosFP and express in cells
- Determine if any truncations must be made, as with the MCPs
- Use PALM microscope to determine organization of these proteins
- Compare this data to MCP organization

Challenges

- The features of the close packed lattice may be slightly too small to visualize with PALM
- Optics must be carefully chosen, aligned, etc. to maximize the signal-to-noise ratio and resolution
- May need to fine tune laser intensities, which determine depth of excitation and activation
- Sample drift may need to be accounted for (e.g. with gold particles)
- Careful truncations of the MCPs may be necessary
- Other monomeric PA-FPs (e.g. PA-GFP, Dendra) may have to be used if mEosFP will not work properly

Further Directions

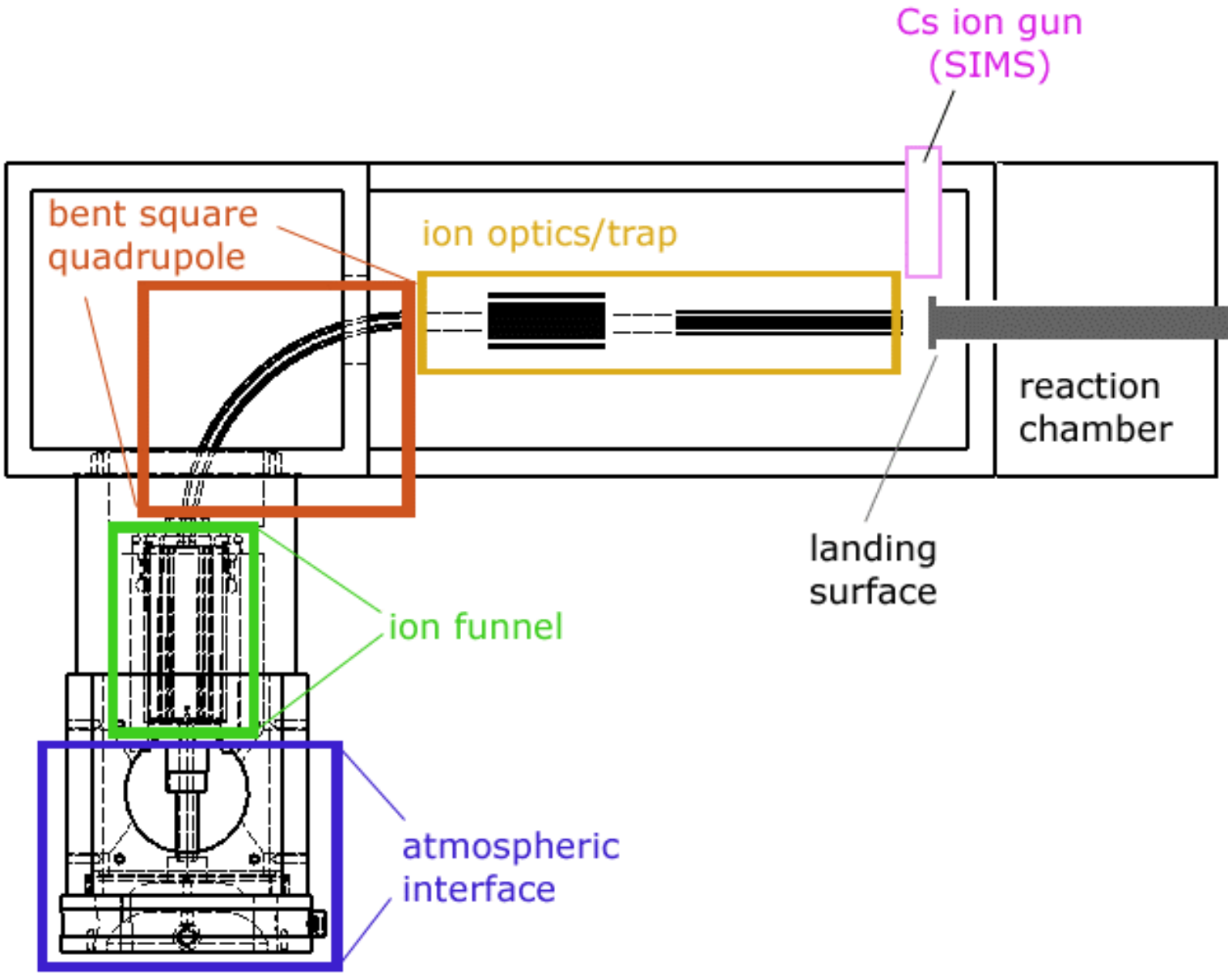
- Extend methodology to receptor proteins involved in cell adhesion and immune responses
- Should provide deeper understanding of receptor cluster organization in general
- May be especially beneficial in future development of polymeric therapeutic drugs



Curr. Opinion Immunol., 2002, 14, 66
Science, 2007, 317, 1183

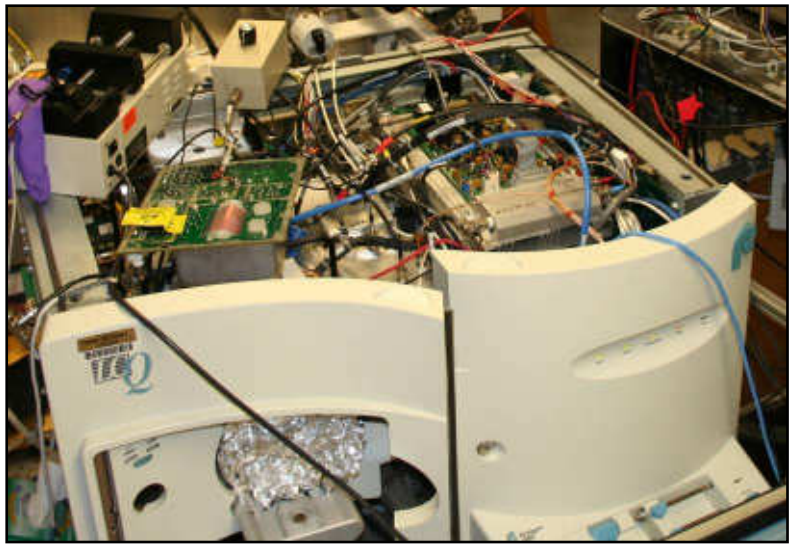
RESEARCH OVERVIEW

New Soft Landing Design

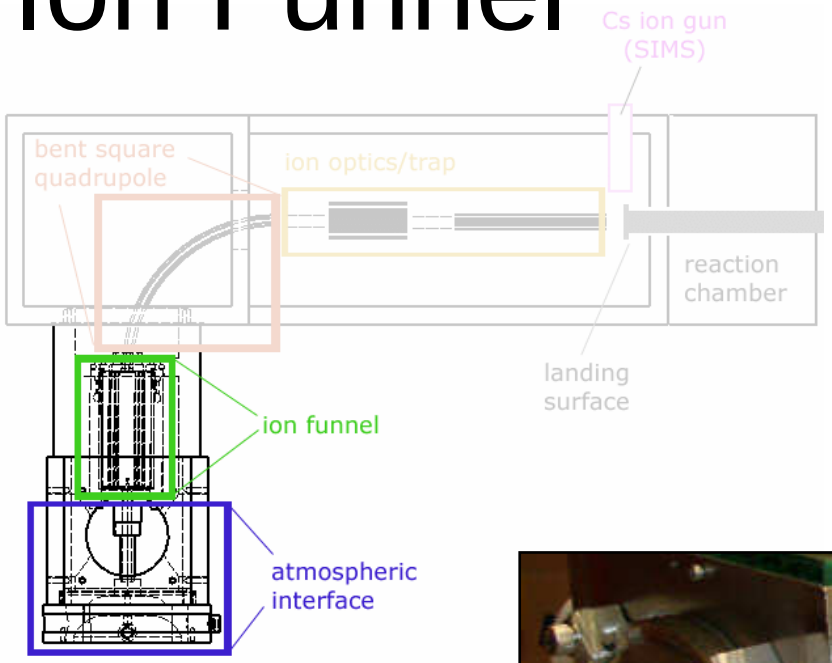


Control Electronics

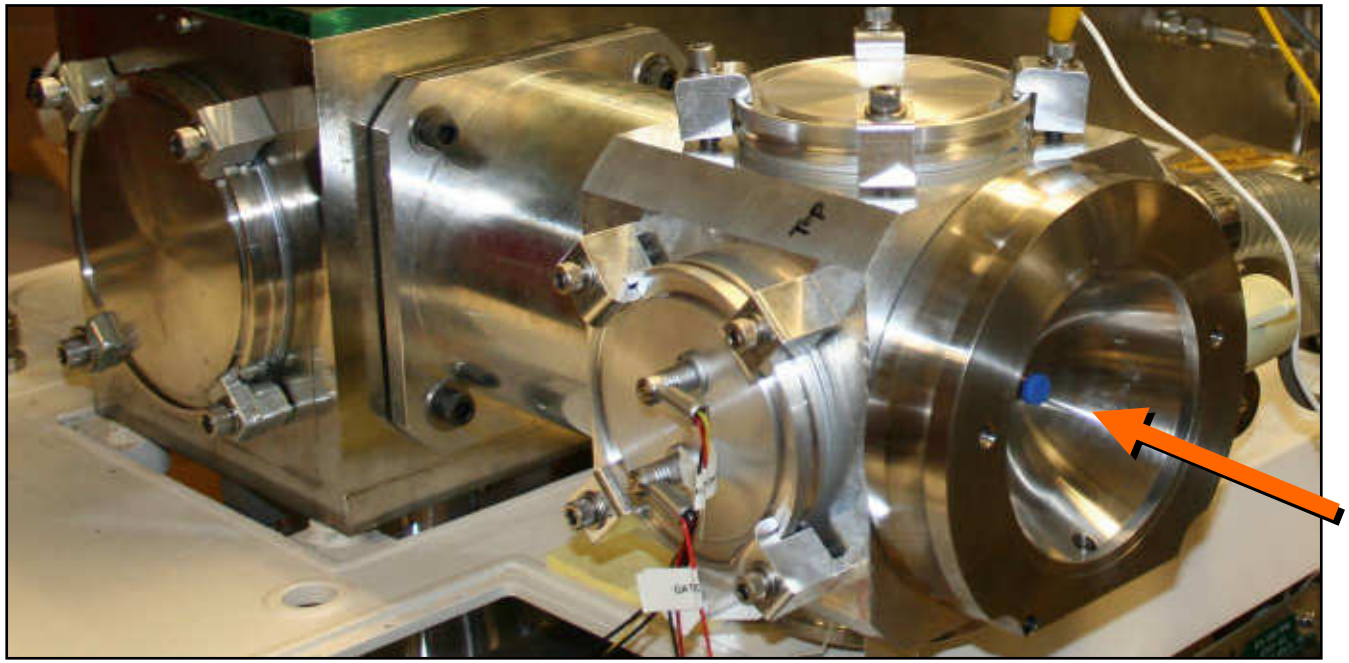
- Thermo LCQ for DC (floats, end caps, detector, etc.), trapping RF and auxiliary AC
- Spectrum Solutions for static DC
- Arbitrary waveform generator and amplifier for ion funnel



Atmospheric Interface & Ion Funnel

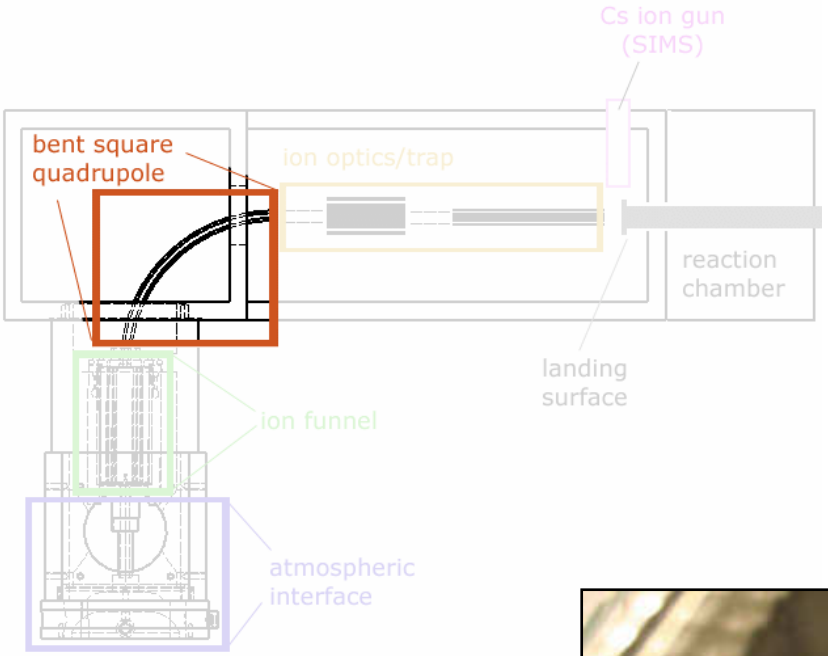


- Heated capillary inlet (500µm id)
- Typical spray current: 200nA
- Funnel: ~680 kHz and 40V_{p-p} RF
- Measure ~1nA at funnel back

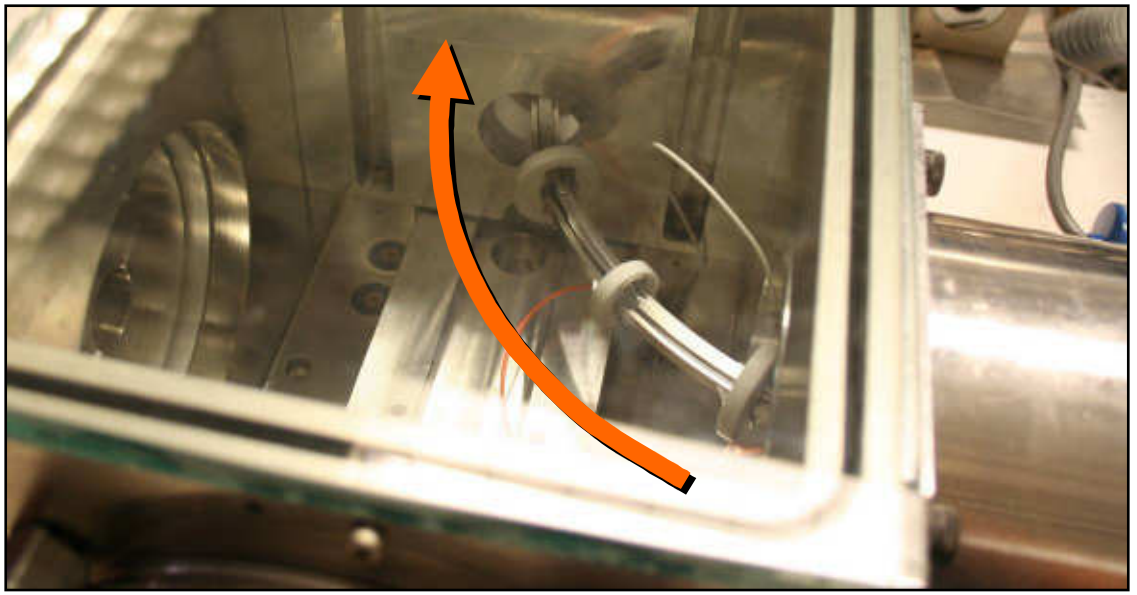


Smith et al, Anal. Chem., **71,** 15, 2957-2964

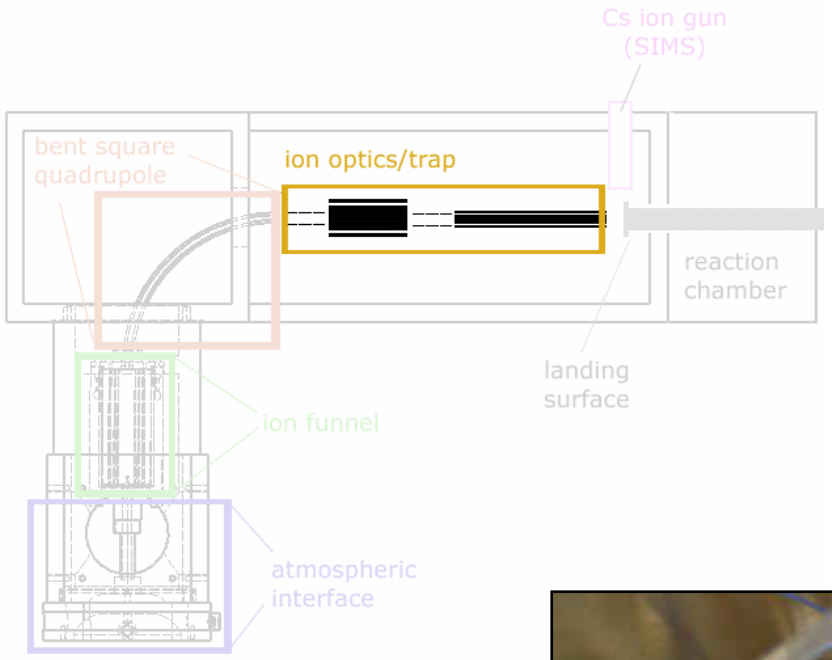
Bent Square Quadrupole



- Distinguishes between ions and neutrals
- From Thermo (Q2 collision cell in triple quad instruments)
- Estimated transmission efficiency: >95%
- RF/DC mass selection will be explored in the near future for high throughput

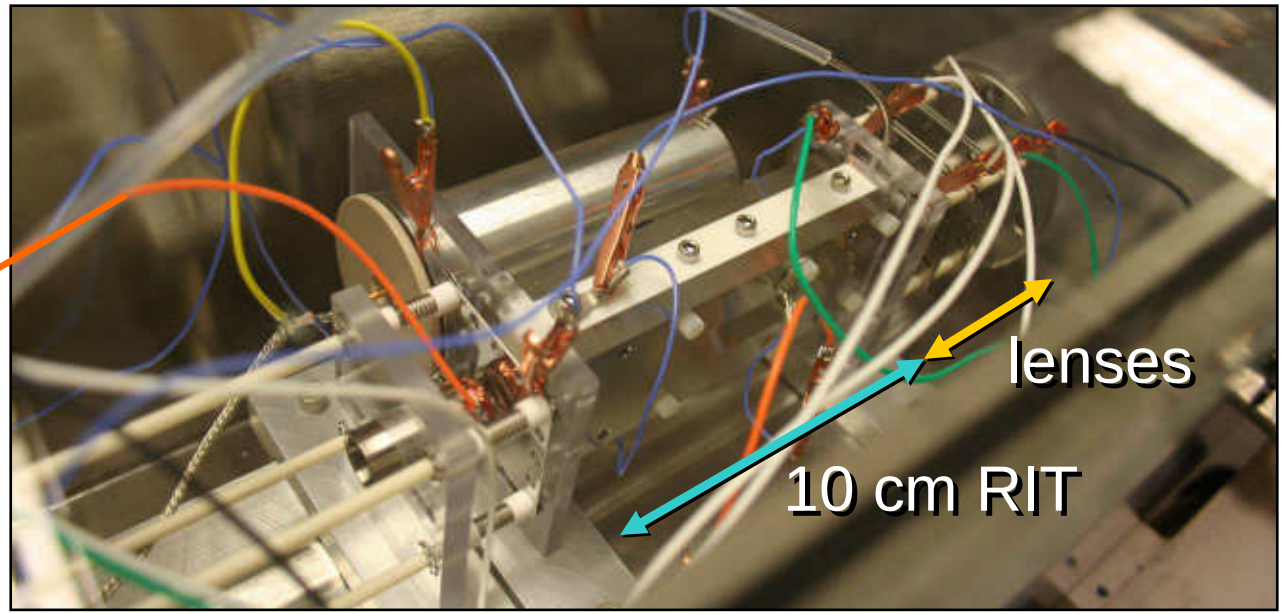


Ion Optics & Trap



- Einzel lenses guide ions into RIT
- Elongated RIT (10 cm)
- RF: 695 kHz , ~5-6kV$_{p-p}$ max

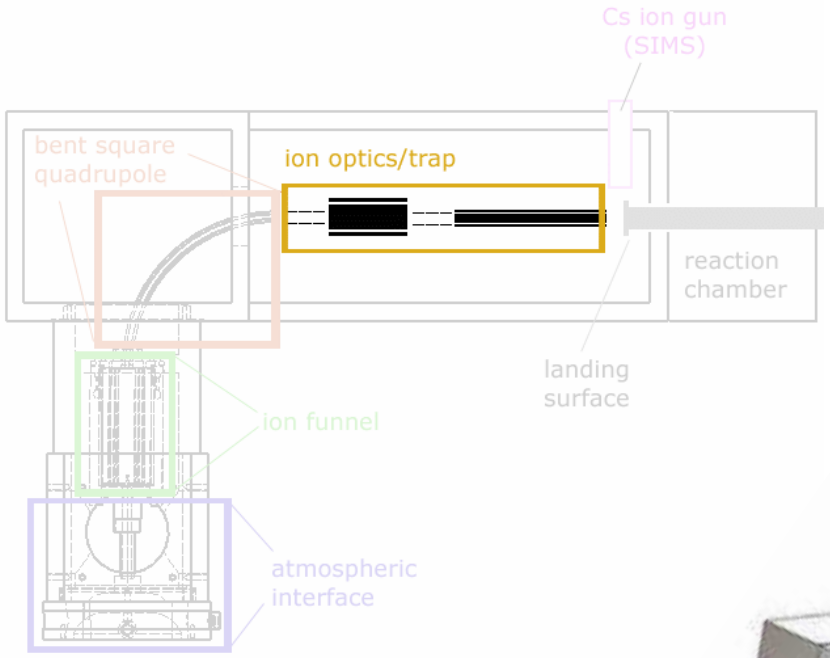
In RF only mode, measure up to 500-600 pA at surface



Cooks et al, Anal. Chem. 76, 2004, 4595 -4605.

10cm Rectilinear Ion Trap

Modes of Operation

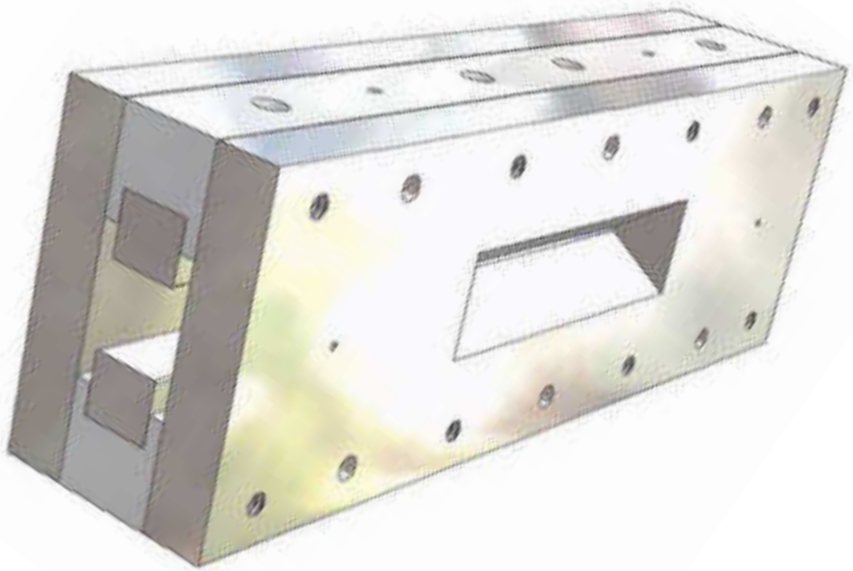


Normal ion trap

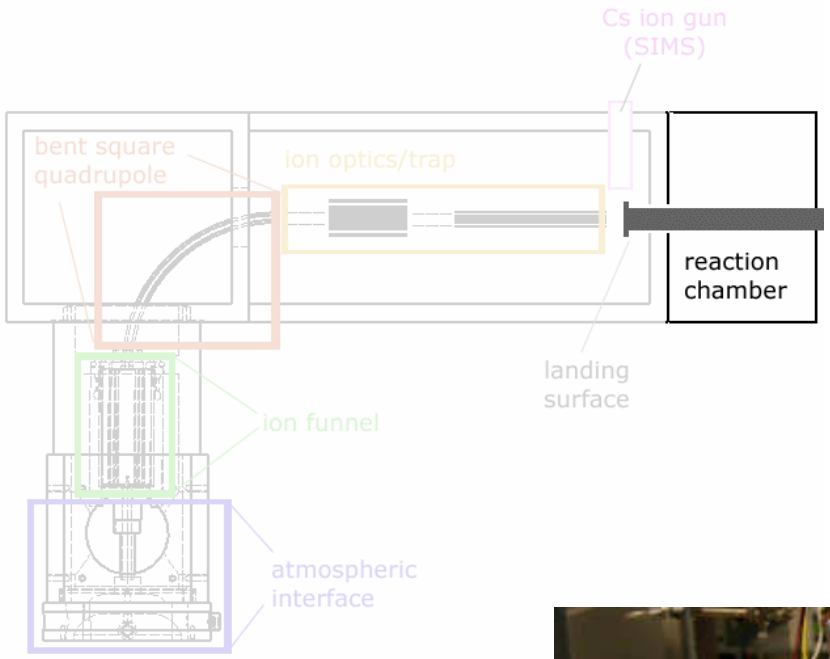
Gate endcap DC voltages and obtain full mass spectrum of generated species

Continuous mass filter

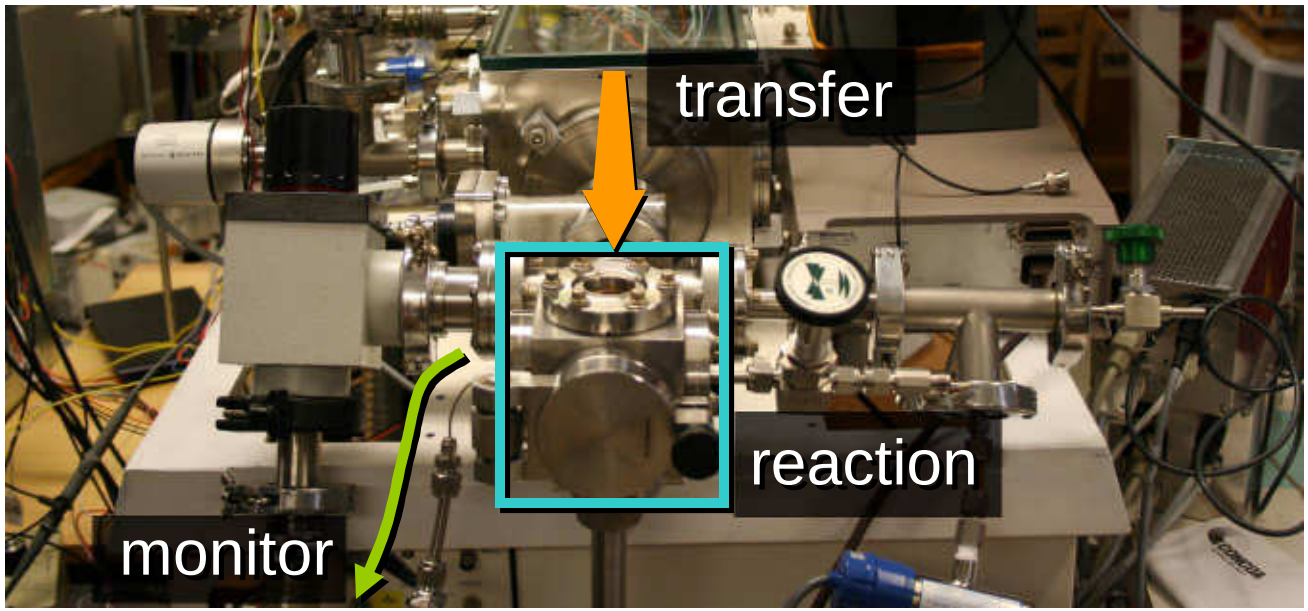
Lower endcap voltages and allow one stable m/z value to pass through, giving maximum landing current



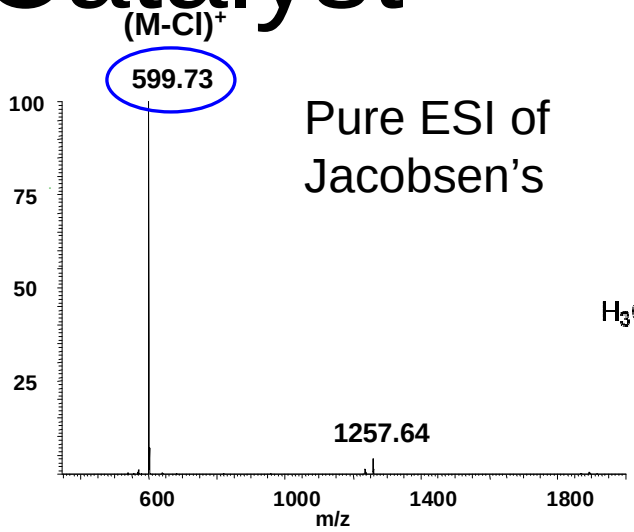
Reaction Chamber



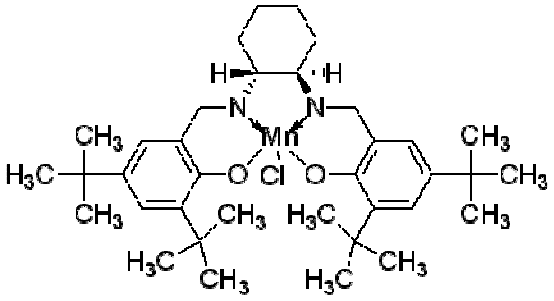
- Landed surface can be transferred to reaction chamber
- Reactant gas mixtures are formed by mass flow controllers
- Reaction mixture sampled out to MS for monitoring product formation and measuring turnover frequency



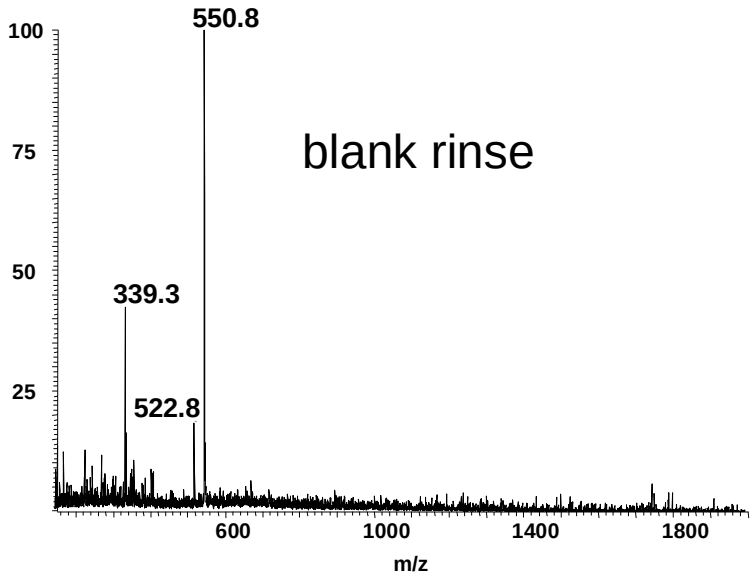
Soft Landing of Jacobsen's Catalyst



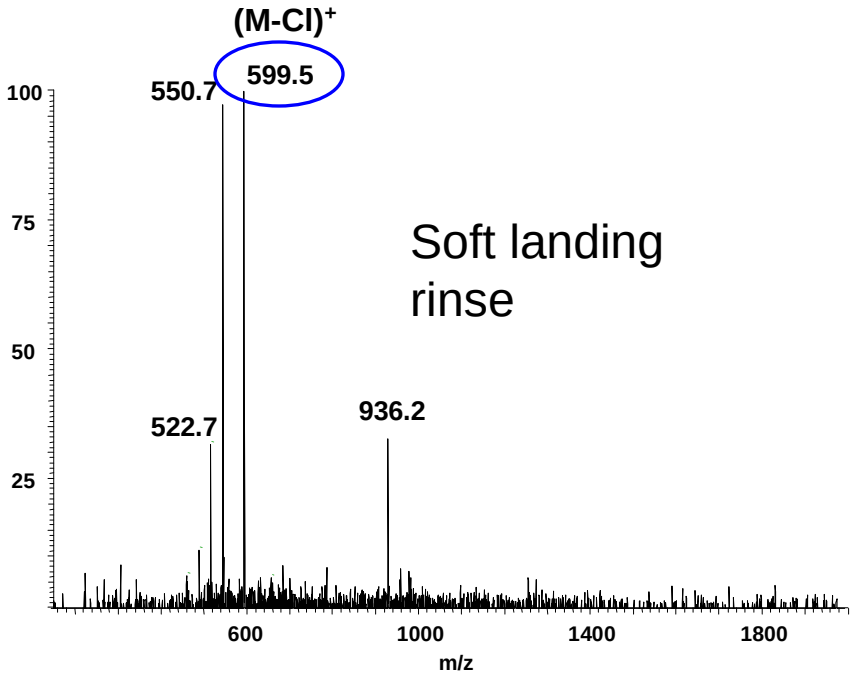
Pure ESI of Jacobsen's



- Spray 10^{-4} M Jacobsen's (MeOH:acetonitrile)
- land ~250pA for 20 min
- RF-only mode



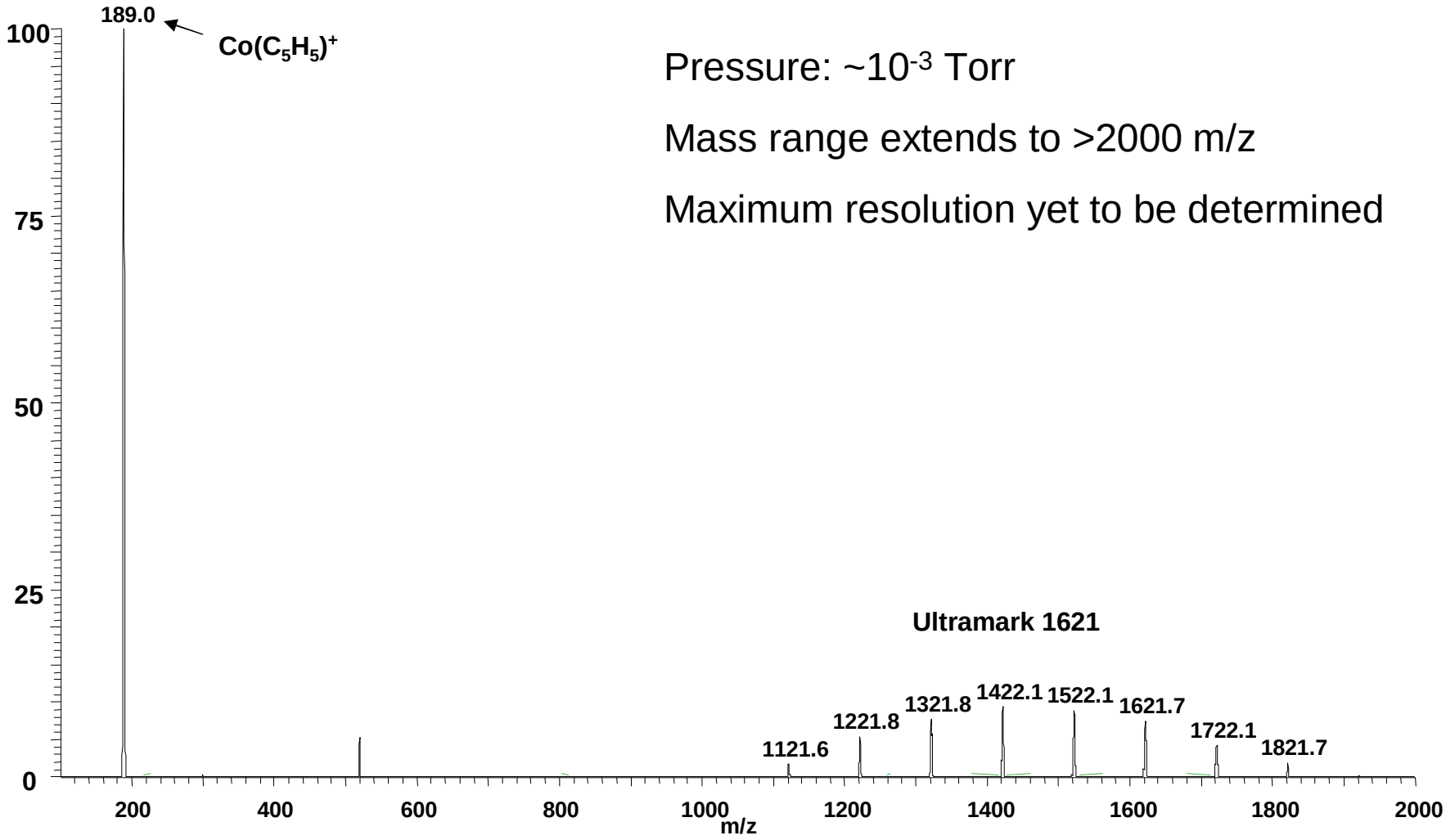
blank rinse



Soft landing rinse

10cm RIT Performance

full mass spectrum



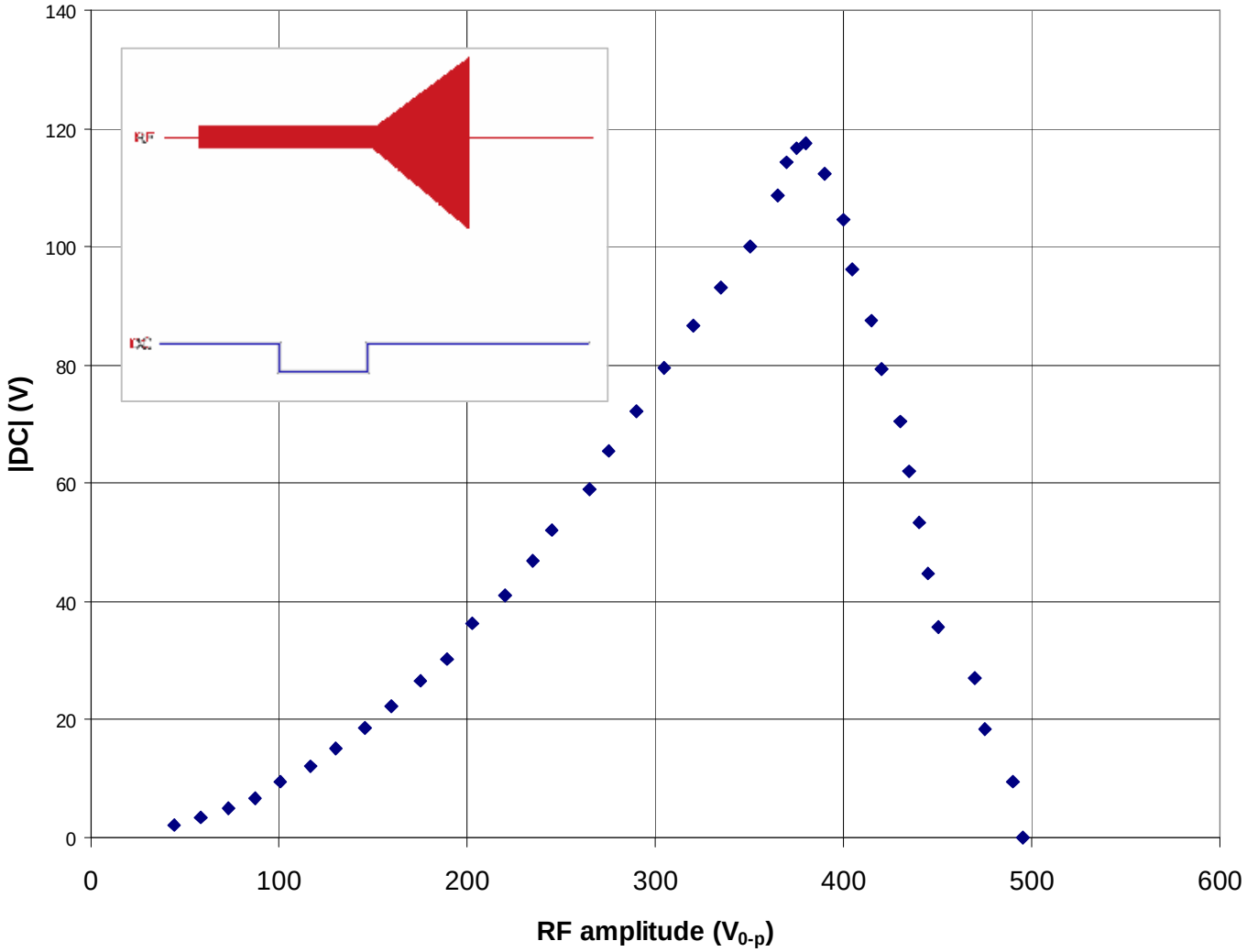
Pressure: ~10^{-3} Torr

Mass range extends to >2000 m/z

Maximum resolution yet to be determined

10cm RIT Performance

RF/DC stability diagram



m/z 600

(Jacobsen's Catalyst)

Ions are trapped, then a negative DC is applied to the y electrodes for ~10 ms.

As the DC voltage is lowered (more negative), the intensity of the m/z 600 peak in the mass spectrum is monitored.

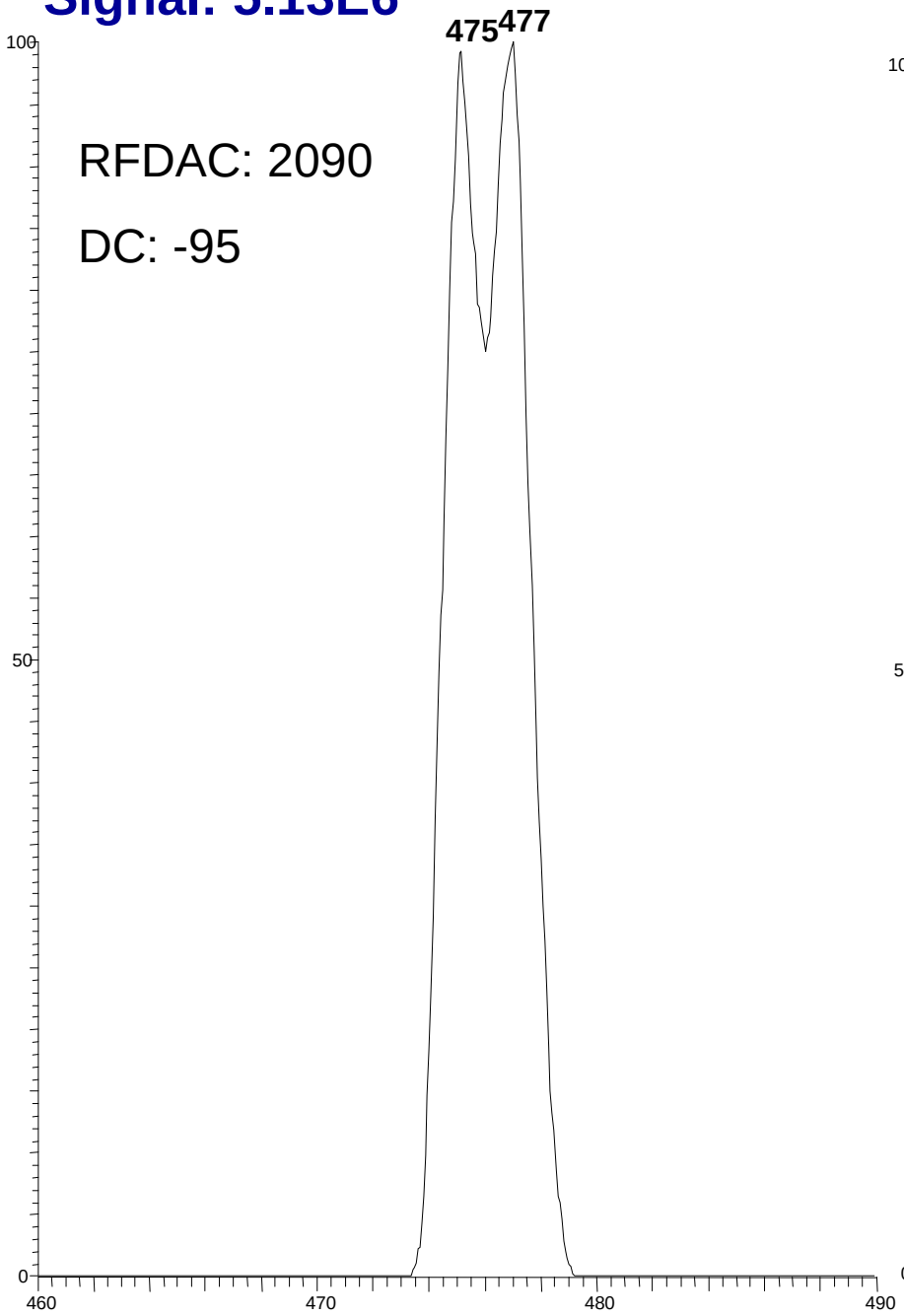
Eventually the DC voltage pushes the ions' working point out of the stability region.

Some peak splitting has occurred, presumably due to non-linear resonances.

Signal: 5.13E6

RFDAC: 2090

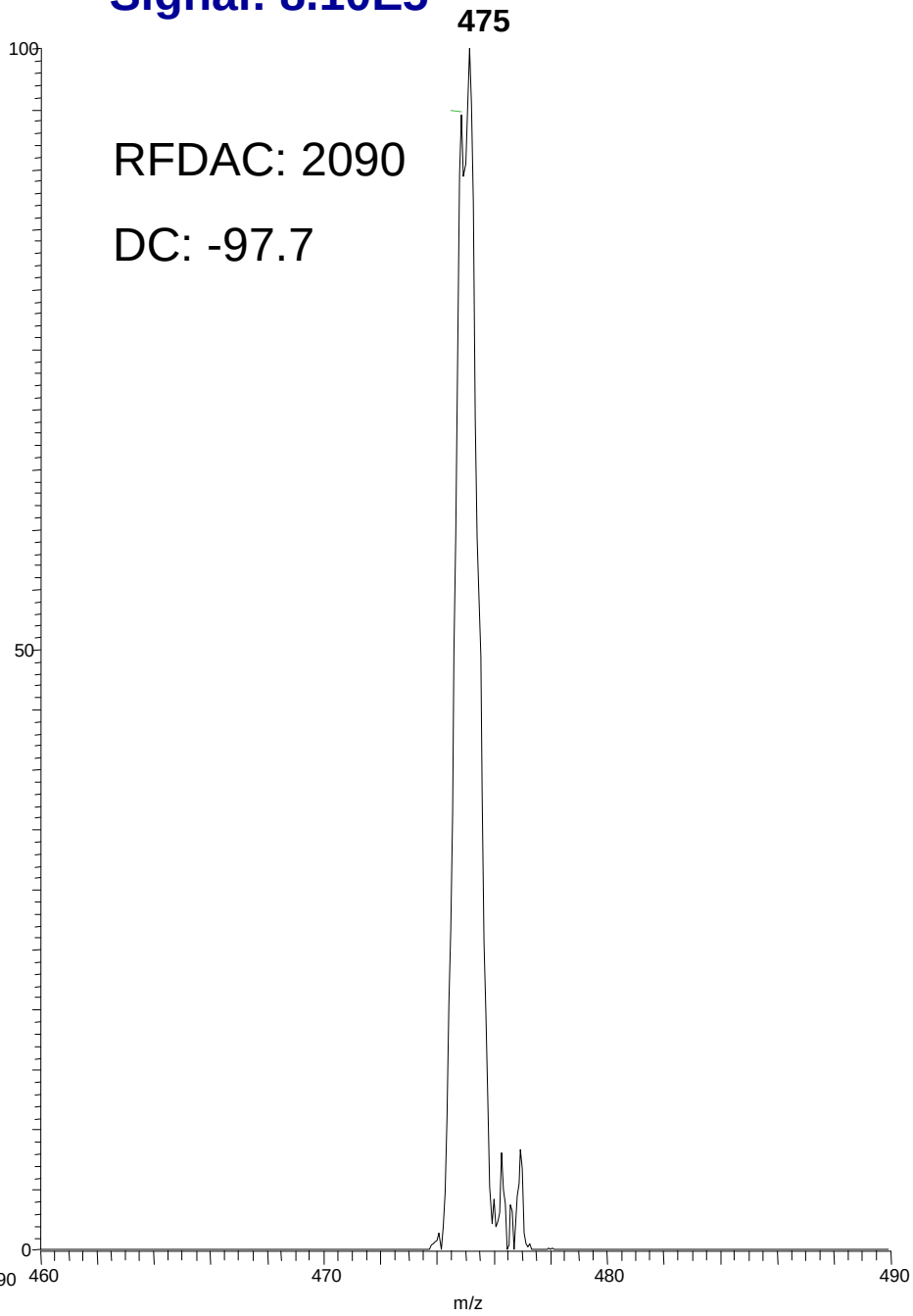
DC: -95



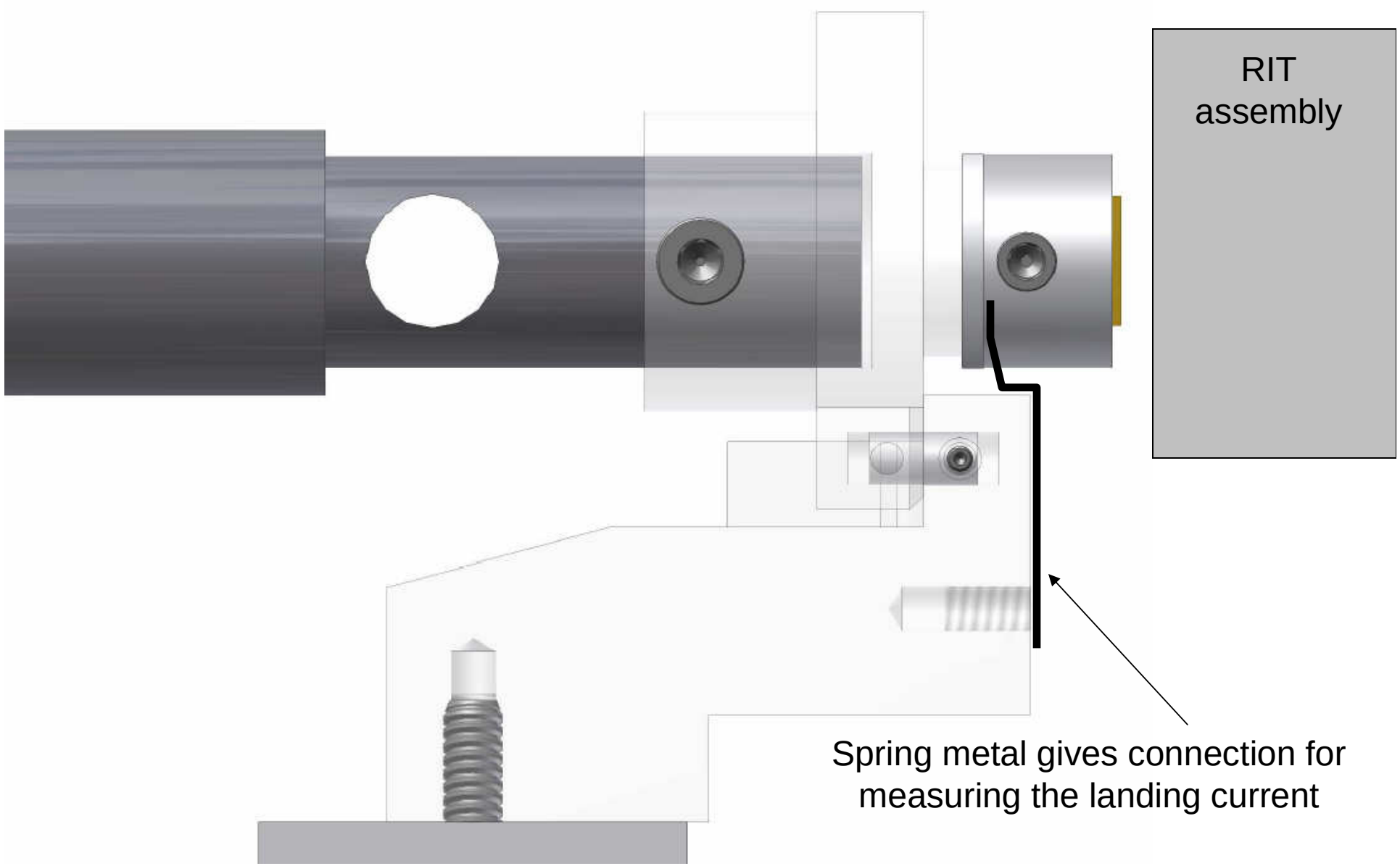
Signal: 8.10E5

RFDAC: 2090

DC: -97.7



Surface Assembly Design



Future Work (Instrumentation)

- Perform continuous landing with trap in RF/DC mode
- Operate square quad in RF/DC mode for possible high throughput landing
- Extend mass range of the instrument by lowering frequencies of RF
- Add SIMS capability with Cs ion gun and ion optics

Future Work (Chemistry)

- Chose model systems to land and monitor
- Find appropriate surfaces for maximizing catalytic activity
- Soft land mixed metal clusters formed from atmospheric heating of inorganic complexes
- Study the effect of various metal and ligand combinations on activity of surfaces