



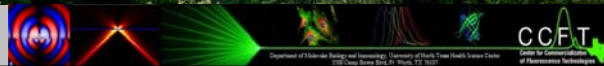
Plasmonics in Fluorescence & Microscopy

Zygmunt (Karol) Gryczynski



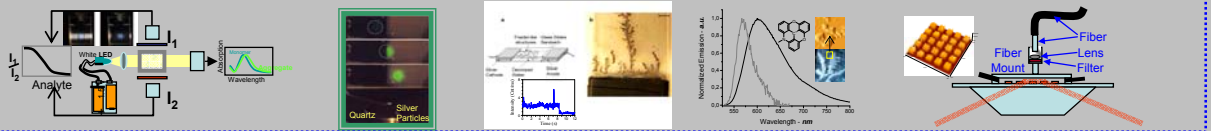
**Center for Commercialization
of Fluorescence Technologies**
University of North Texas Health Science Center at Fort Worth, TX

Principle of Fluorescence Techniques 2010
Chicago, Illinois 2010

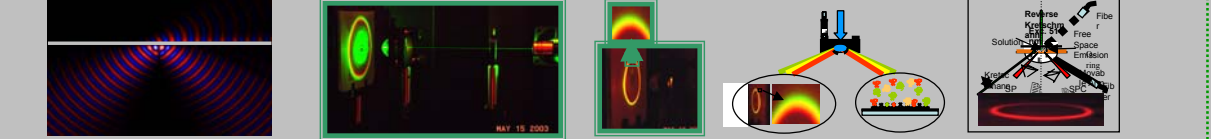


Recent Focus on Nanotechnology

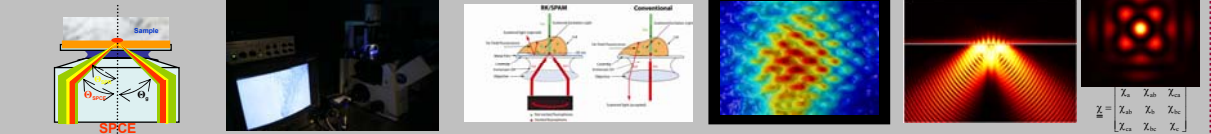
1. Localized Surface Plasmons. Biomedical Applications of Metal Enhanced Fluorescence



2. Traveling Surface Plasmons. Surface Plasmon Coupled Emission and Its Applications



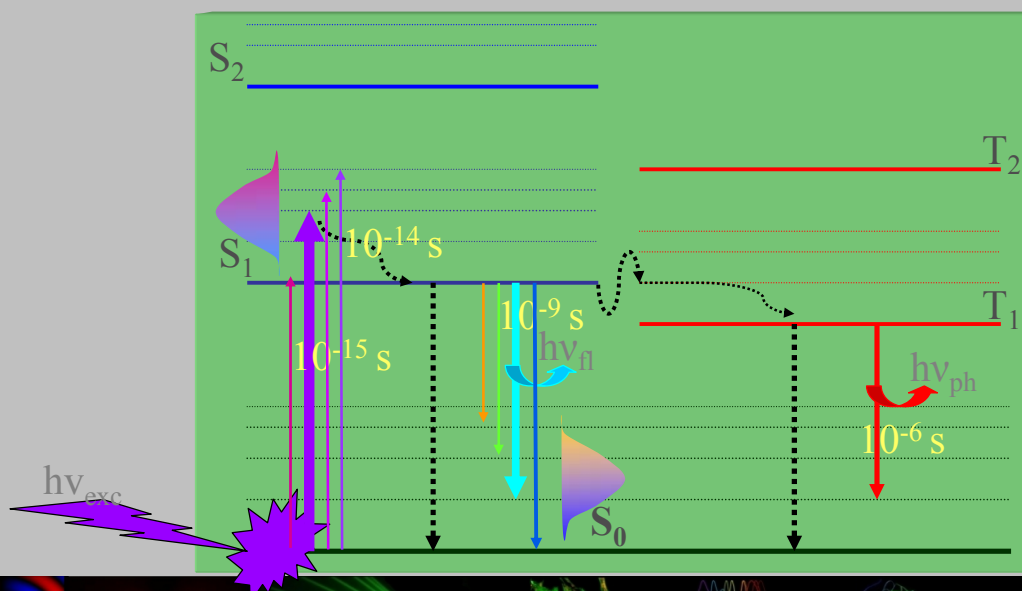
3. Surface Plasmon Assisted Microscopy (SPAM)



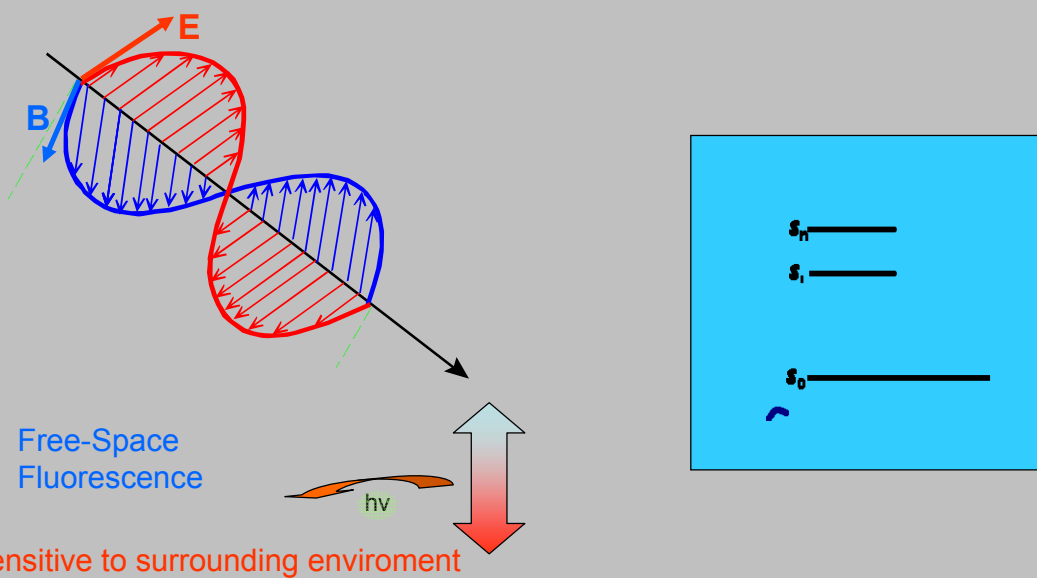
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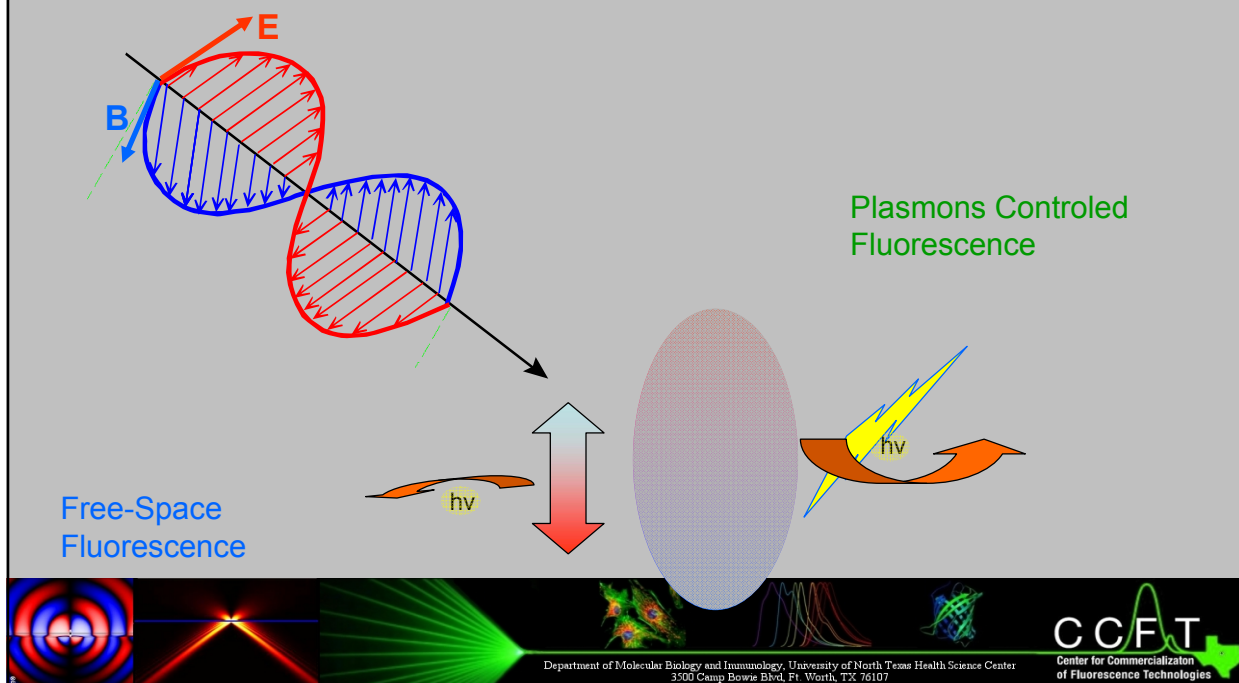
Jablonski Diagram



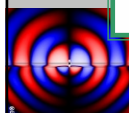
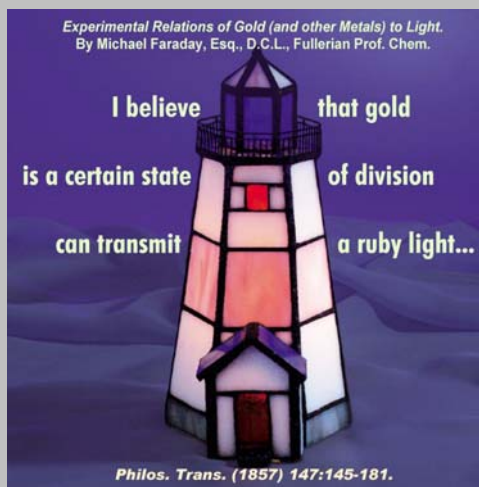
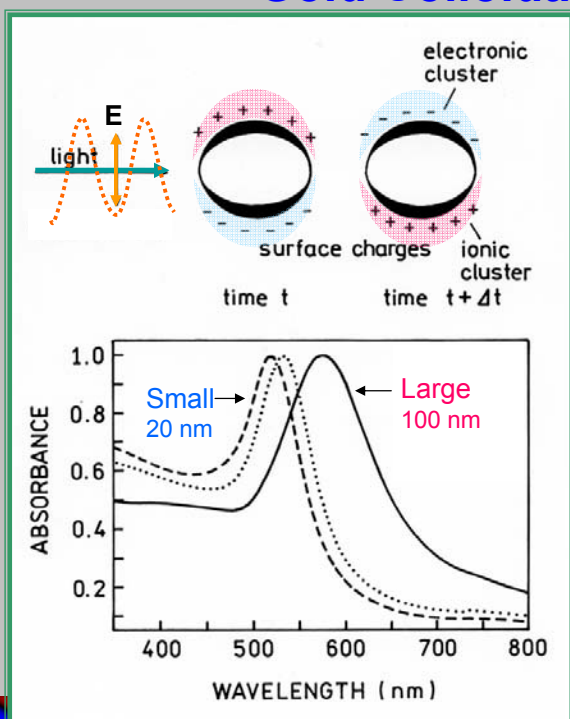
Excitation and Emission



New Directions in Fluorescence Radiating Plasmons Model



Optical Properties of Metal Colloids- Gold Colloidal Spheres

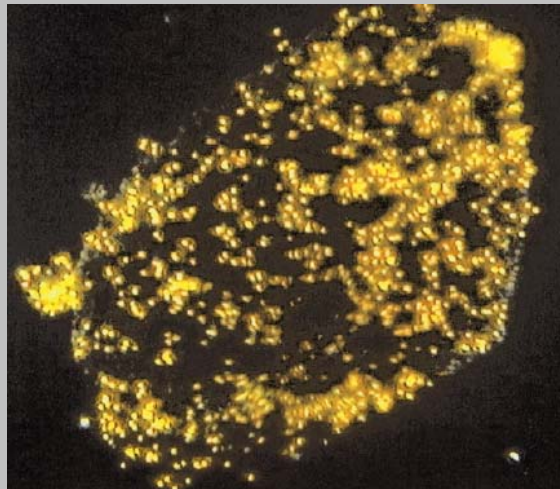


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Can Intrinsic Particle Scattering Have Biomedical Applicability

Cell Labeling



Photograph of epithelial buccal cells with 78-nm gold particles

J. Yguerabide and E. E. Yguerabide, *Anal. Biochem.* **262**, 137–156 (1998)

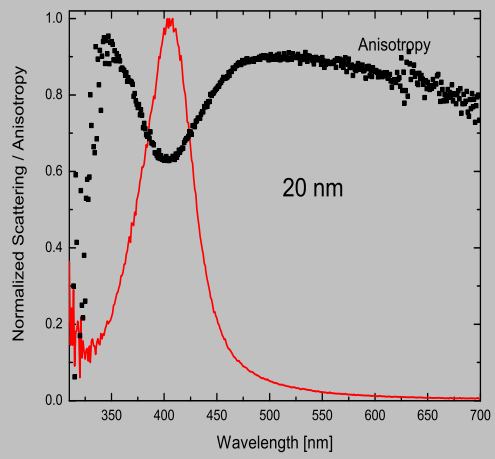
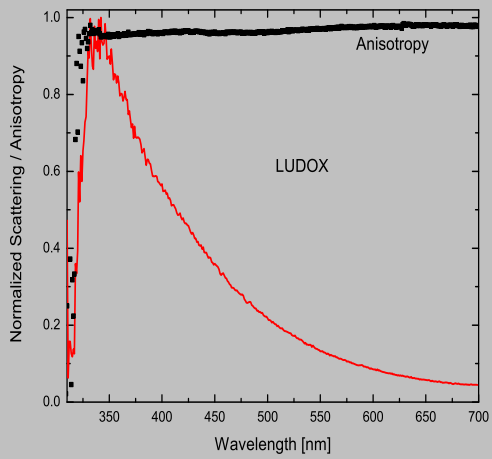
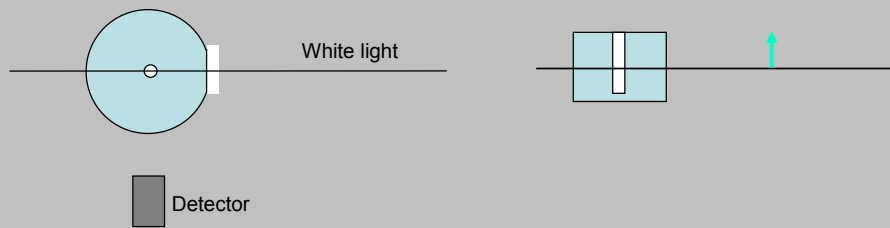
Different Plasmonic Phenomena

Localized Surface Plasmons

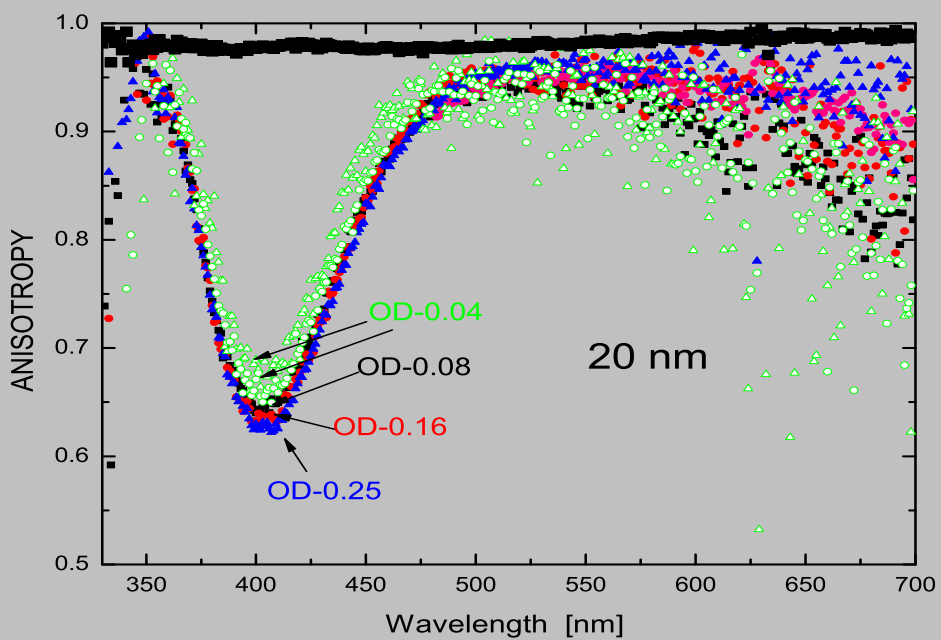
Biomedical Applications of SP



Polarized Light Scattering of Particles



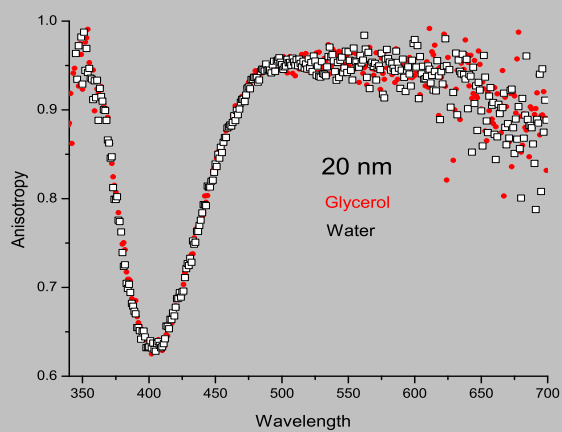
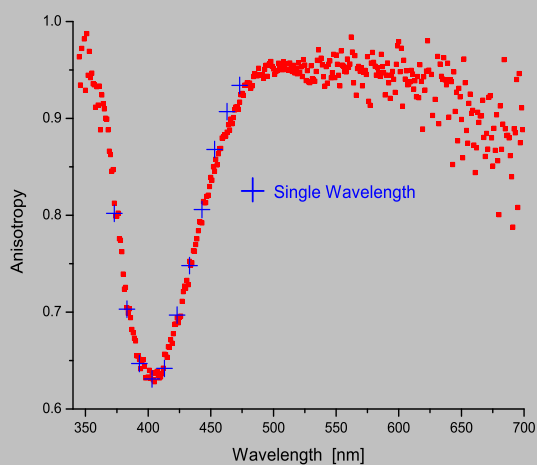
Different Colloids Concentrations



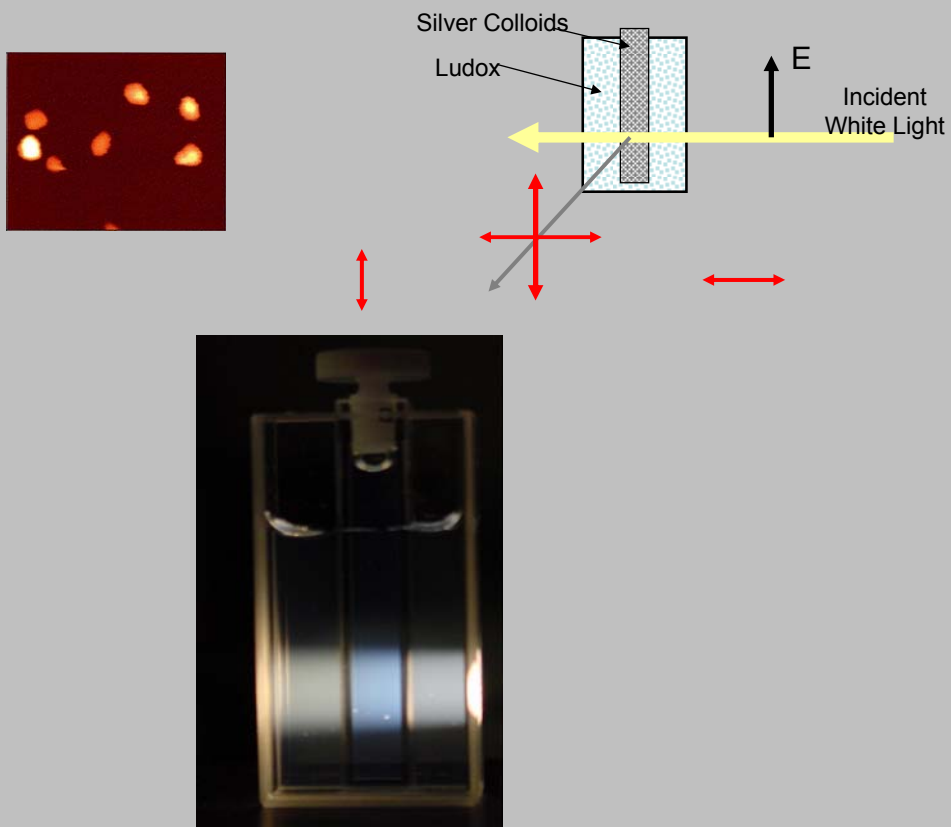
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What About Wavelength or Solvent Viscosity

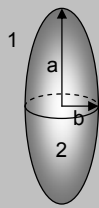


Application of Polarization Dependent Colloid Scattering



How to Explain Polarization Effect?

Polarization of a prolate spheroid in a uniform electric field



$$\begin{cases} x = f\sqrt{\xi^2 - 1}\sqrt{1 - \eta^2} \cos(\varphi) \\ y = f\sqrt{\xi^2 - 1}\sqrt{1 - \eta^2} \sin(\varphi) \\ z = f\xi\eta \end{cases}$$

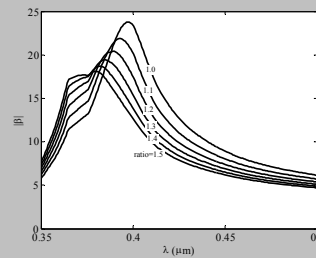
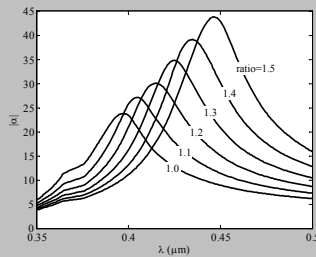
$$\begin{cases} \xi > 0 \\ 0 \leq \eta < 1 \\ 0 \leq \varphi < 2\pi \end{cases}$$

Laplace' equation of a potential V

$$\begin{cases} \nabla^2 V = 0 \\ V = \sum_{n=0}^{\infty} \sum_{m=-n}^n [A_n^m P_n^m(\xi) + B_n^m Q_n^m(\xi)] P_n^m(\eta) e^{im\varphi} \end{cases}$$

polarizabilities along the long and short axes

$$\alpha = f^3 \frac{4\pi\epsilon_1(\epsilon_1 - \epsilon_2)\xi_0}{3[\epsilon_1\xi_0 Q_1'(\xi_0) - \epsilon_2 Q_1(\xi_0)]} \quad \beta = f^3 \frac{8\pi\epsilon_1(\epsilon_2 - \epsilon_1)\xi_0\sqrt{\xi_0^2 - 1}}{3[\epsilon_1(\xi_0^2 - 1)Q_1'(\xi_0) - \epsilon_2\xi_0 Q_1(\xi_0)]}$$

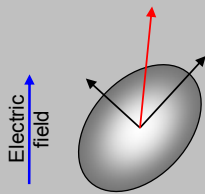


Interference of surface plasmon resonances causes enhanced depolarized light scattering from metal nanoparticles. (2007) Calander, N., Gryczynski, I., Gryczynski, Z. *Chem. Phys. Lett.* **434**, 326-330.

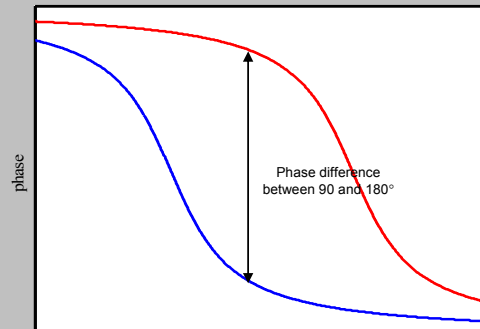
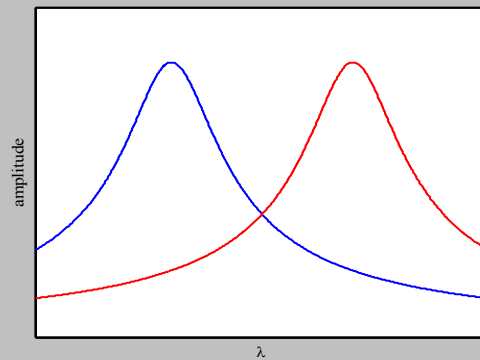
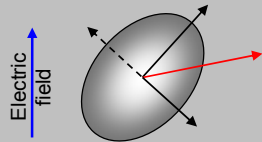
Depolarized light scattering from silver nanoparticles. Gryczynski, Z., Lukomska, J., Lakowicz, J.R., Matveeva, E.G., Gryczynski, I. (2006) *Chem. Phys. Lett.* 421, 189-192.

Phase difference of two resonances may exceed 90° in between,
if they are enough far apart

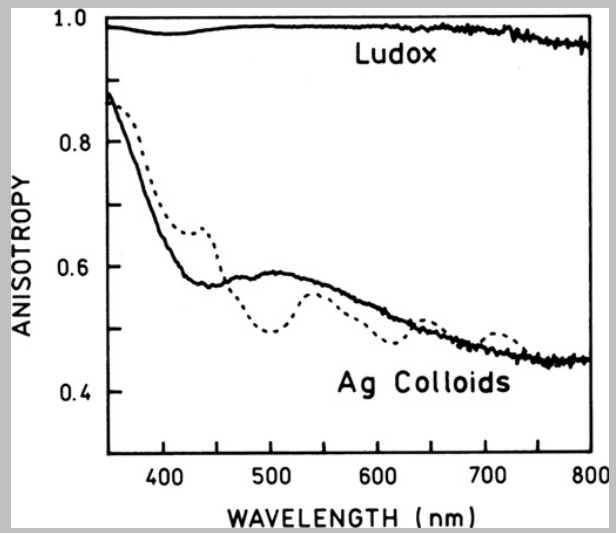
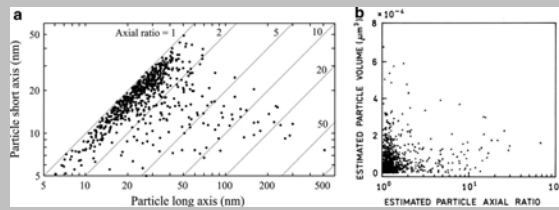
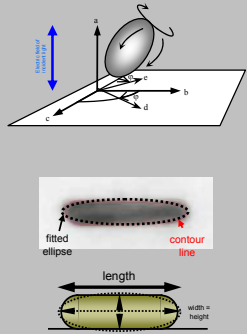
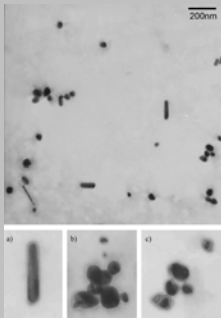
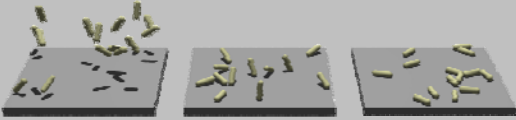
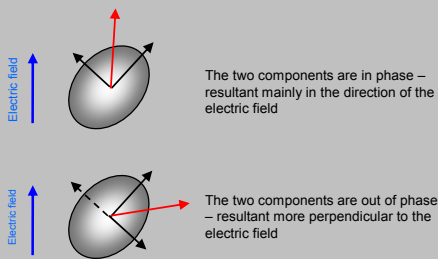
The two components are in phase –
resultant field mainly in the direction of the
electric field

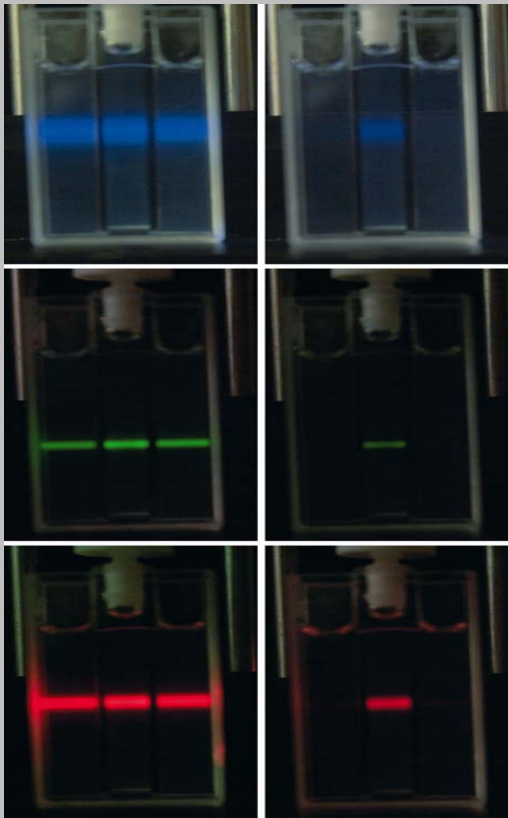


The two components are out of phase –
resultant field more perpendicular to the
electric field



Estimating Scattering from Shape Distribution

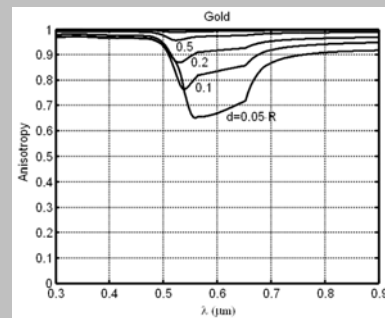
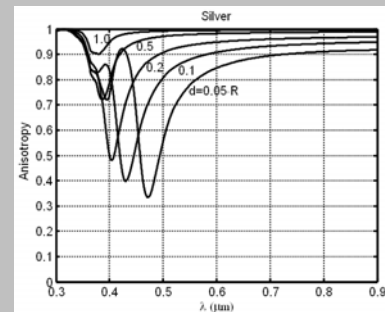
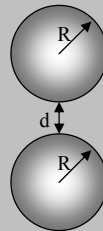
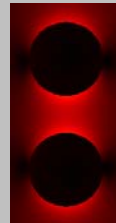
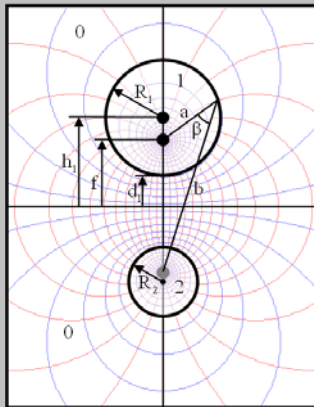




1. Z. Gryczynski, J. Lukomska, J. R. Lakowicz, E. G. Matveeva, I. Gryczynski, Depolarized light scattering from silver nanoparticles, *Chemical Physics Letters* **421** (1-3), 189-192, (2006)
2. N. Calander, I. Gryczynski, and Z. Gryczynski. *Interference of surface plasmon resonances causes enhanced depolarized light scattering from metal nanoparticles*. *Chemical Physics Letters* **434** (4-6), 326-330, (2007)
3. S. Klitgaard, T. Shtoyko, N. Calander, I. Gryczynski, E. Matveeva, J. Borejdo, M. Neves-Petersen, S. Petersen, and Z. Gryczynski, *Long wavelength depolarized light scattering from silver nanoparticles*. *Chemical Physics Letters* **443** (1-3), 1-5, (2007).
4. K. Drozdowicz-Tomsia, F. Xie, N. Calander, I. Gryczynski, K. Gryczynski, E. M. Goldys, *Depolarized light scattering from colloidal gold nanoparticles*. *Chemical Physics Letters*, **468** (1-2), 69-74, (2009).

Theoretical approach

Solving Laplace's Equation
in Bi-spherical coordinates



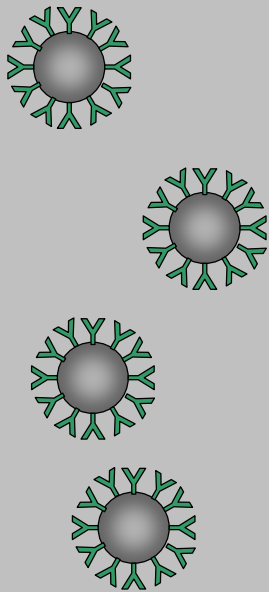
$$V = Ez + f \sqrt{2(\cosh(\alpha) - \cos(\beta))} \sum_{n=0}^{\infty} P_n(\cos(\beta)) \left[A_n \sinh\left(\left(n + \frac{1}{2}\right)\alpha\right) + B_n \cosh\left(\left(n + \frac{1}{2}\right)\alpha\right) \right]$$

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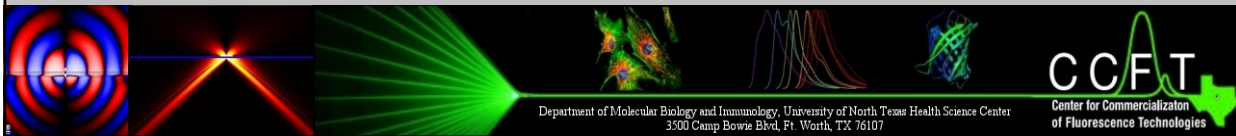
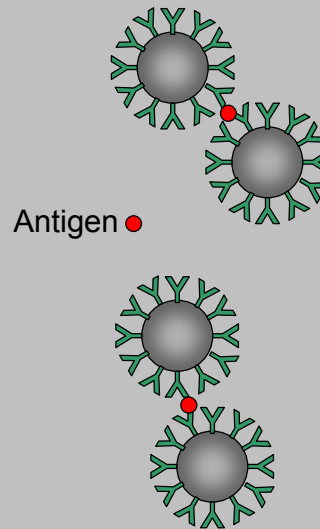
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Application

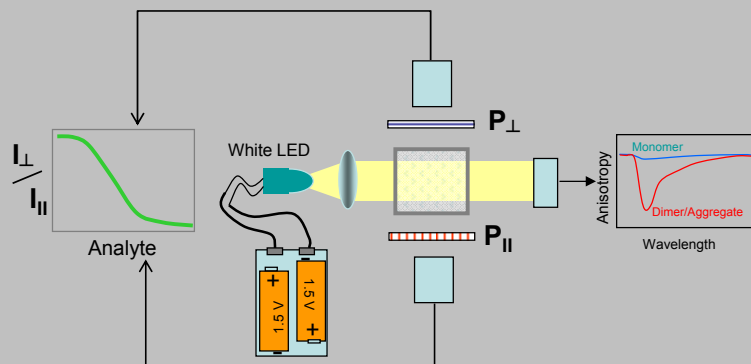
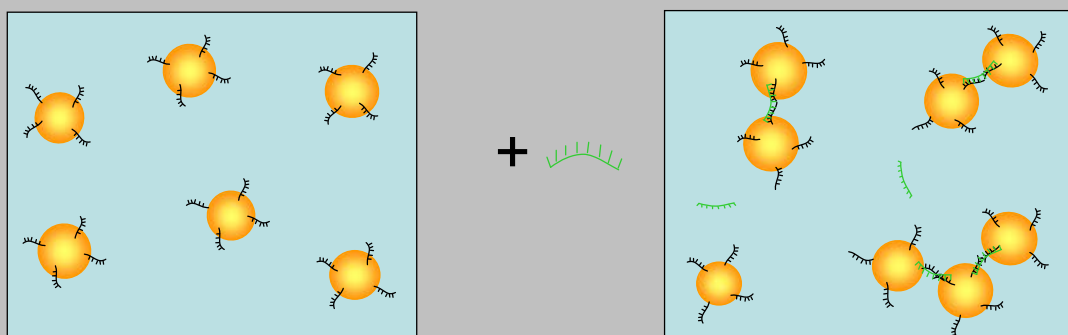
No antigen \Rightarrow no depolarization



Antigen present \Rightarrow depolarization



Wavelength-Ratiometric Scattering Sensor Based on Metallic Colloids



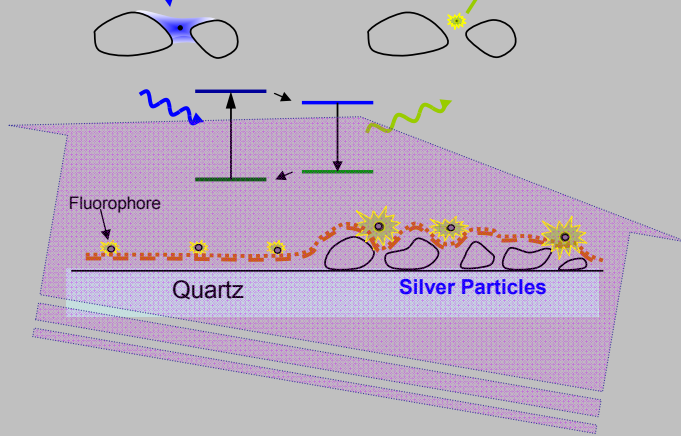
Metal Enhanced Fluorescence (MEF)

Metal Enhanced Fluorescence – Localized Plasmons

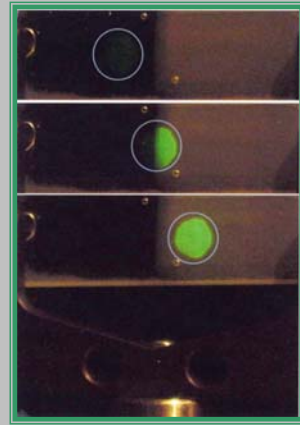
Light efficiently couples in

- stronger EM field
- stronger excitation

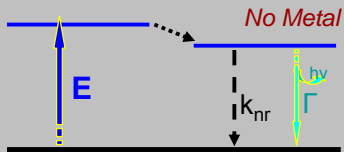
- light efficiently couples out
- shorter lifetimes



Metal Enhanced Fluorescence (MEF)

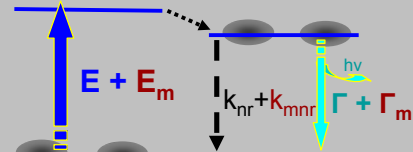


Quartz Silver Particles



$$Q_0 = \Gamma / (\Gamma + k_{nr})$$

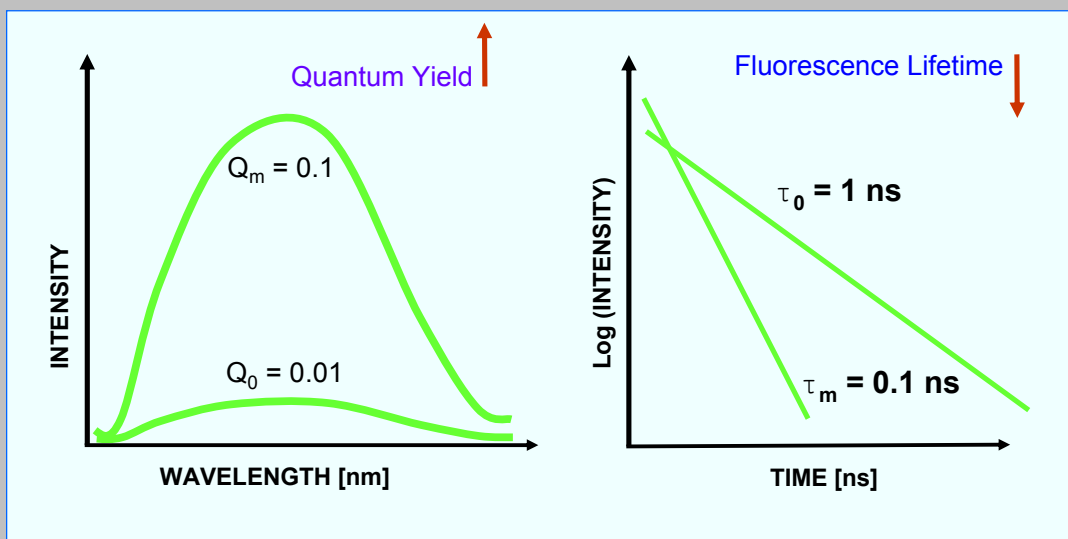
$$\tau_0 = 1 / (\Gamma + k_{nr})$$

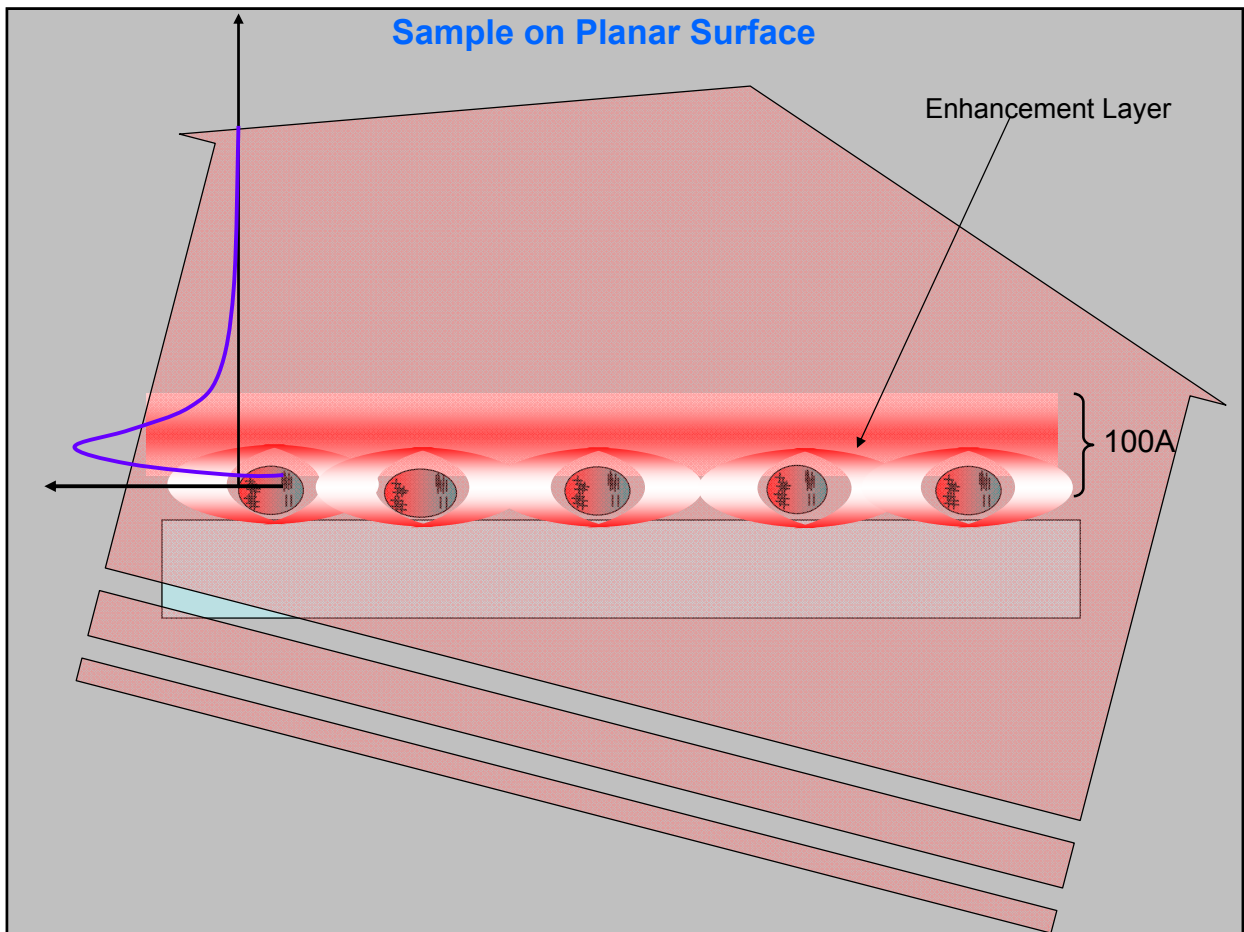


$$Q_m = (\Gamma + \Gamma_m) / (\Gamma + \Gamma_m + k_{mnr} + k_{nr})$$

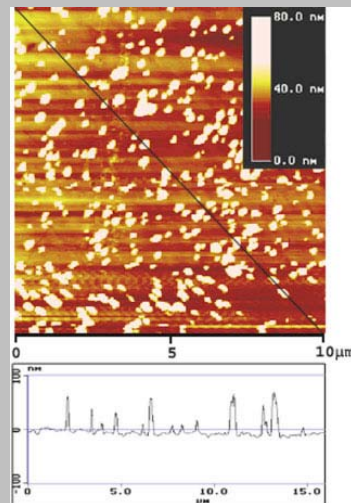
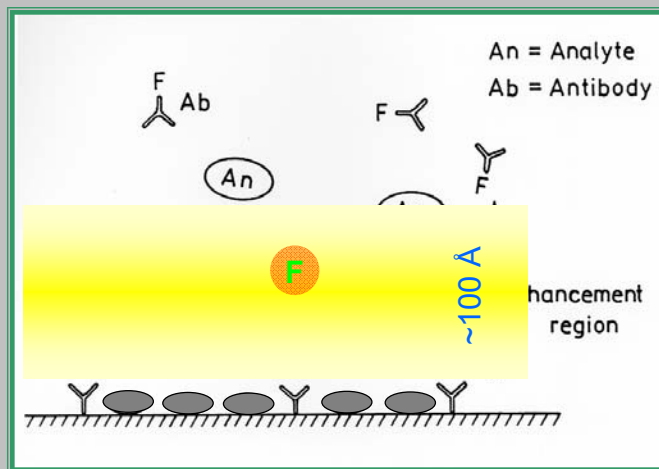
$$\tau_m = 1 / (\Gamma + \Gamma_m + k_{mnr} + k_{nr})$$

Metallic Surfaces can Create Unique Fluorophores with High Quantum Yields and Short Lifetimes



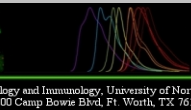
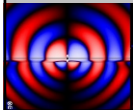
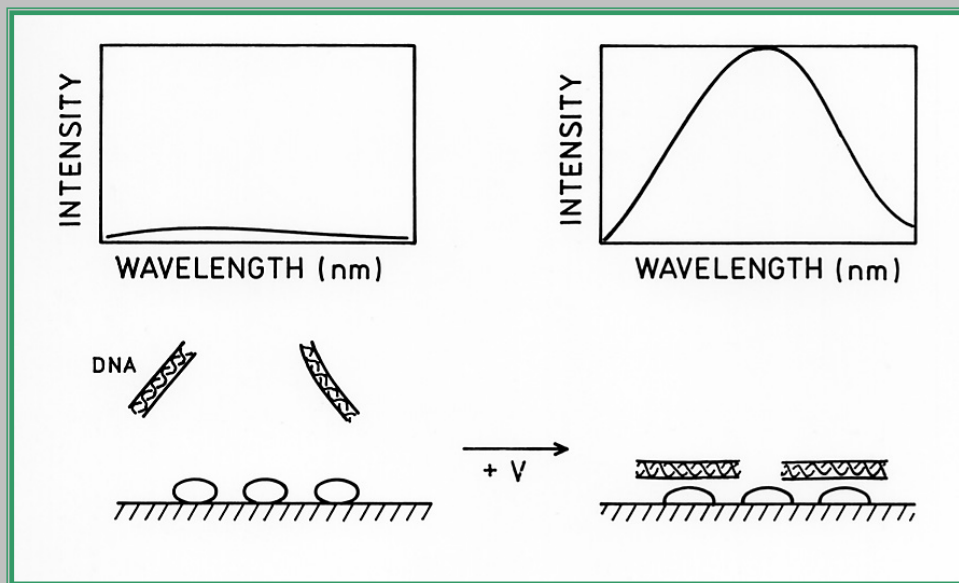


Fluorescence Immunoassays with Non-Fluorescent Chromophores



- Easy preparation
- Difficult to control

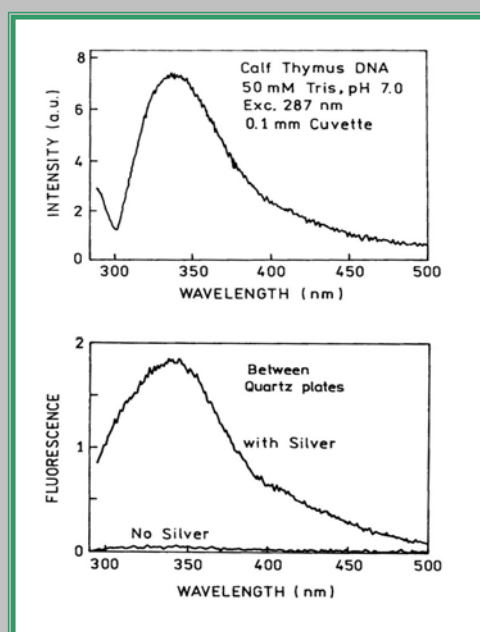
Intrinsic Emission from Unlabeled DNA



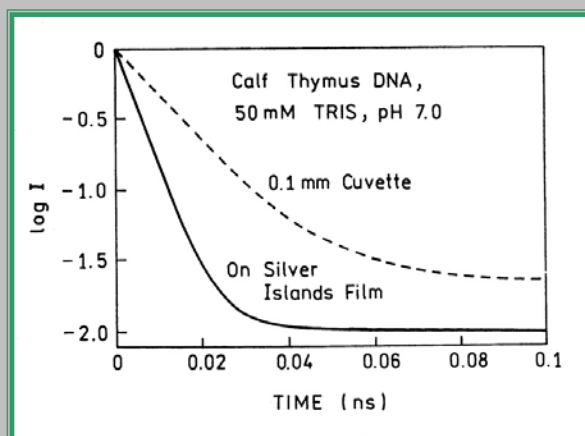
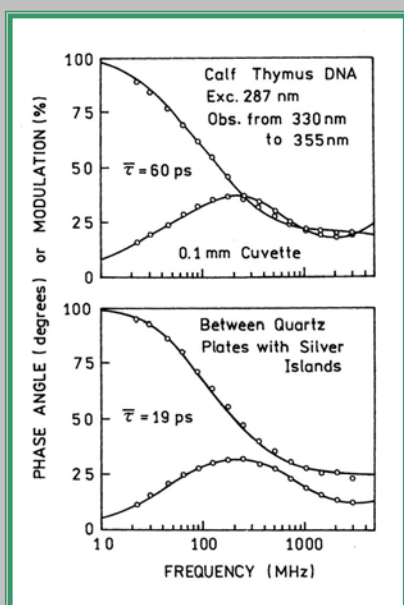
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Emission Spectra of Double Helical DNA in the Presence and Absence of Silver Island Films



Frequency-Domain Intensity Decays of Double Helical DNA in the Absence and Presence of a Silver Island Film



How Enhancement Depends on Distance ?

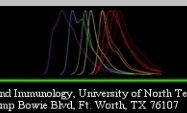
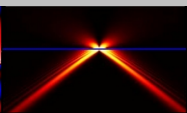
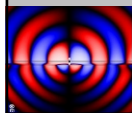
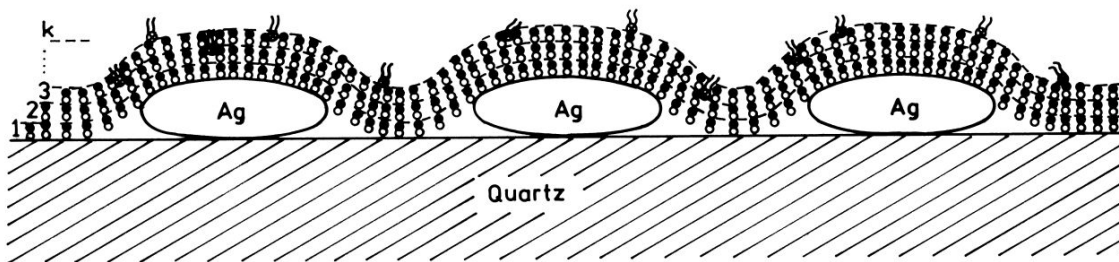
How to experimentally measure enhancement?



Distance Dependence of Enhanced Fluorescence of Cy3 and Cy5-Labeled Oligonucleotides

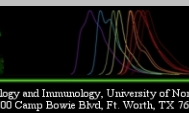
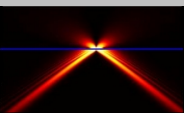
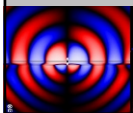
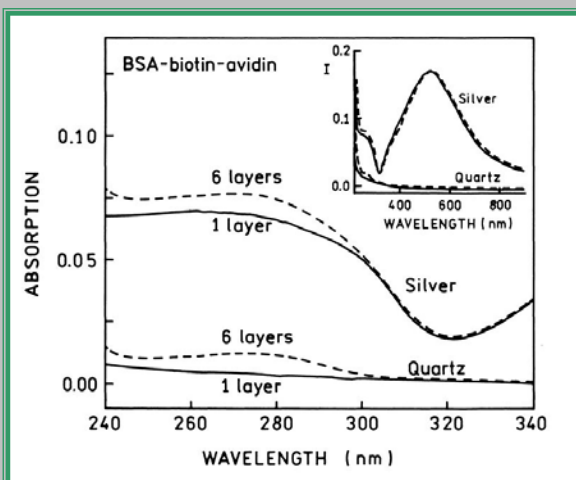
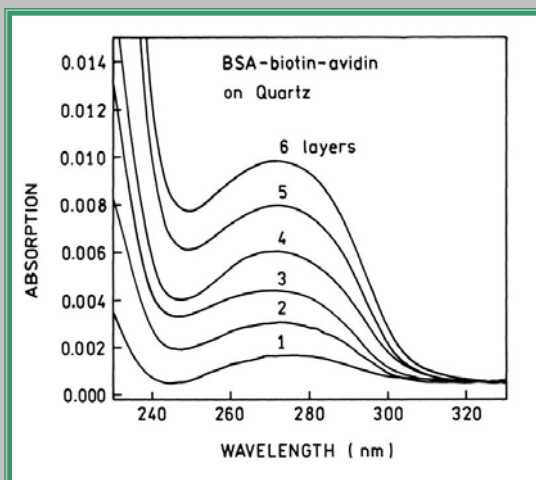
Thickness is about 90Å per
BSA-Avidin Layer

- BSA - biotin
- avidin
- ⌘ DNA(Cy3)-biotin or DNA(Cy5)-biotin

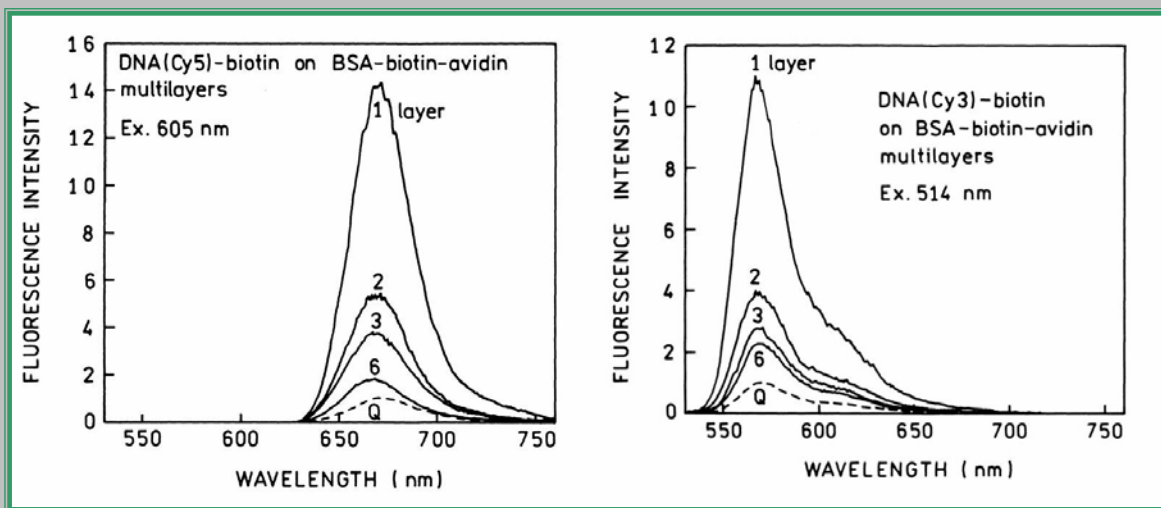


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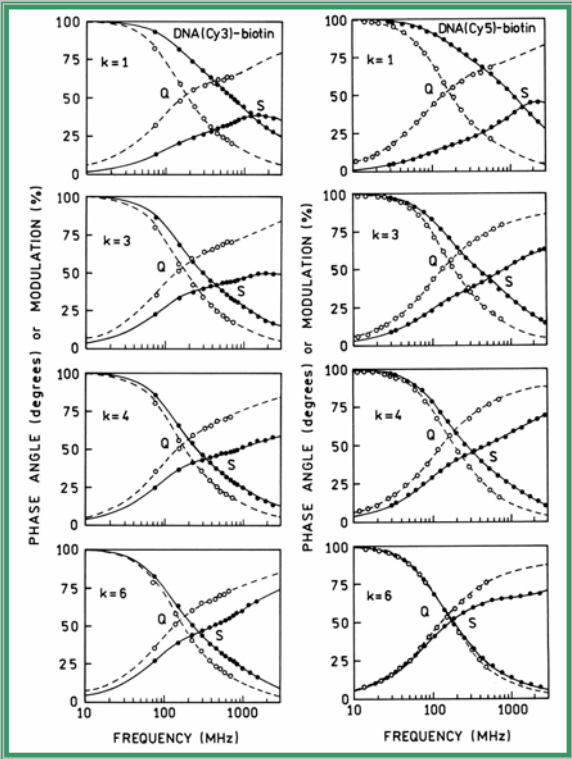
Absorption Spectra Show Complete Coverage for Each BSA-Biotin-Avidin Layer



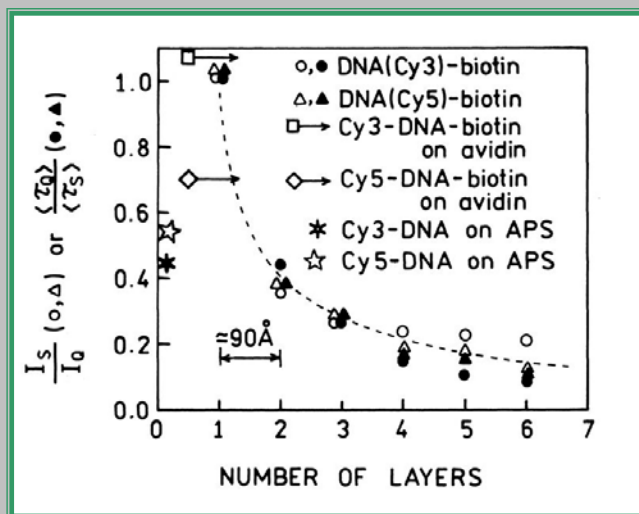
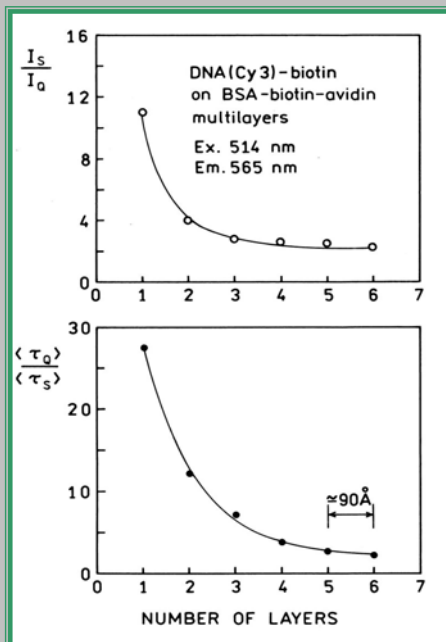
Dependence of Emission Intensity on Distance from Silver Particles



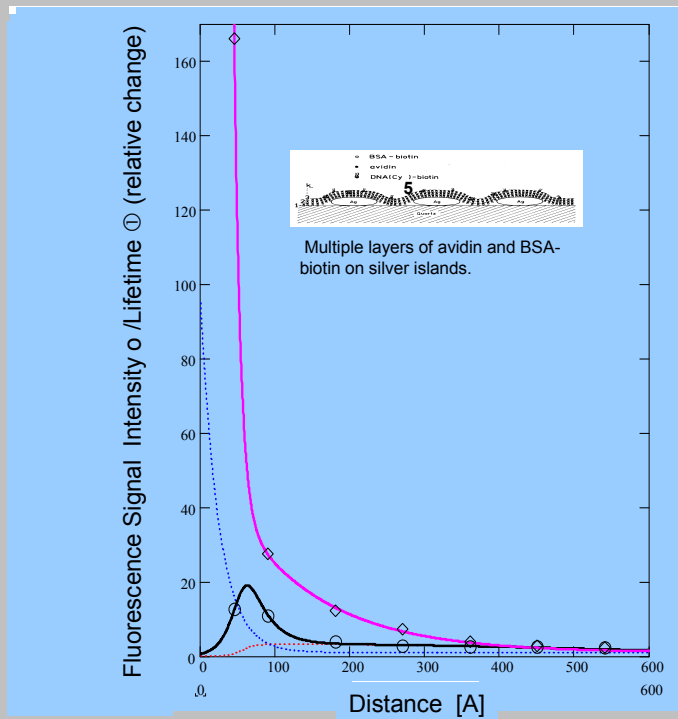
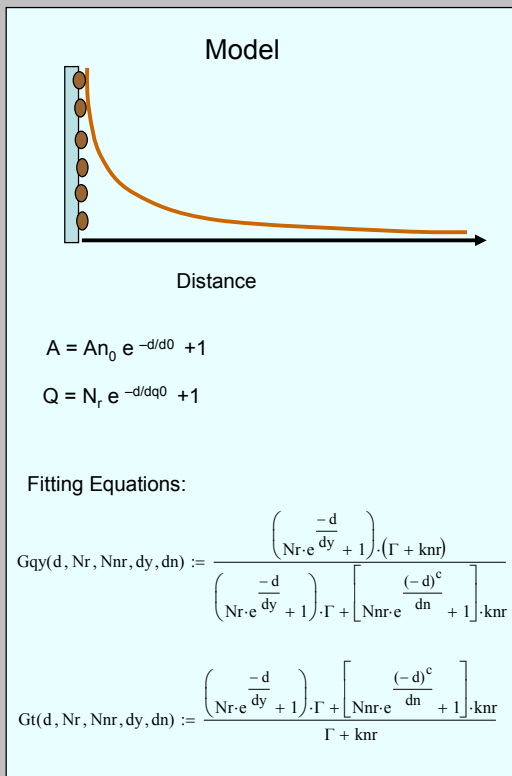
Shortest Lifetimes are Found for the Shortest Distance with BSA-Biotin-Avidin Layer



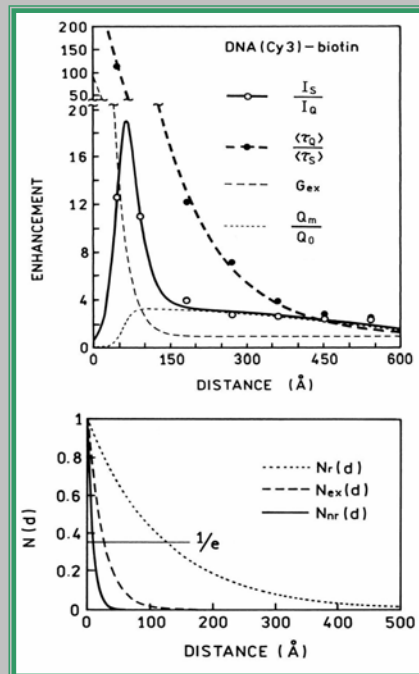
Relative Intensity and Lifetimes of DNA(Cy-dyes) on Quartz and SIFs



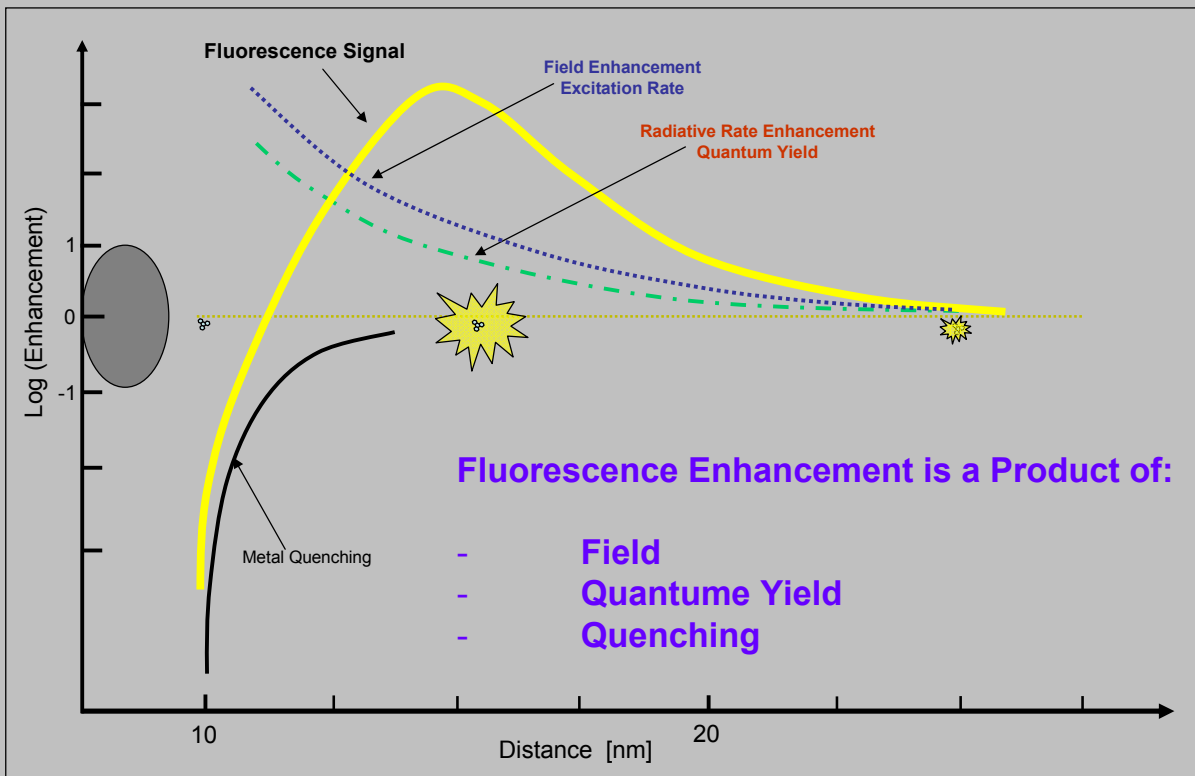
Model



Distance Dependence of Excitation (ex) Radiation (r) and Non-Radiative (ns) Decay



Fluorescent Properties Near Metal Particle



Fluorescence Enhancement is a Product of:

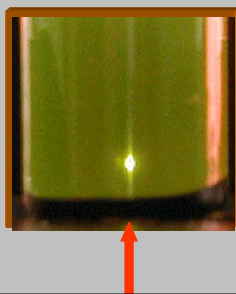
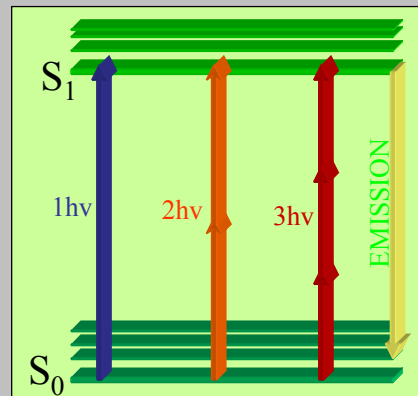
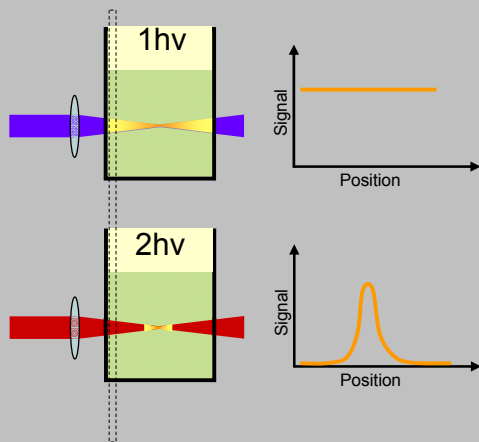
- Field
- Quantum Yield
- Quenching

How to separate field enhancement and quantum yield enhancement?

Use non-linear process like $2h\nu$
Or
Observe only single molecule



Multi-Photon Excitation - Ultra Bright Fluorophores



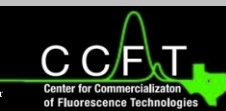
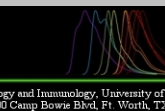
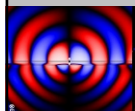
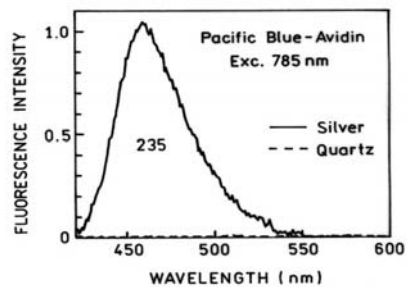
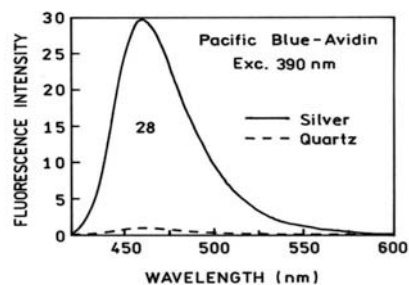
$$Q = \frac{\Gamma_r}{\Gamma_r + \Gamma_{nr}} \quad \tau = \frac{1}{\Gamma_r + \Gamma_{nr}}$$

$$Q_m = \frac{N_r \Gamma_r}{N_r \Gamma_r + N_n \Gamma_{nr}} \quad \tau_m = \frac{1}{N_r \Gamma_r + N_n \Gamma_{nr}}$$

$$G_{\omega} = \frac{Q_m}{Q} = \frac{N_r (\Gamma_r + \Gamma_{nr})}{N_r \Gamma_r + N_n \Gamma_{nr}} \quad G_r = \frac{\tau_m}{\tau} = \frac{\Gamma_r + \Gamma_{nr}}{N_r \Gamma_r + N_n \Gamma_{nr}}$$

$$G_{fl}^{(1)} = G_{exc} \cdot G_{em} = G_{exc} N_r \frac{\tau_m}{\tau} \quad G_{fl}^{(2)} = G_{exc}^2 \cdot G_{em} = G_{exc}^2 N_r \frac{\tau_m}{\tau}$$

$$\frac{G_{fl}^{(2)}}{G_{fl}^{(1)}} = G_{exc}$$



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3500 Camp Bowie Blvd, Ft. Worth, TX 76107

Table. Calculated enhancement factor for the labeled avidin

| Probe | $G_{fl}^{(1)}$ | $G_{fl}^{(2)}$ | G_{exc} | $\Gamma(ns^{-1})^b$ | $\Gamma_m(ns^{-1})^b$ | N_r |
|--------------|----------------|------------------|------------------|---------------------|-----------------------|-------|
| Pacific Blue | 28 | 235 | 25 | 0.32 | 11 ^c | 35 |
| | | | | | | |
| Lissamine | 16 | 200 ^a | 9.5 ^a | 0.53 | 23 ^c | 45 |
| | | | | | | |
| Texas Red | 46 | 200 ^a | 418 ^a | 0.16 | 71 ^c | 455 |
| | | | | | | |

^aCalculated assuming two-photon excitation displays a 200-fold increases in probe fluorescence on the silver island films.

^bCalculated using $\Gamma = Q/\langle\tau\rangle$

^cCalculated assuming the quantum yield $Q = 1.0$ on the silver island films

Conclusion

Picosecond Laser Diode Can Be Used for Two-Photon Excitation

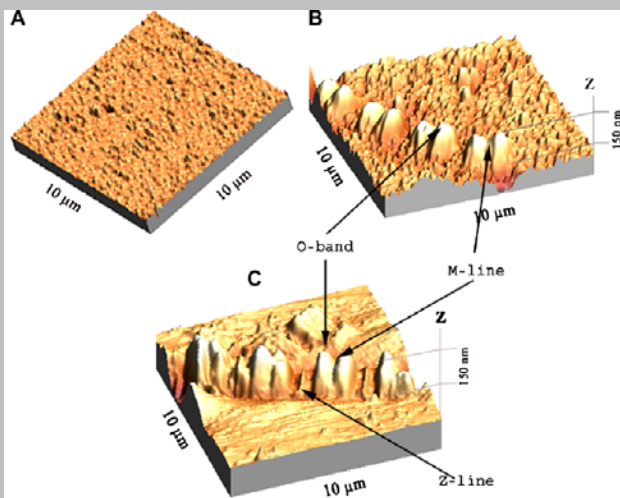
Fluorescence properties of labeled proteins near silver colloid surfaces (2003). Maliwal B.P., Malicka J., Gryczynski I., **Gryczynski Z.**, Lakowicz J.R., Biopolymers (Biospectroscopy), **70**, 585-594.

Is the enhancement real or could it be a concentration artifact?

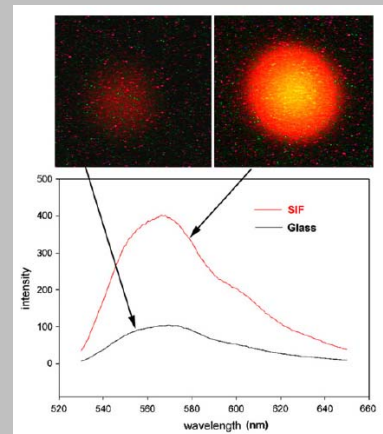
How to design experiment where the signal will NOT depend on dye deposition?



Application of MEF to Muscle Study

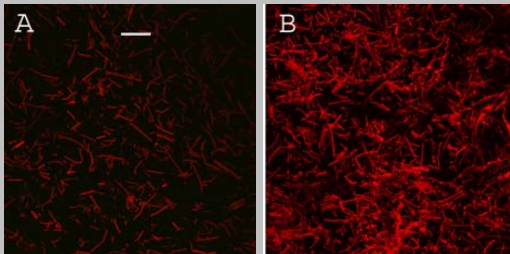


- (A) AFM image of glass coated with SIF
- (B) myofibrils on glass coverslips coated with SIF
- (C) myofibrils on glass coverslips



Enhancement of fluorescence by SIF. Myofibrils (1 mg/mL) labeled with 0.1 μ M Rh-phalloidin were placed on a glass coverslip (top left) and on a coverslip coated with SIF (top right). The spectra were measured at a 45 angle in a Varian Eclipse spectrofluorometer. The vertical scale is in arbitrary units (a.u.). The spectrum of SIF in the absence of muscle is less than 10 a.u. at all wavelengths.

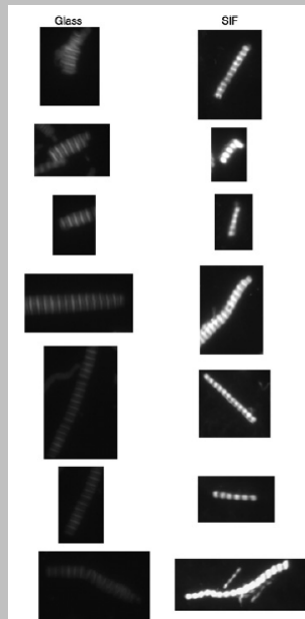
Increased Brightness on SIFs



Confocal image of myofibrils contributing to the fluorescence of

(A) myofibrils on glass coverslip;

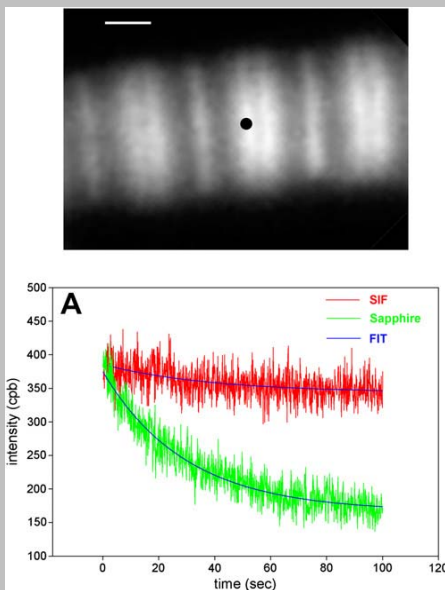
(B) myofibrils on glass covered with SIF. Bar is 10 μ m. Exposure is the same in each panel.



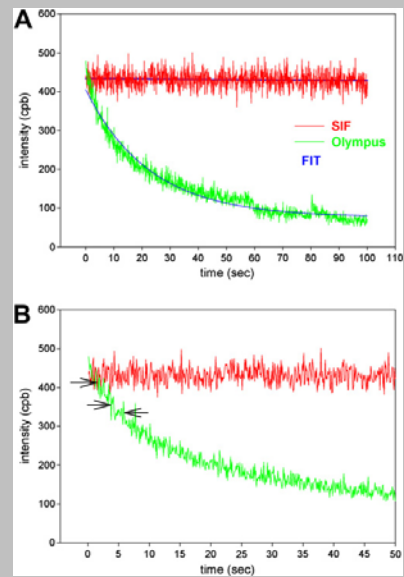
Enhancement of fluorescence by SIF using TIR fluorescence. Myofibrils were placed on glass coverslip (left column) or on glass coverslips coated with SIF and detected by TIRF. The representative images, from the faintest to the most intense, are shown. Exposure times and camera gain are the same for each panel.



Decreasing Photobleaching by Silver Island Films: Application to Muscle



Comparison of the rate of photobleaching of the myofibrillar overlap zone on uncoated sapphire (green) and on sapphire coated with SIF (red)



Comparison of the photobleaching of myofibrillar overlap zone on uncoated Olympus TIRF coverslips (green) and on Olympus TIRF coverslips coated with SIF (red)

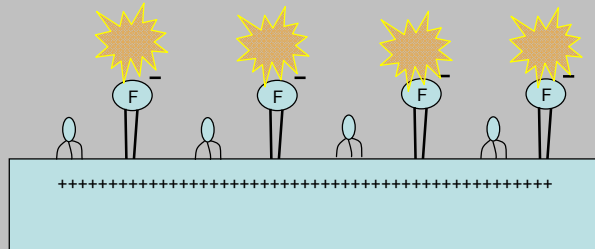
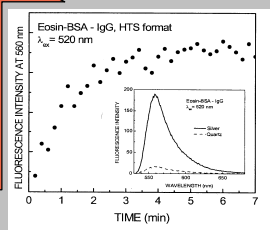
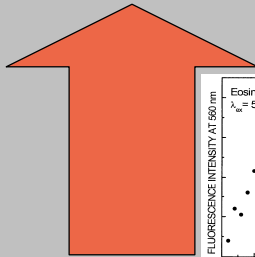
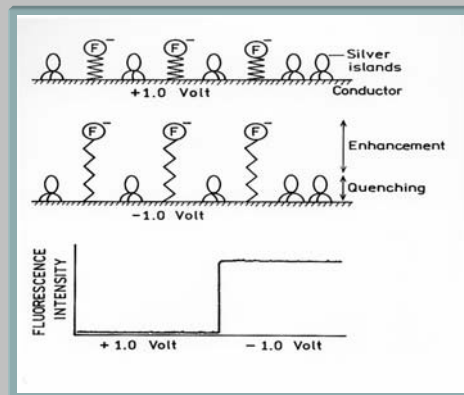
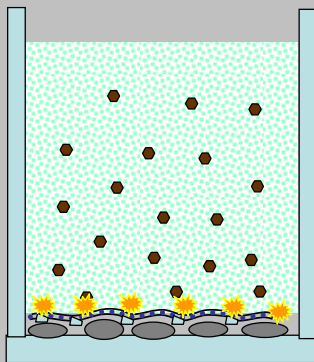
Anal. Biochem. (2007) **366**, 228-236

Biomedical Applications of Localized Plasmons

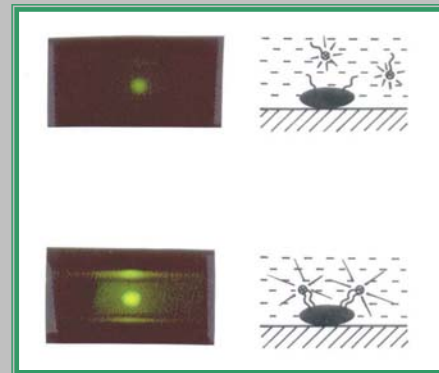
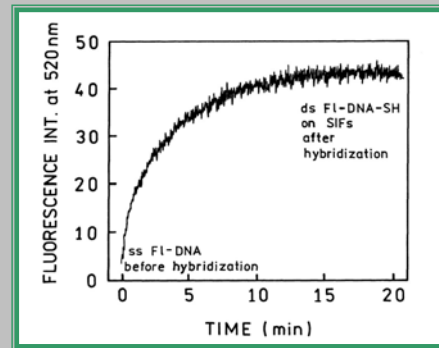
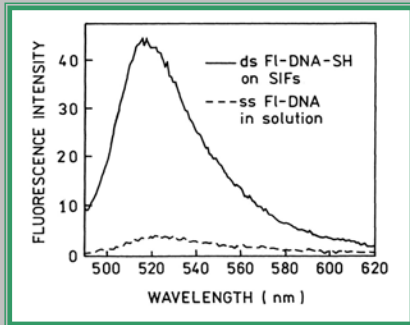
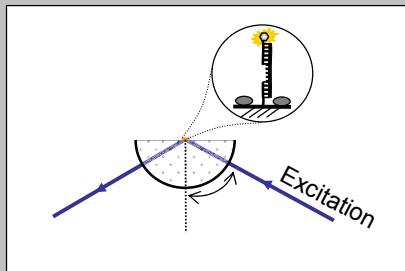


Fluorescence Assays Utilizing MEF

HTS Format Assay

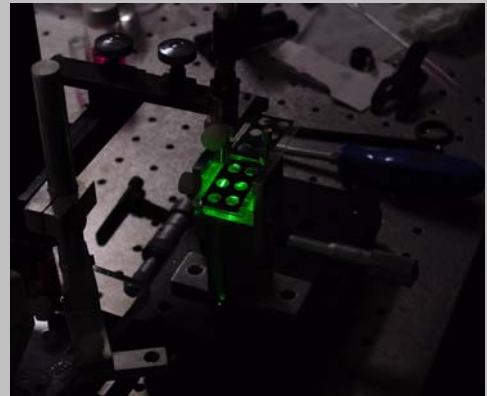
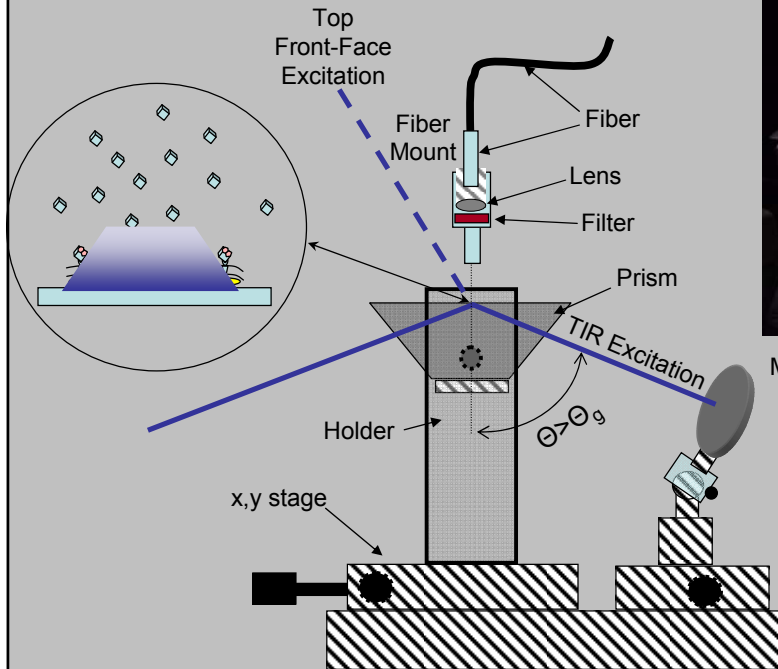


DNA Hybridization Can be Measured Using Silver – Enhanced Fluorescence



Use of surface plasmon-coupled emission to measure DNA hybridization (2004). Malicka J., Gryczynski I., Gryczynski Z., Lakowicz J.R., J. Biomol. Screening, 9(3), 208-215.

TIRF Immunoassay on a Silver Island Surface



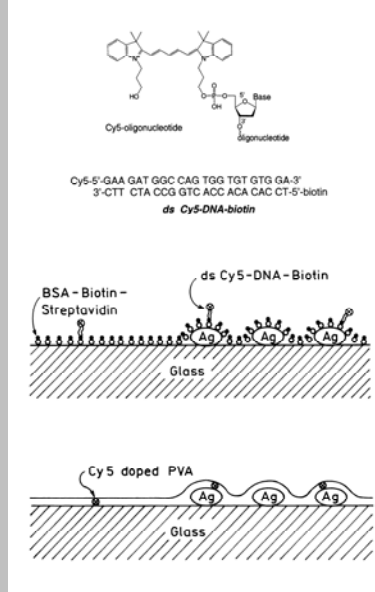
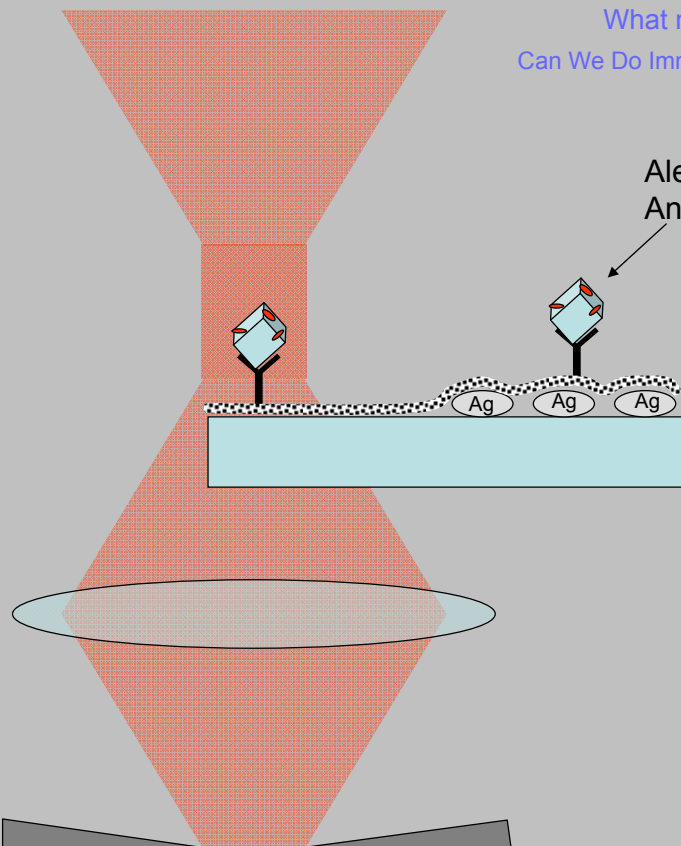
Mirror

Metal-enhanced fluorescence immunoassays using total internal reflection and silver island-coated surfaces (2004). Matveeva E., Gryczynski Z., Malicka J., Gryczynski I., Lakowicz J.R., *Anal. Biochem.*, **334**, 303-311.

Coupled plasmon effects for the enhancement of fluorescent immunoassays. (2007) Anne Barnetta, Evgenia G. Matveeva, Ignacy Gryczynski, Zygmunt Gryczynski, Ewa M. Goldys. *Physica B*. **394**, 297-300.

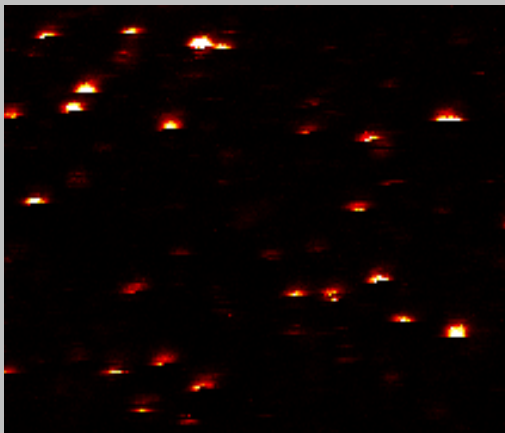
Scanning Confocal System. Capability of Separating 500 nm Spots

What maximum enhancement to expect ?
Can We Do Immunoassay on a Single Molecule Level ?

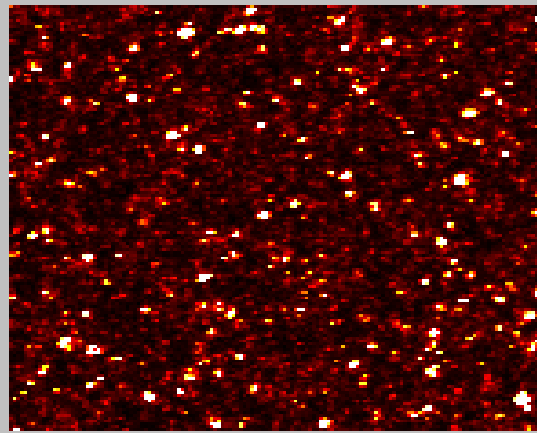


Monolayer of Labeled Antibodies

On Glass
(Intensity Scale 0-10)

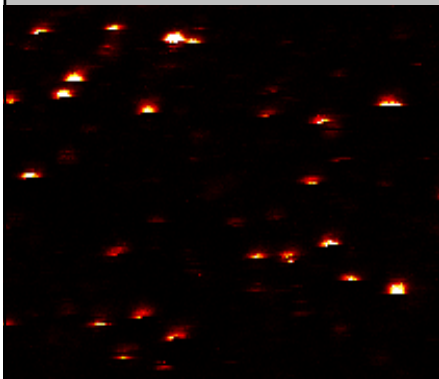


On Silver Island Film
(Intensity Scale 0-100)

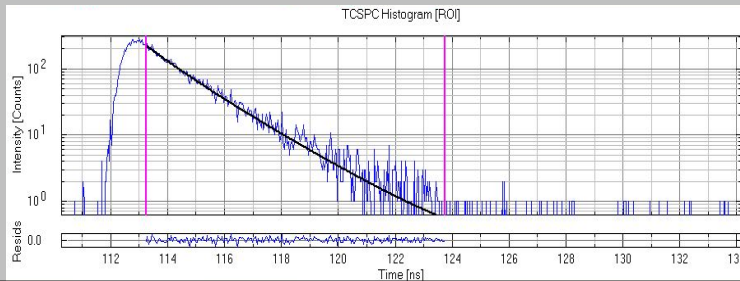
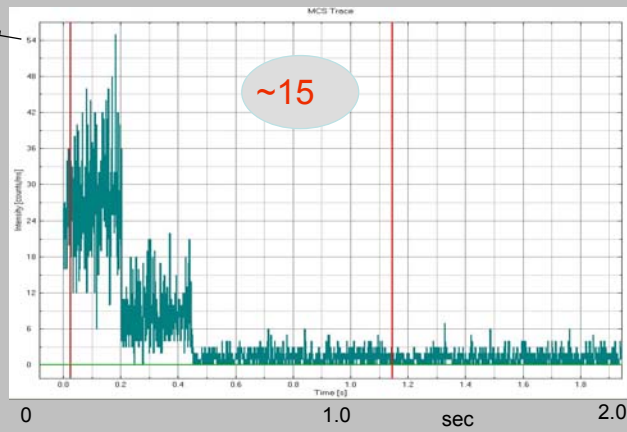


Future of Biomedical Assays – Assay Based on Single Molecule

Alexa647 c=1pg/ml **GLASS** $\lambda=635\text{nm}$ Filter HQ685nm/70

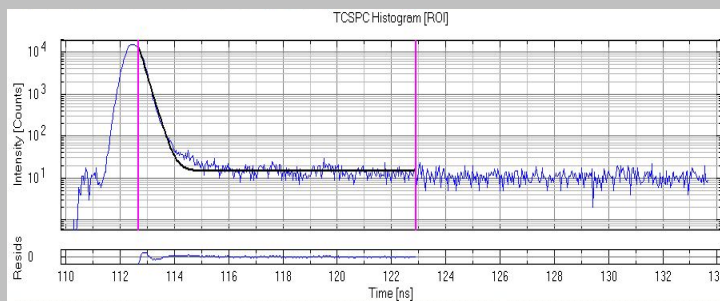
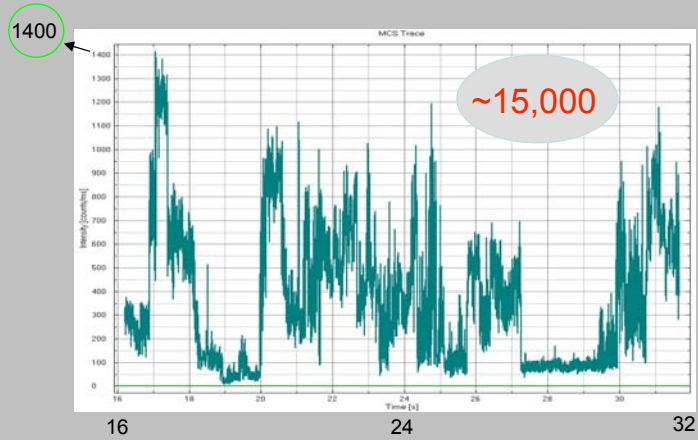
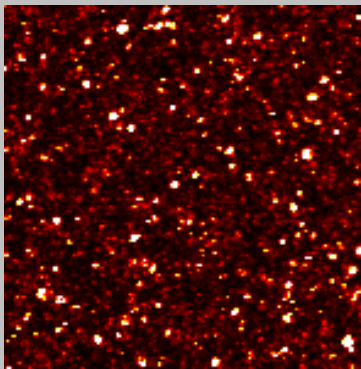


54



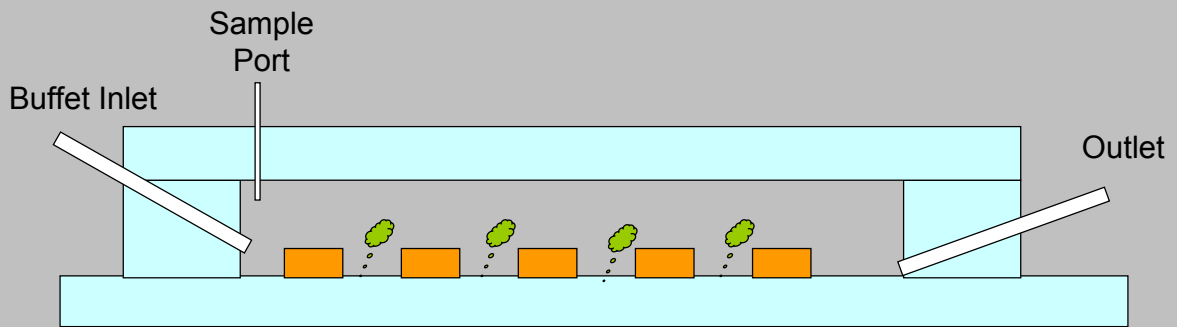
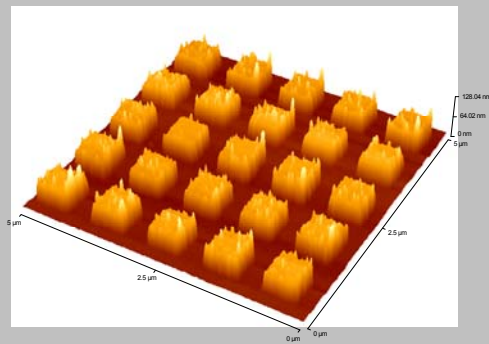
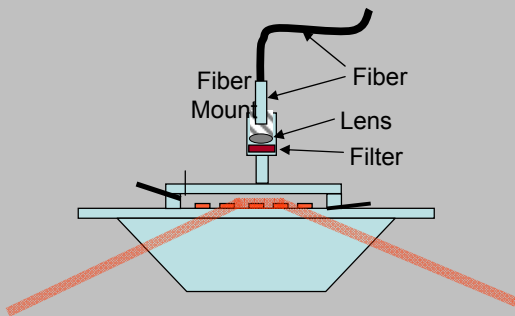
| | |
|----------|--------|
| Ampl. 1 | 140.59 |
| Lifet. 1 | 1.777 |
| Ampl. 2 | 82.2 |
| Lifet. 2 | 0.968 |
| Backgr. | 0.16 |

Alexa647 c=1pg/ml **SILVER** $\lambda=635\text{nm}$ Filter HQ685nm/70



| | |
|----------|----------|
| Ampl. 1 | 13360.91 |
| Lifet. 1 | 0.201 |
| Backgr. | 14.5 |

Assay Based on Nanostructures and Microfluidics

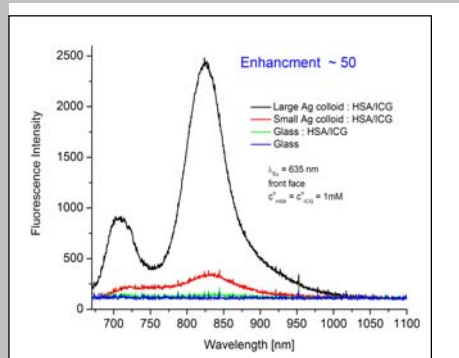
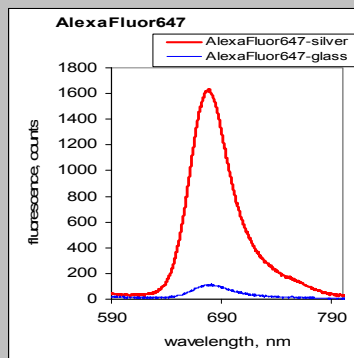
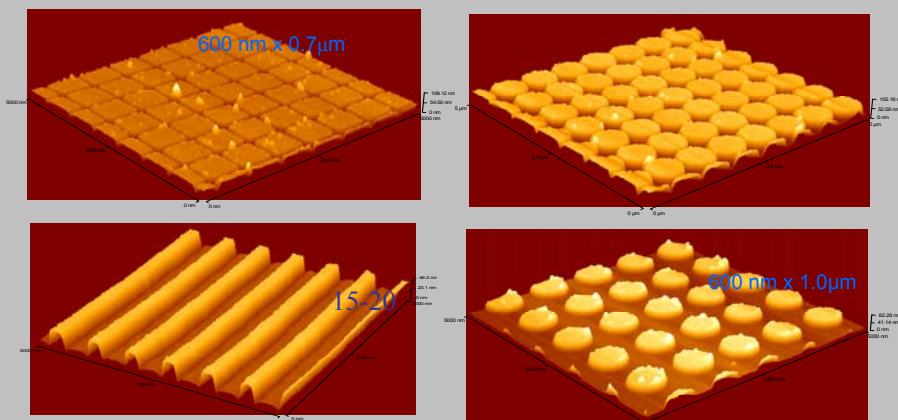


Can we improve enhancement?

New Structures

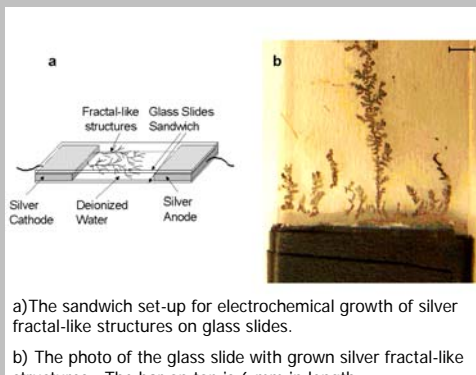


New Structures



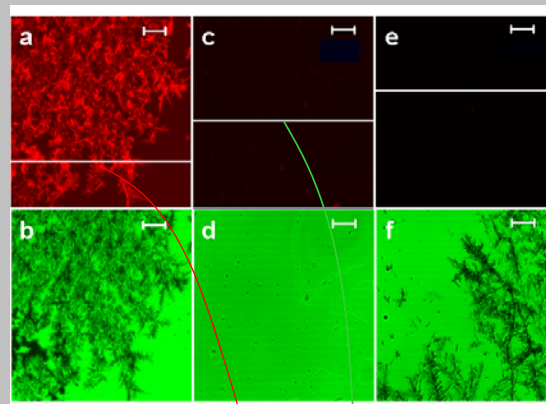
New Structures

Fluorescence Enhancement on Silver Fractal-Like Structures



a) The sandwich set-up for electrochemical growth of silver fractal-like structures on glass slides.

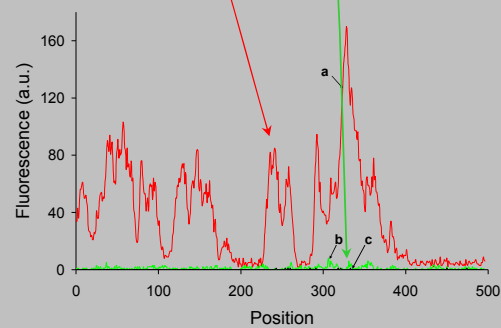
b) The photo of the glass slide with grown silver fractal-like structures. The bar on top is 6 mm in length.



The fluorescence intensities due to the immunoassay measured at the cross-section of the glass slide with fractal-like structures shown in Figure a as a white line.

The fluorescence intensities due to the immunoassay measured at the cross-section of the clean glass slide shown in Figure c as a white line.

The fluorescence intensities due to non-specific binding measured at the cross-section of the glass slide with fractal



JACS (2007) **129**, 12117-12122

Anal. Biochem. (2008) **80** (6), 1962-1966

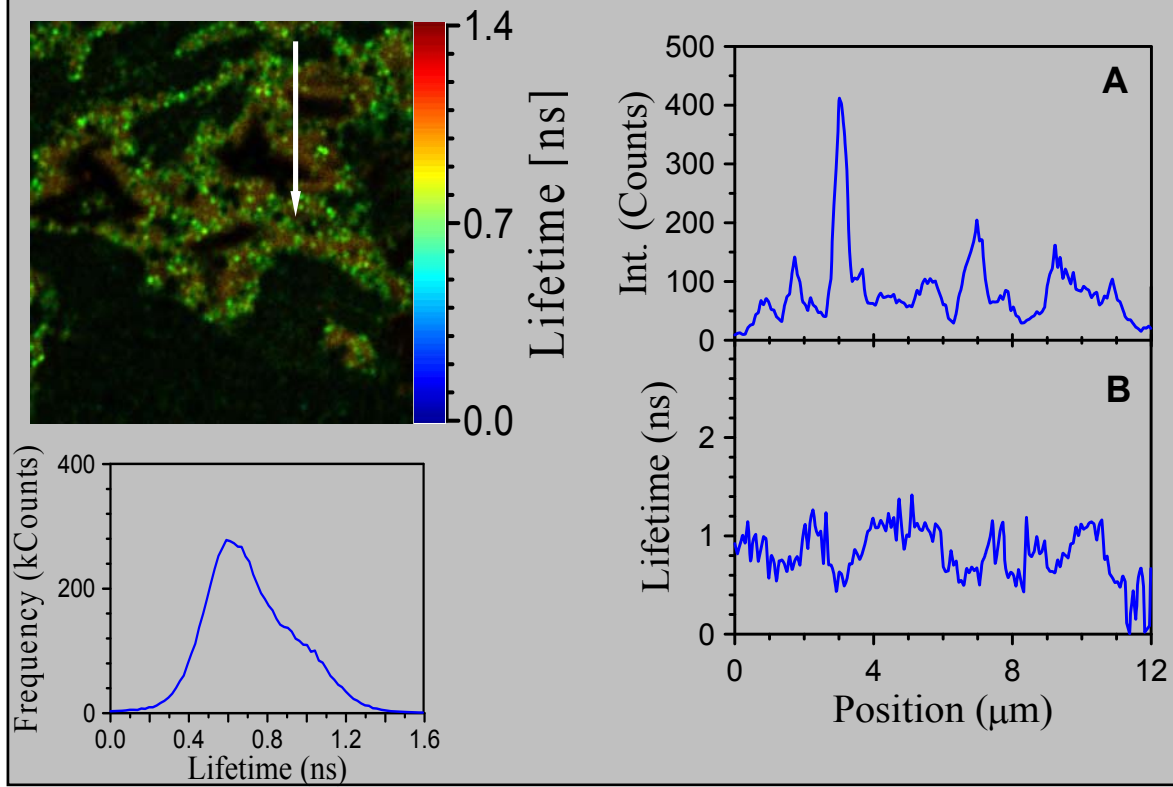
What could be better now?

FRACTALS / GOLD MIRROR



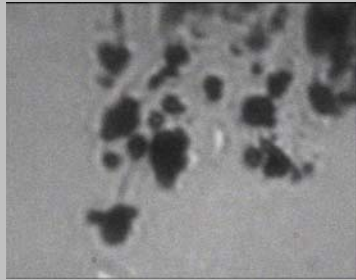
Model Immunoassay

The enhancement can be ~1000 fold

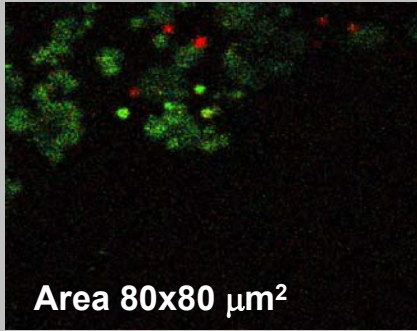


SM on FRACTALS

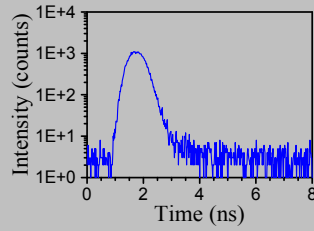
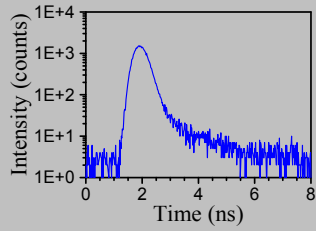
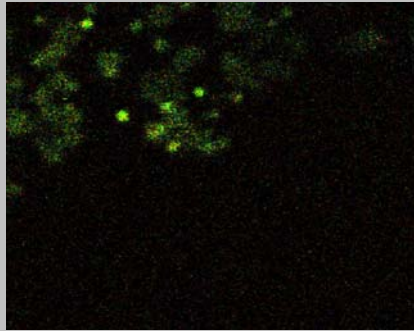


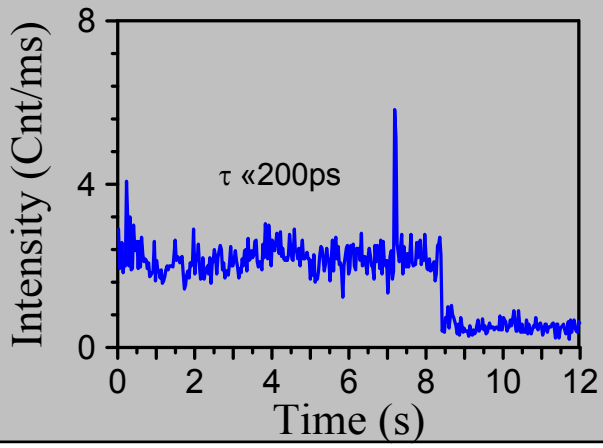
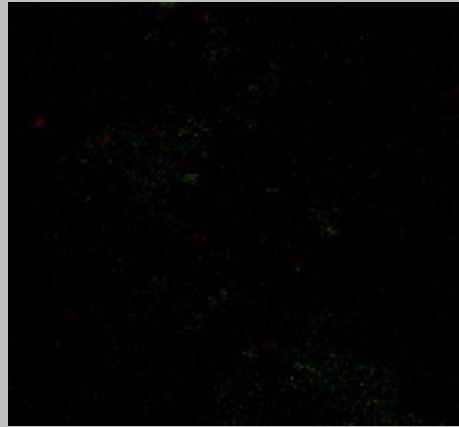
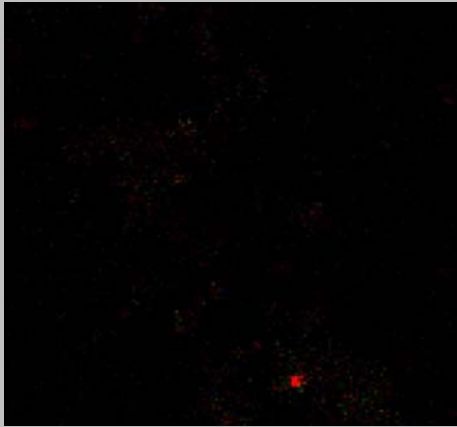


Laser power 2 μW
1 filter 665lp + 640lp RAZOR



Area 80x80 μm^2





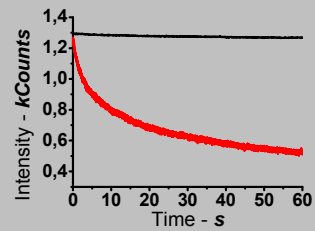
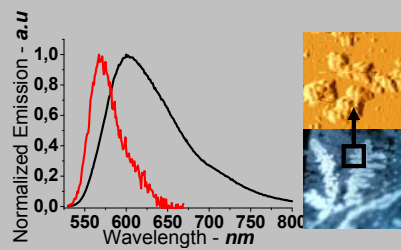
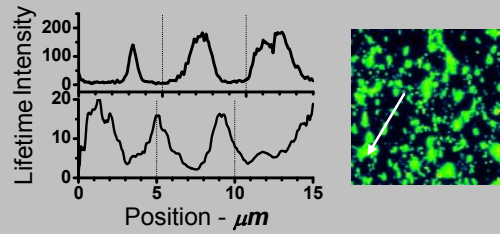
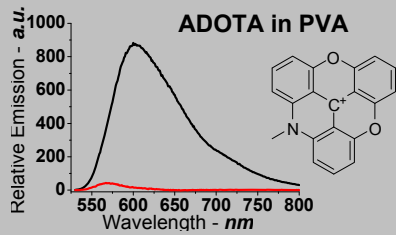
Intensity 0.2 μW
2xfilters 665lp + 640lp RAZOR

Self Assembling Colloidal Structures

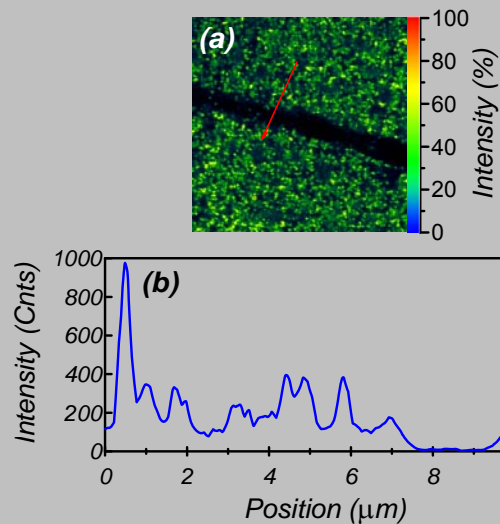
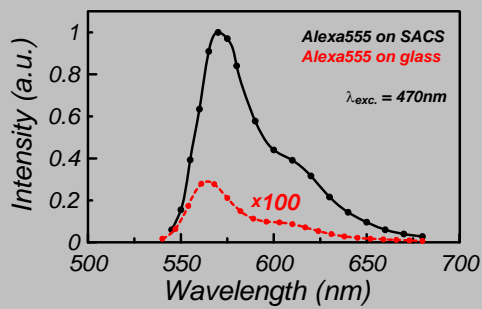
SACS



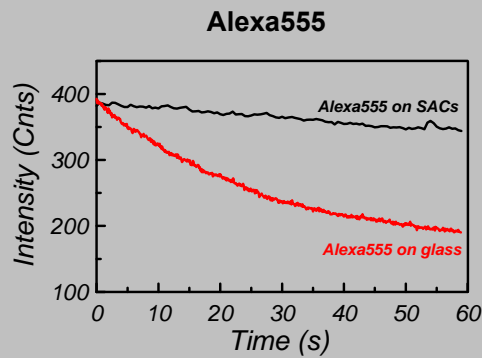
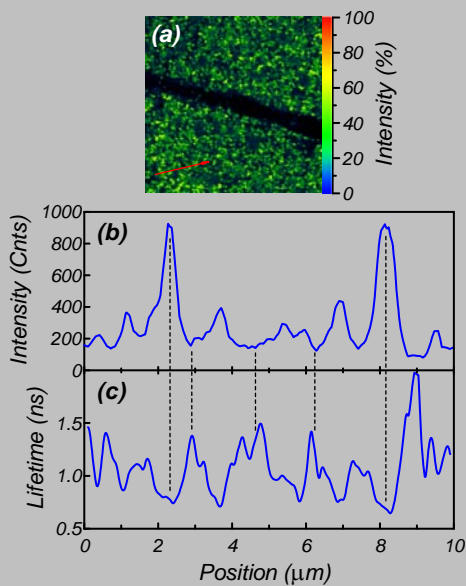
Metal Enhanced Fluorescence – Localized Plasmons Self-Assembled Colloids (SACs) on Gold Film



Self-Assembled Colloids (SACs) on Silver Film Alexa 555

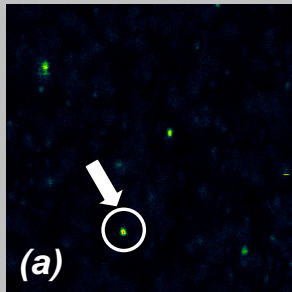


Metal Enhanced Fluorescence – Localized Plasmons Self-Assembled Colloids (SACs) on Silver Film

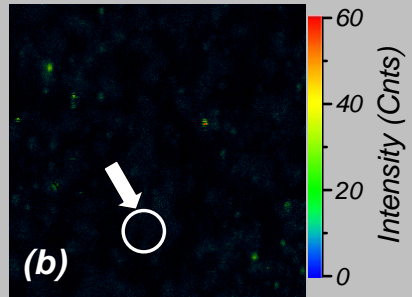


Metal Enhanced Fluorescence – Localized Plasmons Self-Assembled Colloids (SACs) on Silver Film

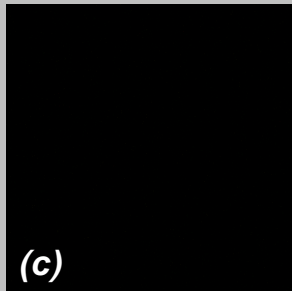
SM on SACs
Light int. 70nW (before photobl.)



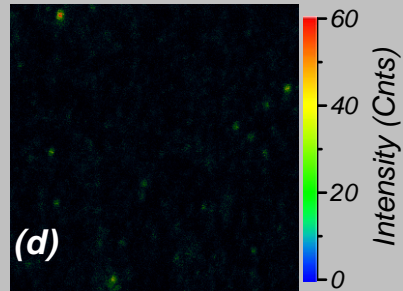
SM on SACs
Light int. 70nW (after photobl.)



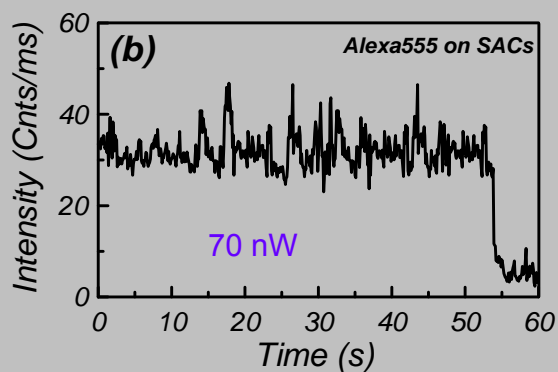
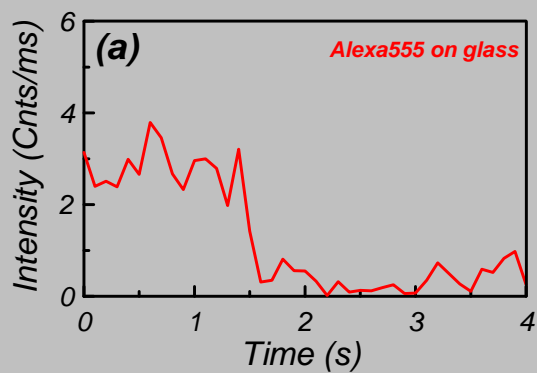
SM on glass
Light int. 70nW

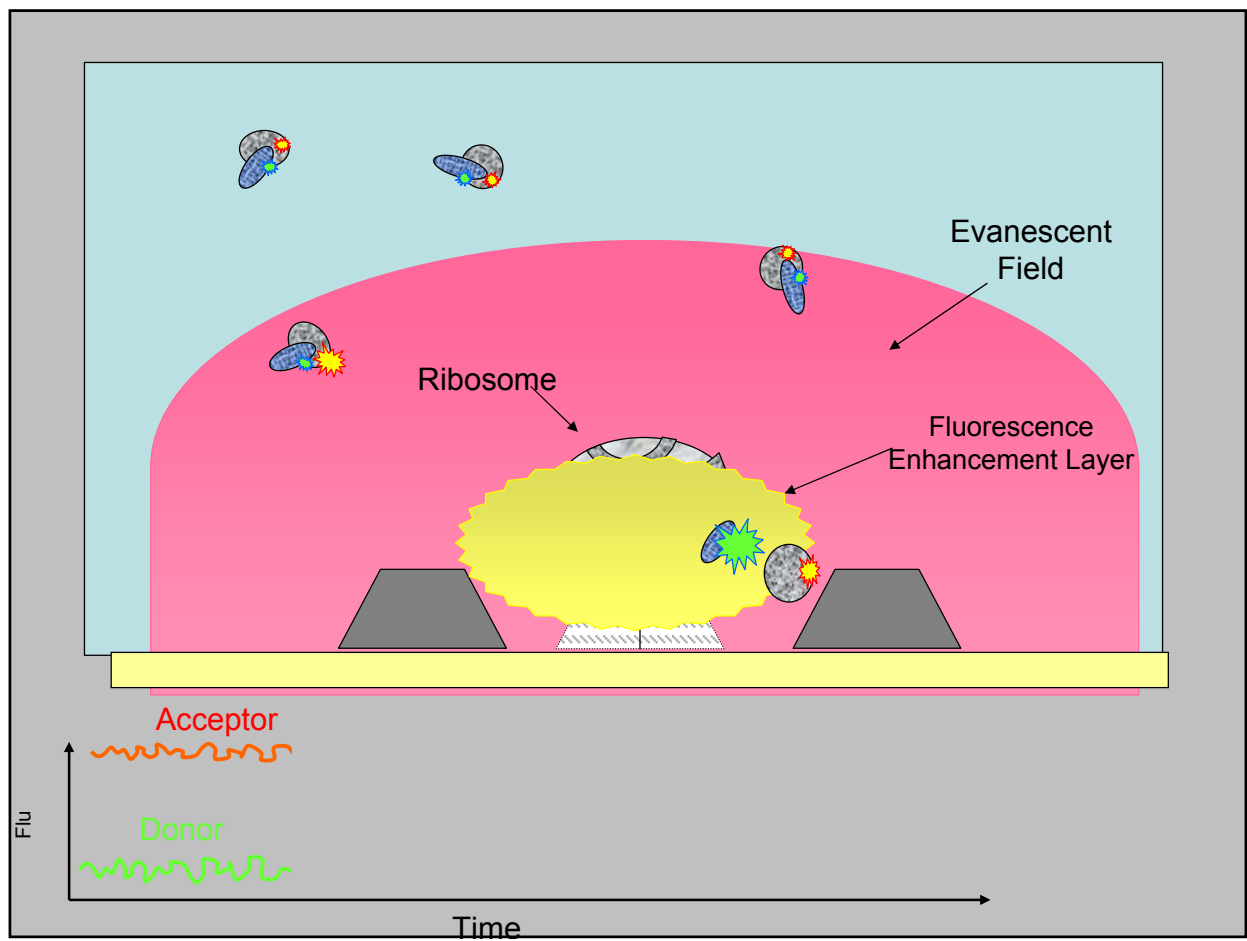


SM on glass
Light int. 3μW



Metal Enhanced Fluorescence – Localized Plasmons Self-Assembled Colloids (SACs) on Silver Film



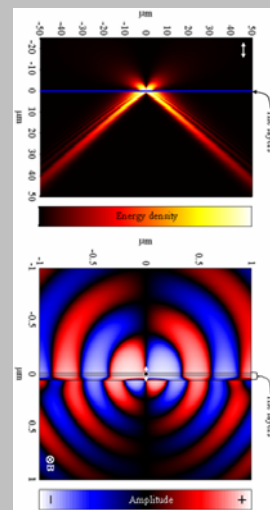
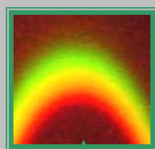
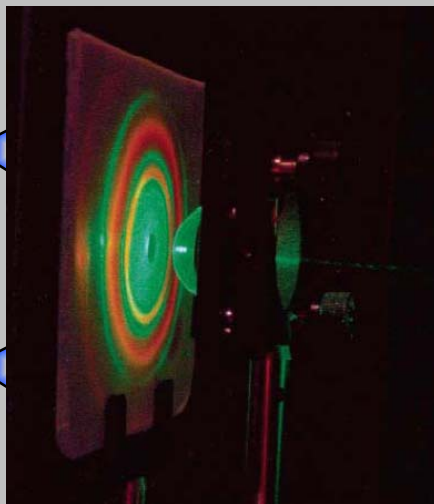


Traveling Surface Plasmons

SPCE

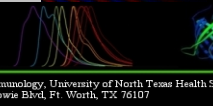
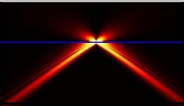
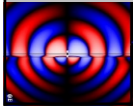
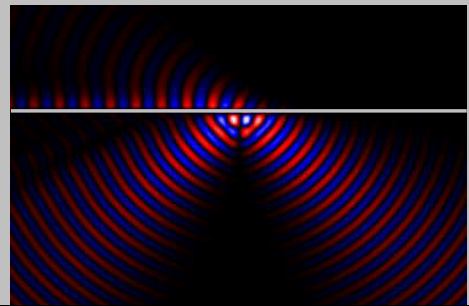
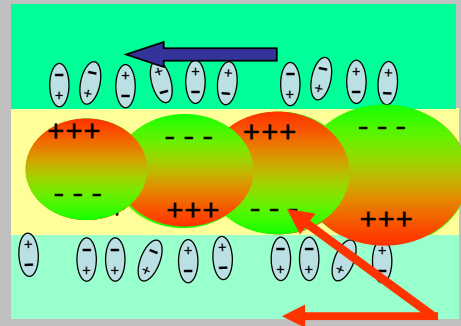
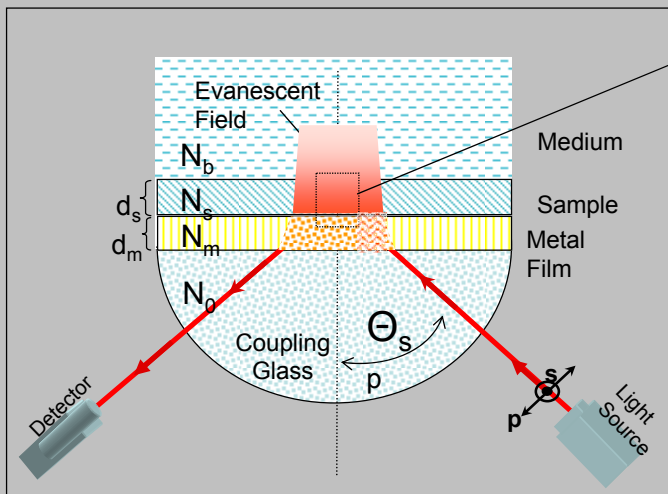
New Concept of Fluorescence

- Fluorophore emits in random directions



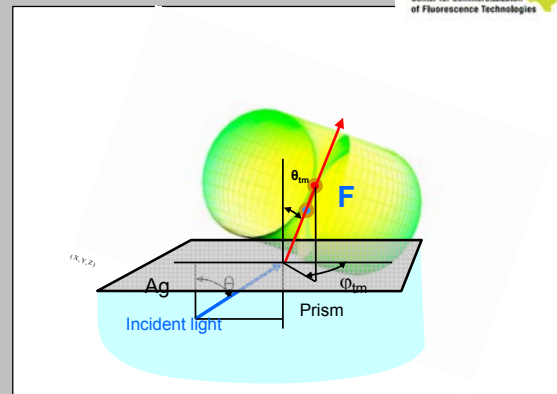
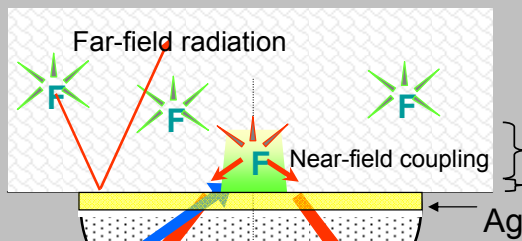
Wavelength resolution of SPCE

Surface Plasmons

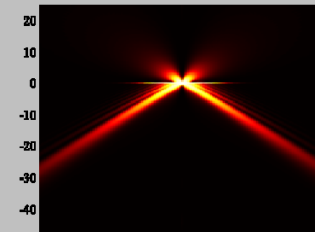
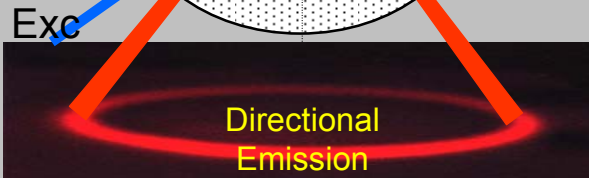


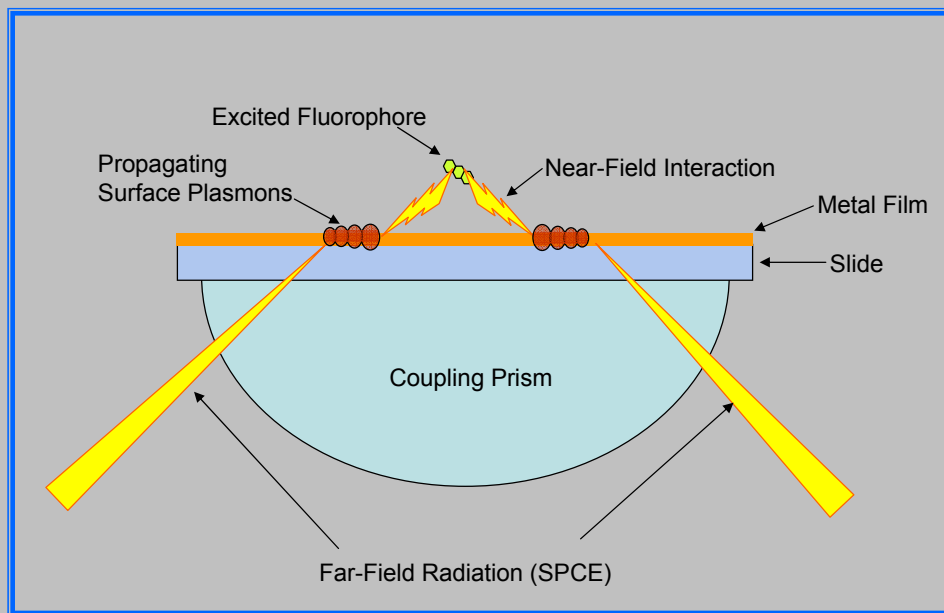
Near-Field Coupling of Fluorophore to SPP

Surface Confinement of
Excitation and Emission



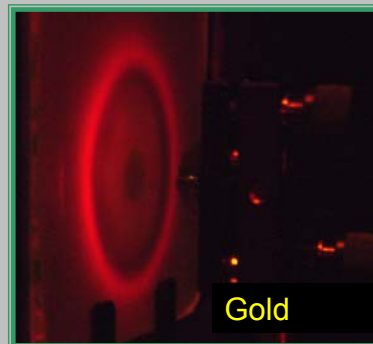
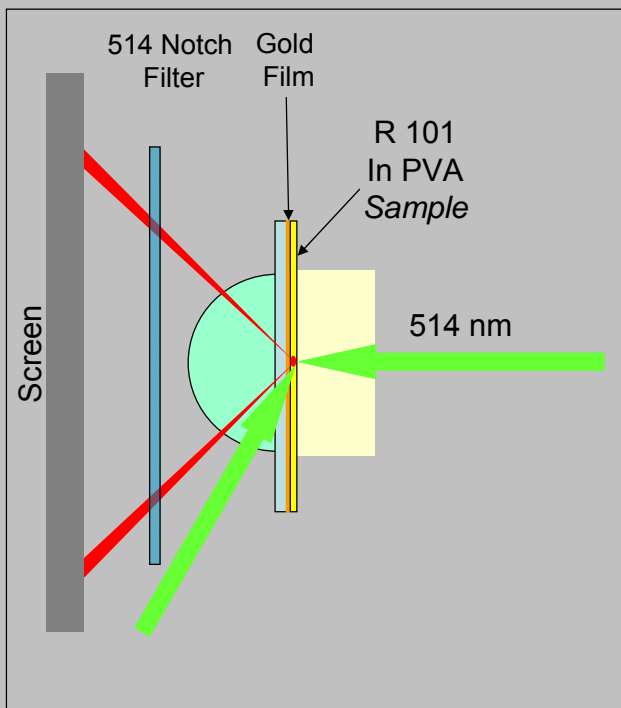
50 nm coupling range
5 nm metal quenching





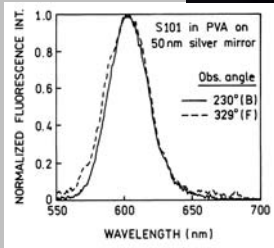
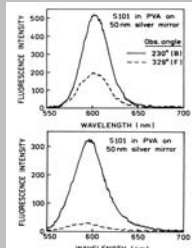
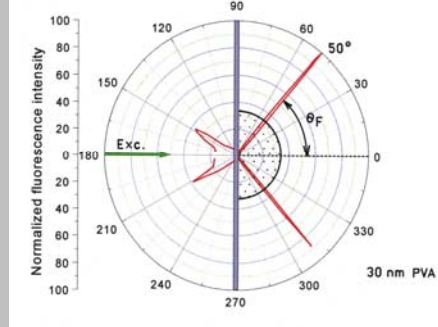
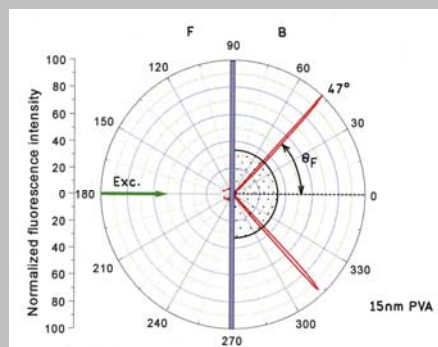
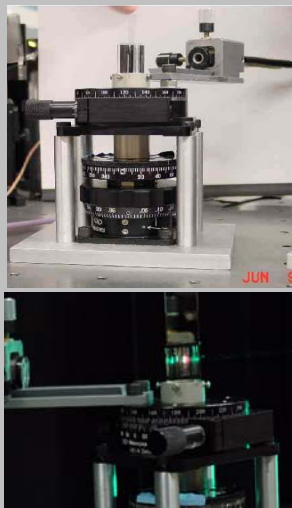
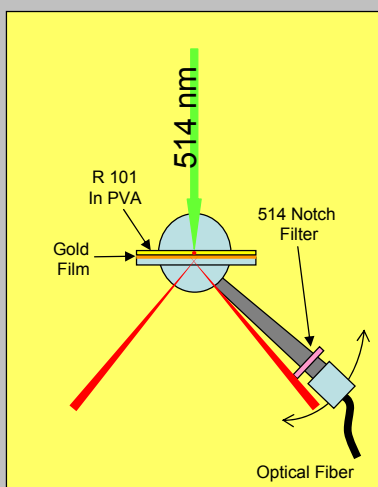
Schematic of process involved in creating the directional emission (SPCE)

Examples of SPCE - Different Metals



Surface-plasmon-coupled emission: New technology for studying molecular processes. Gryczynski Z., Gryczynski I., Matveeva E., Malicka J., Nowaczyk K., Lakowicz J.R., in Cytometry: New Developments (Methods in Cell Biology, Vol. 75) 4th edition, Darzynkiewicz Z., Roederer M. and Tanke H.J. (Eds), Academic Press, 2004, pp. 73-104

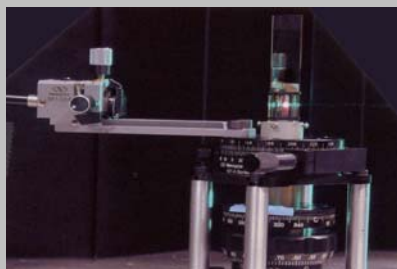
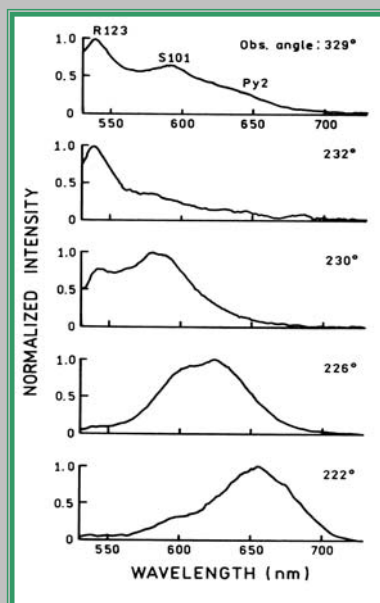
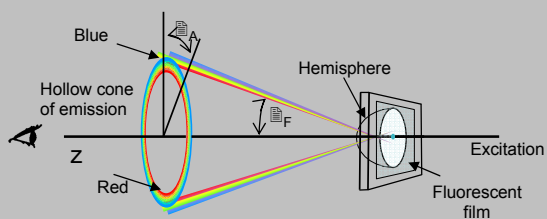
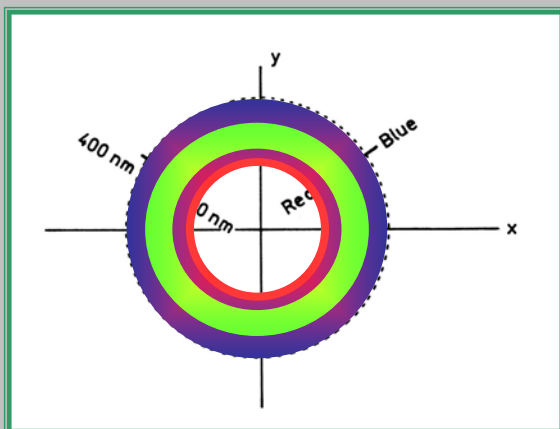
SPCE Angular Dependence



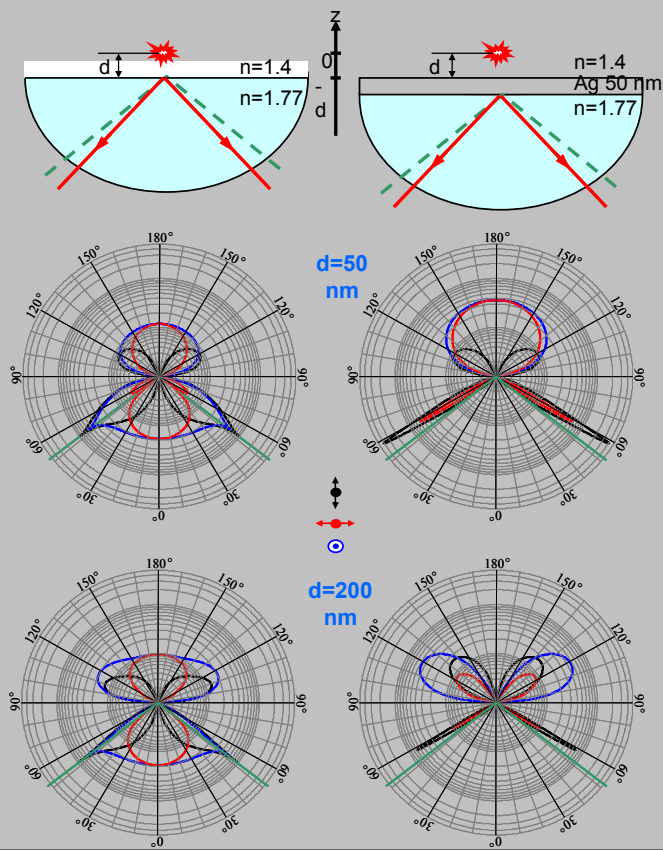
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Mixture of Fluorophores – Wavelength Resolution



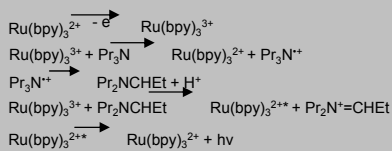
Radiation Diagrams



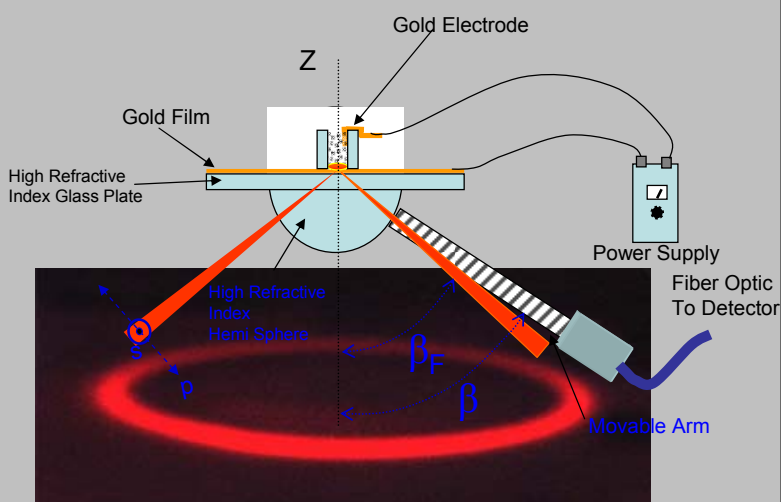
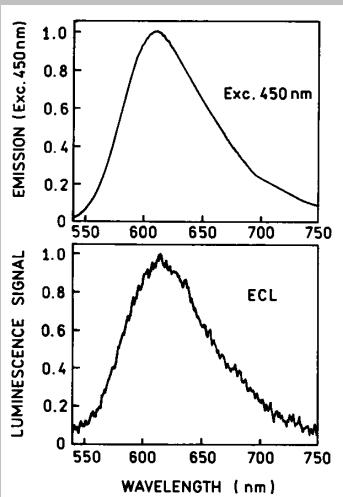
Does the SPCE effect depends on fluorescence origin

Schematic for Measurements of ELC Directionality

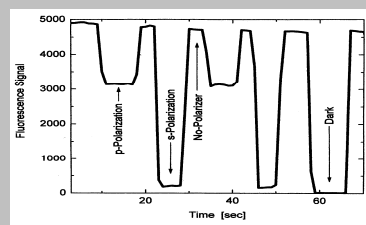
Chemical Mechanism

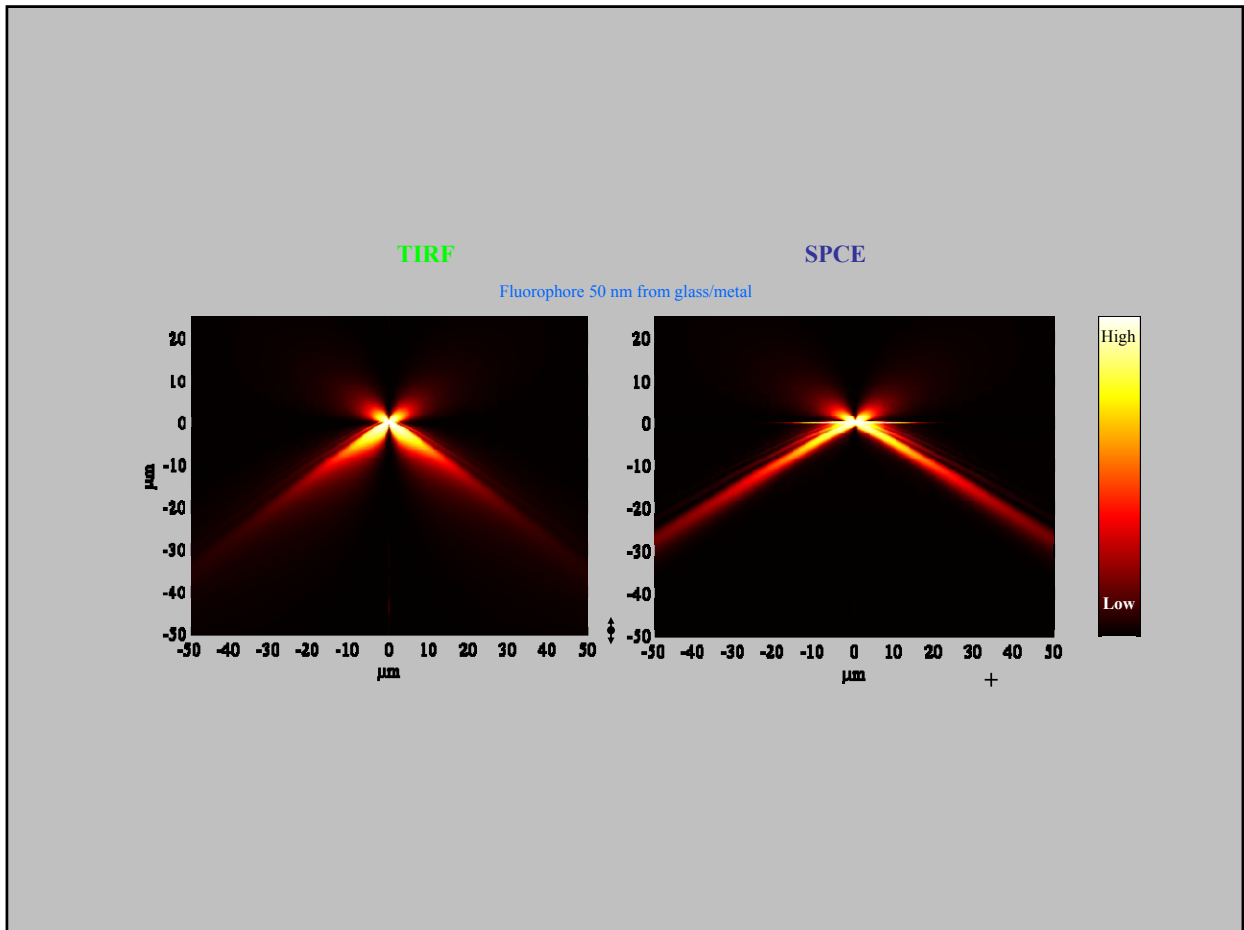


(Where Pr = CH₃CH₂CH₂- and Et = CH₃CH₂-)

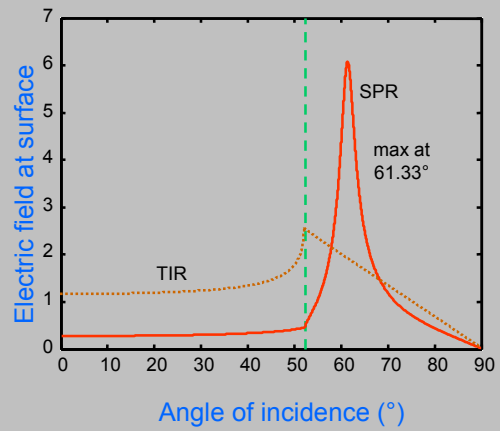
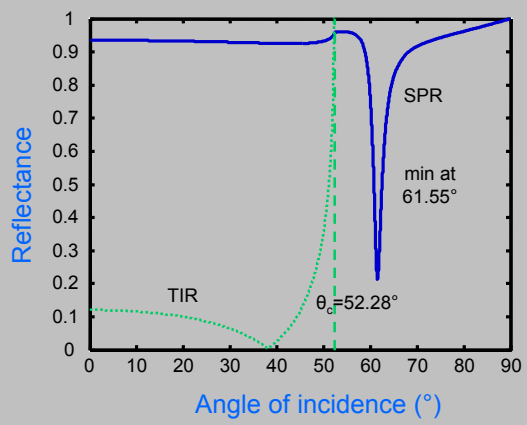


Polarization
of
ELC

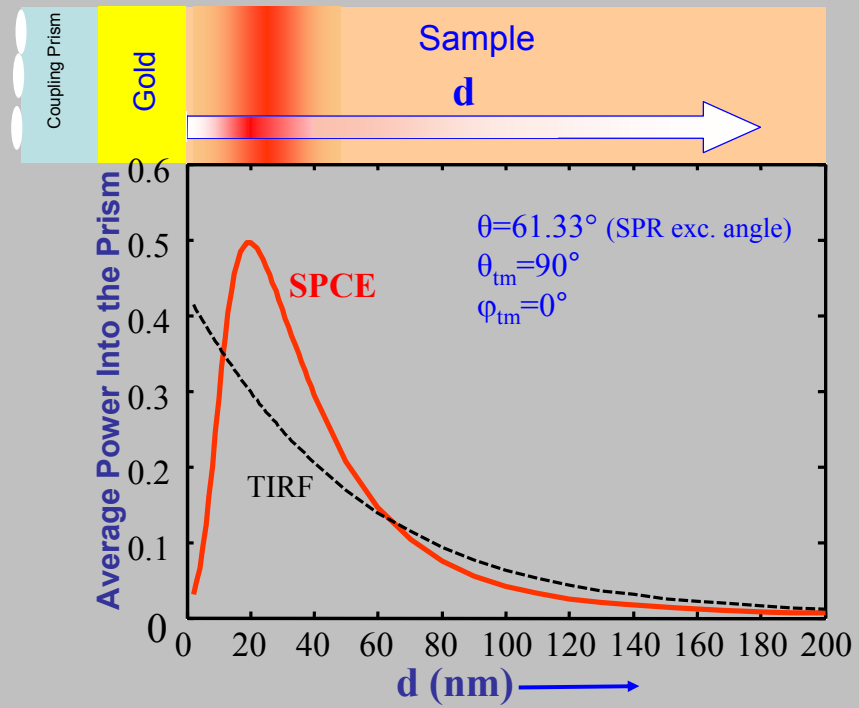




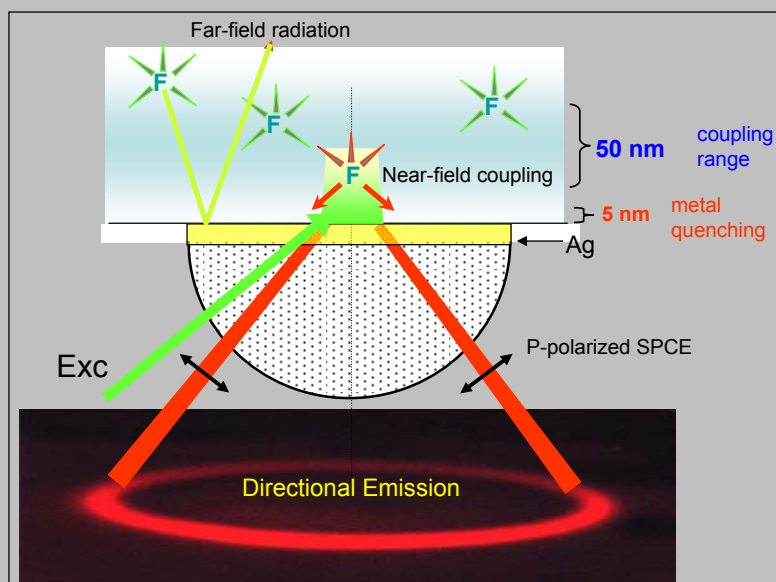
Calculated Reflectance and Electric Fields TIR and SPR Conditions What is the Effective Distance from the Interface



Effective Depth Penetration for SPCE



Surface Plasmon Assisted Microscopy (SPAM) Effective Volume



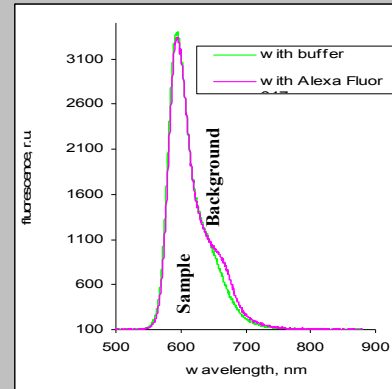
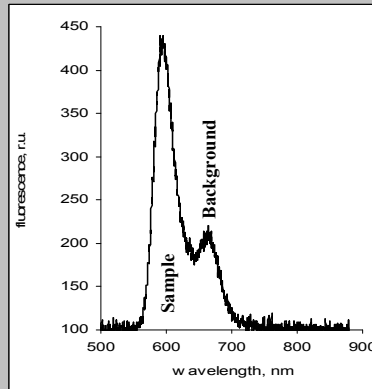
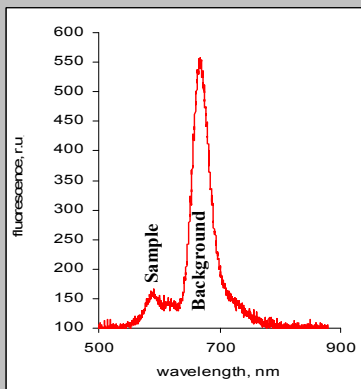
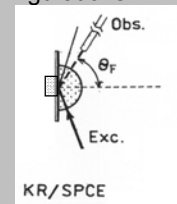
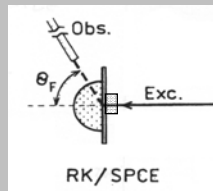
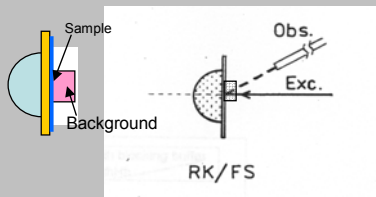
Concept of surface plasmon-coupled emission (SPCE). F is a fluorophore. The excitation energy of the fluorophore couples to the surface plasmons and radiates to the glass prism in a form of the ring. Far-field radiation is reflected by the metal surface.



Background Suppression by SPCE

Fluorescent Background

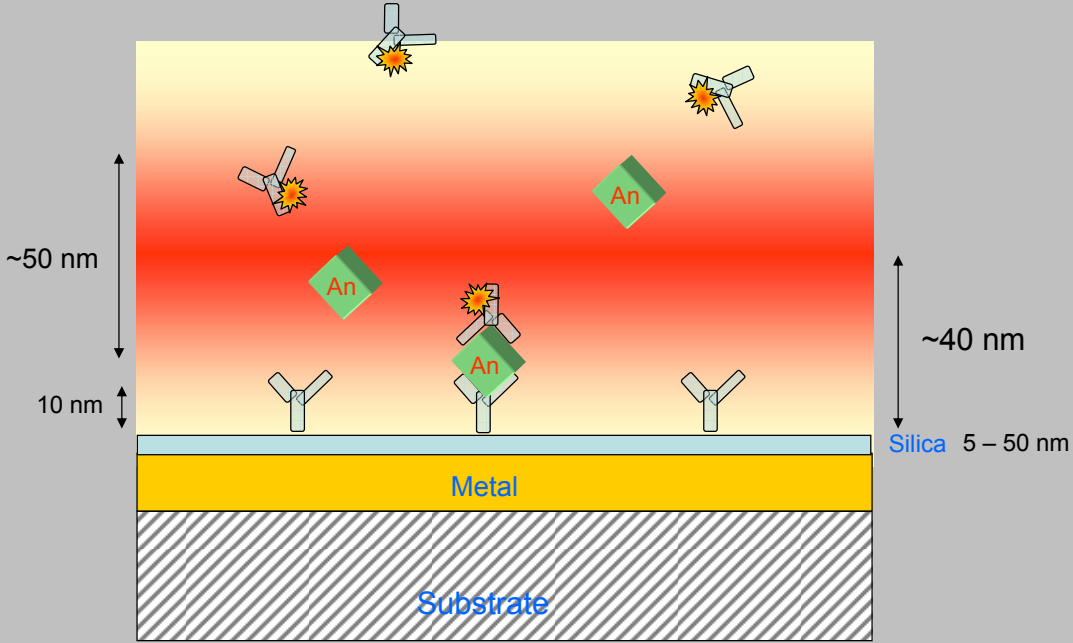
Emission spectra of the Rhodamine Red-X labeled anti-rabbit antibodies bound to the Rabbit IgG immobilized on the 50 nm silver mirror surface in presence of fluorescent background (anti-Mouse antibodies labeled with Alexa Fluor 647) in solution measured at different configurations.



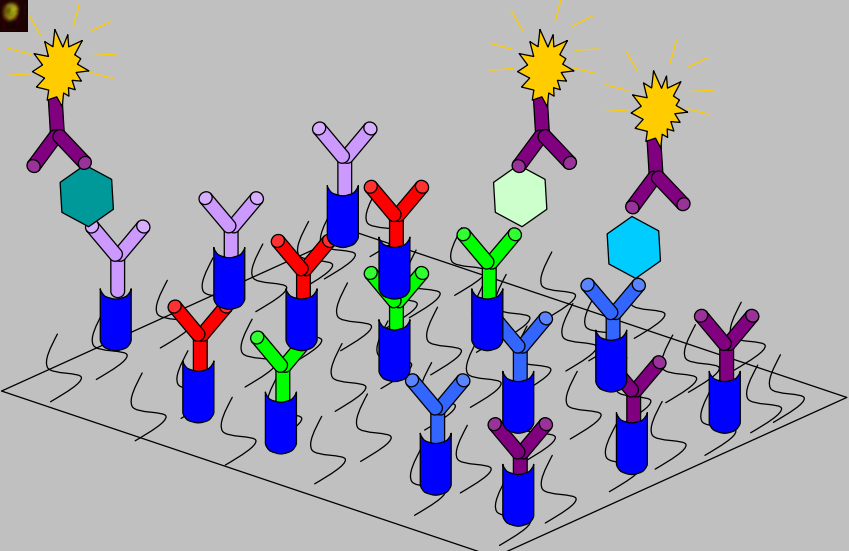
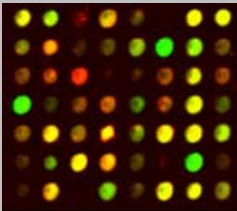
Biomedical Applications of Traveling Plasmons



Fluorescence Immunoassays on Surface



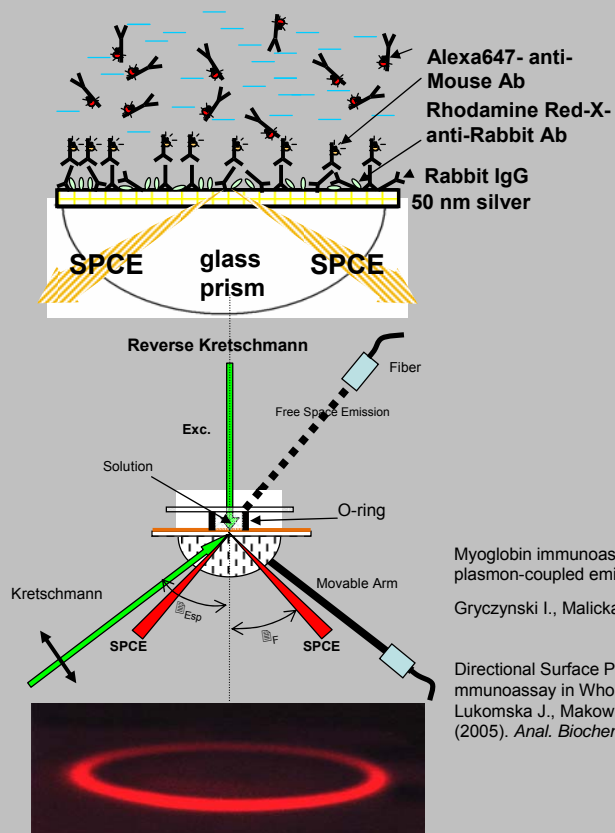
SPCE-based Assays



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Model Immunoassay with SPCE

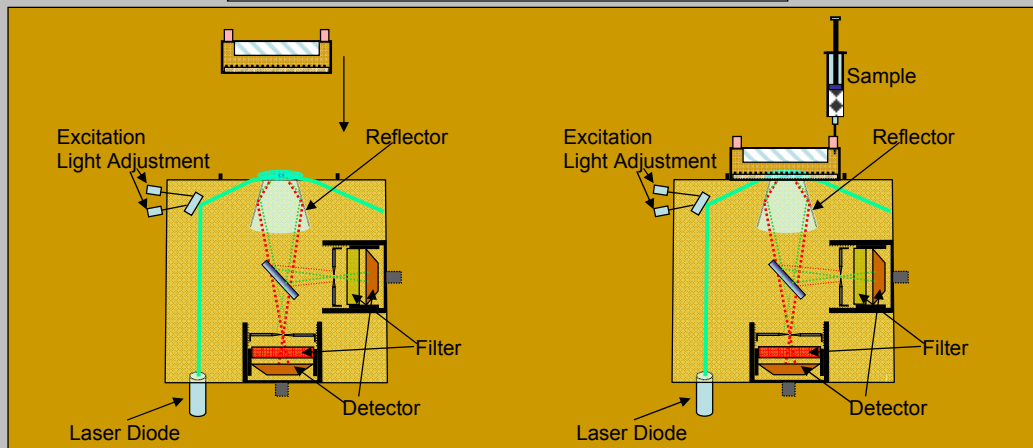
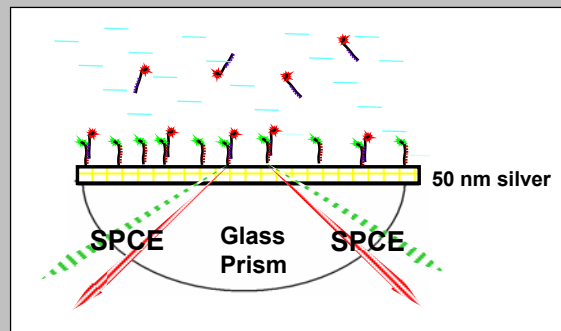


Myoglobin immunoassay utilizing directional surface plasmon-coupled emission (2004). Matveeva E., Gryczynski Z., Gryczynski I., Malicka J., Lakowicz J.R., *Anal. Chem.*, **76**, 6287-6292.

Directional Surface Plasmon-Coupled Emission – Application for an Immunoassay in Whole Blood. Matveeva E.G., Gryczynski Z., Malicka J., Lukomska J., Makowicz S., Berndt K.W., Lakowicz J. R., Gryczynski I. (2005). *Anal. Biochem.*, **344**(2), 161-167.

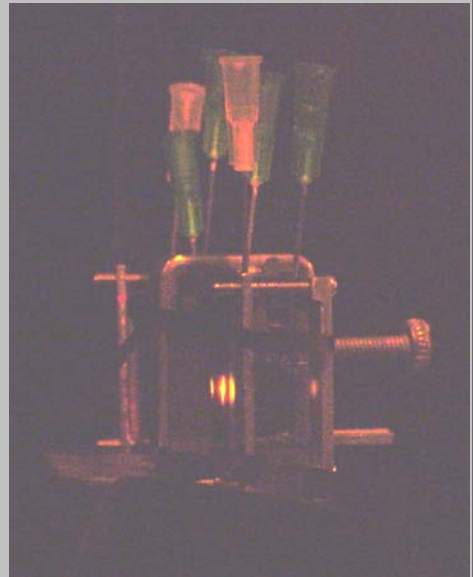
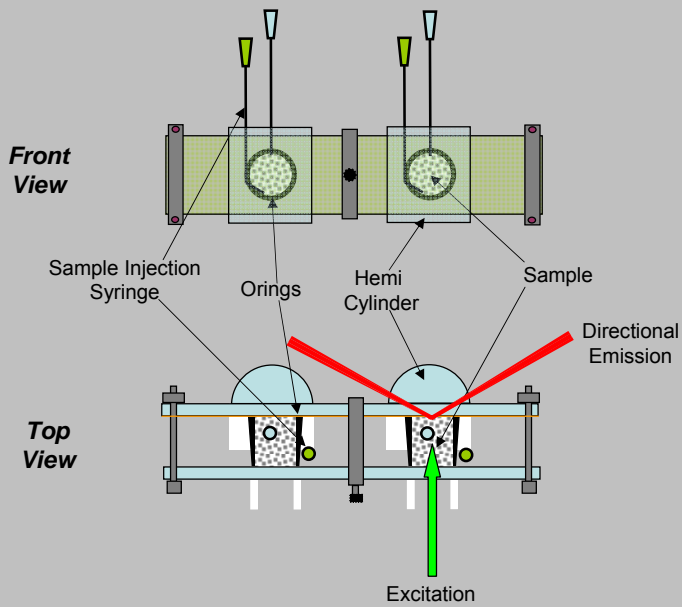
Model Hybridization Assay with SPCE

Ratiometric Detection of Wavelength Resolved SPCE
Collaboration with Dr. D. Stewart, Omm Sci.



SPCE Assay Instrumentation

Binding Kinetics



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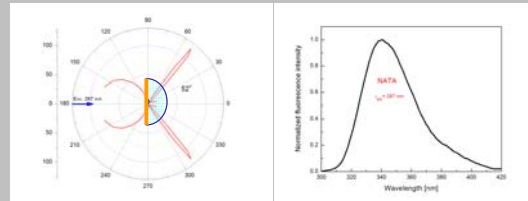
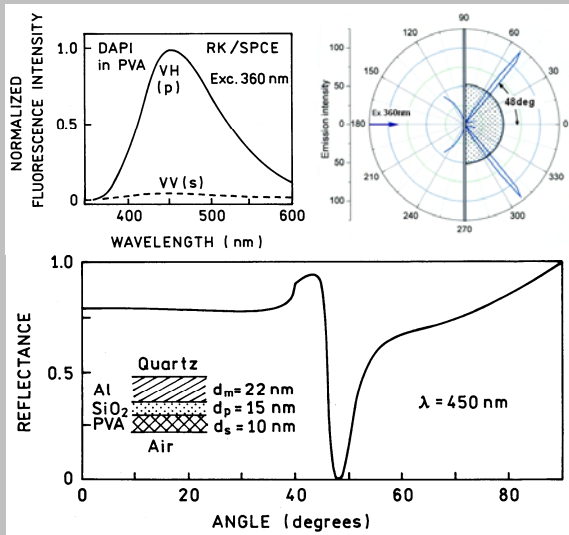
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UV - Detection and Label-Free

Aluminum on Sapphire

Proteins - Amino Acids; Tryptophan & Tyrosine

DNA/RNA - Bases; Adenine, 2AP, DAPI

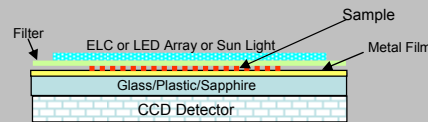
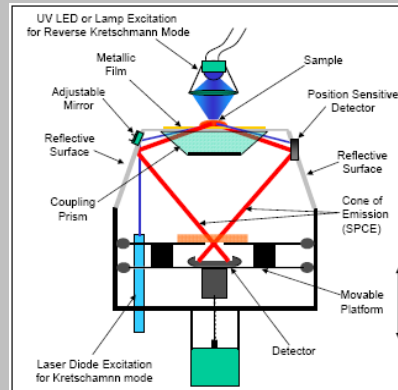
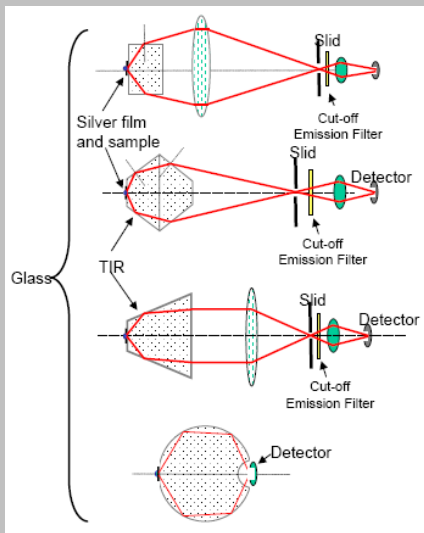
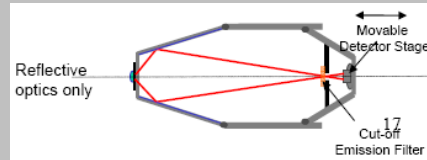
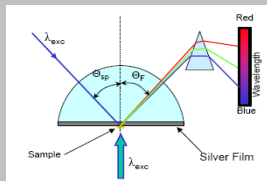


Previously Reported



Examples of Simple Devices Based on SPCE Technologies

To be a field deployable technology to has to be simple and resistant to severe conditions



SPCE Microscope - Tool For Direct Detection of Conformational Changes



Modern Microscopy

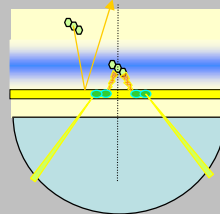
- (1) Breaking the Limits of Optical Resolution
- (2) Detection and Monitoring of Single Molecule

In the detection of single molecule background fluorescence is the biggest obstacle

- Stimulated emission depletion (STED) (Klar et al. 2000)
- Photo activated probes (proteins) (Betzig et al. 2006)
- Near-field scanning optical microscopy (NSOM) (de Lange et al. 2001)
- Zero-mode waveguide (Levene et al. 2003; Wenger et al. 2007)
- Confocal total internal reflection microscopy (Star & Thompson 2001; Thompson & Pero 2006)
- Standing evanescent wave (Chung et al. 2007)
- Surface plasmon-coupled emission (SPCE) (Borejdo et al. 2006)
- Standing SP (E. Chung et al., 2007)

What are the advantages of SPCE?

- Surface confined coupling
- Background suppression



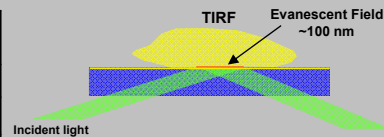
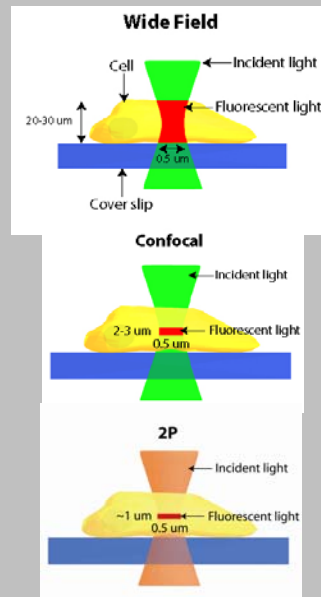
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How to Minimize Excitation Volume

The observational volume of **conventional wide-field microscopes** is way too large. For example, in 1 cm long muscle cell there are ~100 billion myosins in the typical observational volume. Even if we observe with diffraction limited optics (high NA objective, laser illumination) the number of observed molecules is ~million.

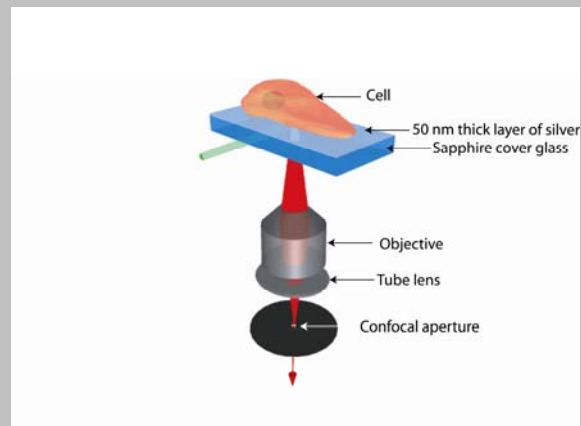
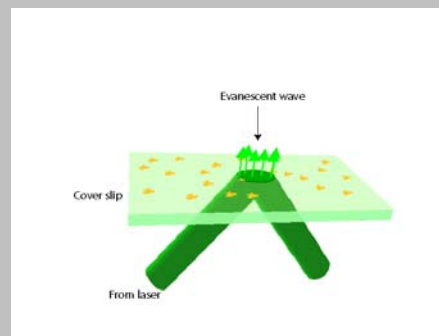
- The introduction of small observational volumes defined by confocal detection made it possible to limit the thickness to few micrometers (2-3 μm) and eliminate much background (**confocal microscope**).
- **Two-photon (2P) microscope** can reduce the thickness by factor of 2 but this is still too large to define a single molecule.
- **Confocal TIRF** can further effectively reduce the thickness by factor of about 50.
- **SPCE** effectively reduce the volume by factor of 2 compare to confocal TIRF and in the same time **reduce background**



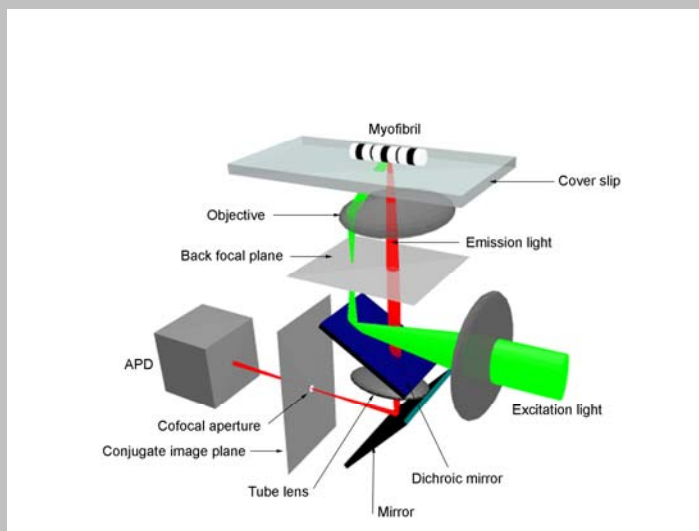
| Method | Exp volume (L) | ~# of xbs in the volume | Degree of labeling (%) | ~# of observed (fluorescent) xbs |
|------------|---------------------|-------------------------|------------------------|----------------------------------|
| Wide field | 10^{-14} | 10^6 | 100 | 10^6 |
| Confocal | 3×10^{-16} | 2×10^4 | 2 | 400 |
| 2P | 2×10^{-16} | 10^4 | 2 | 200 |
| TIRF/Con | 4×10^{-18} | 200 | 2 | 4 |
| SPCE | 2×10^{-18} | 100 | 2 | 2 |

A new method is required !!!

- The method described here, a combination of Surface Plasmon Coupled Emission (SPCE) and confocal microscopy (referred to here as Confocal SPCE) offers hope to see a single molecule in a cell.
- The principle is shown schematically at right. The light which impinges on a silver-plated cover-slip made from sapphire at SPR angle, penetrates the silver and resonates with surface plasmons to create extremely bright fluorescence (~50 x brighter than fluorescence excited by TIR).



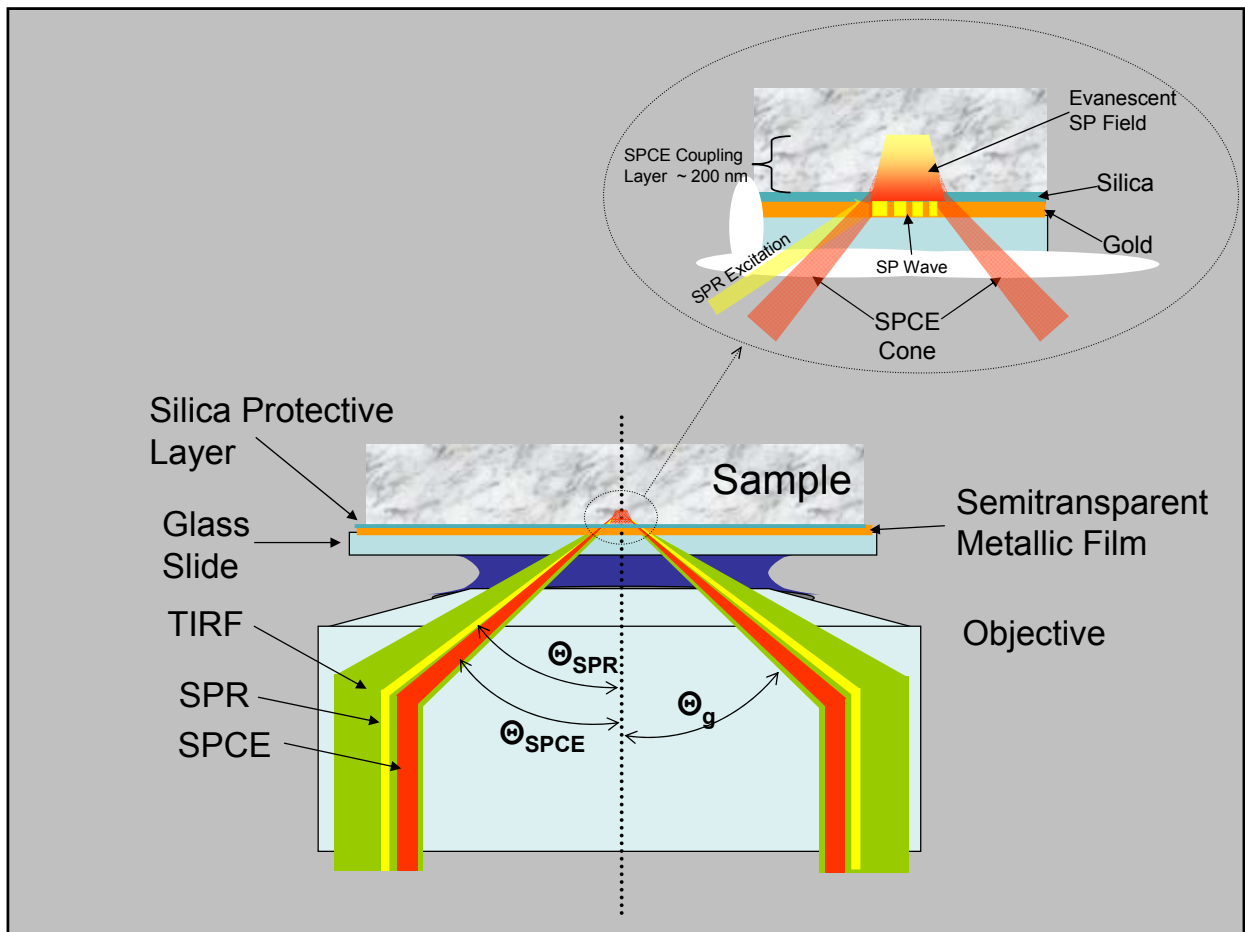
Principle of the method

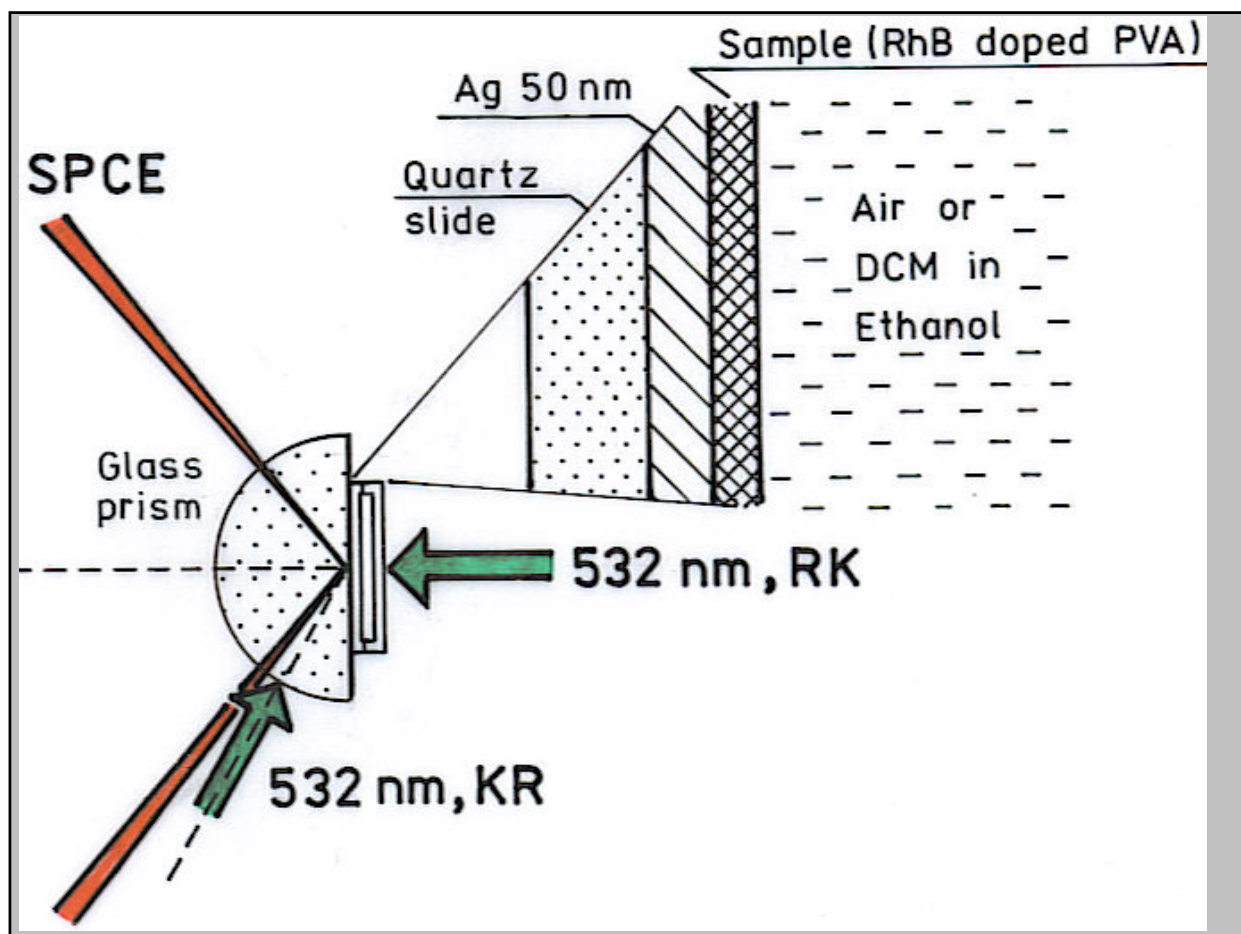


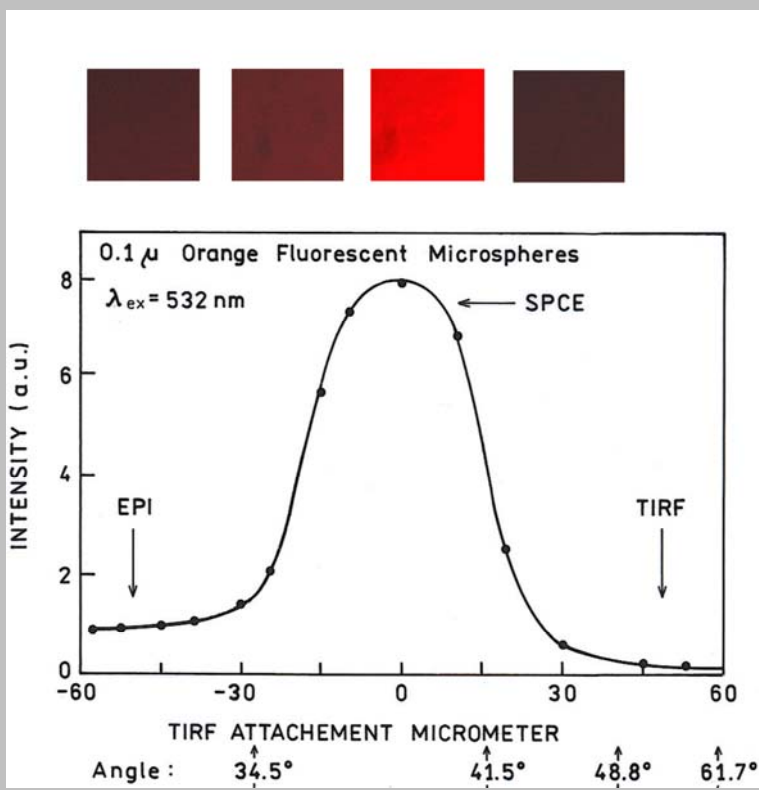
| Method | Experimental volume (L) | ~Number of xbs in the volume | Degree of labeling (%) | ~Number of observed (fluorescent) xbs |
|--------|-------------------------|------------------------------|------------------------|---------------------------------------|
| SPCE | 10^{-18} | 100 | 2 | 2 |

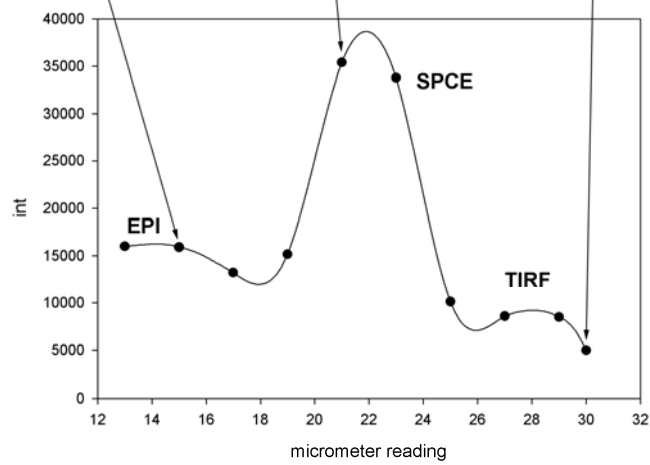
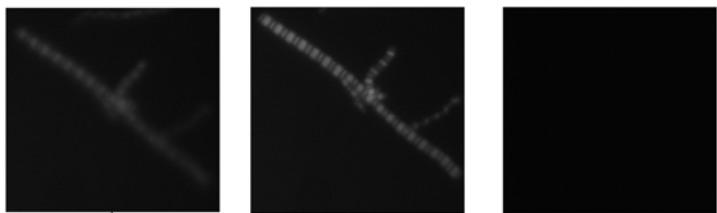
Principles of SPCE Microscopy

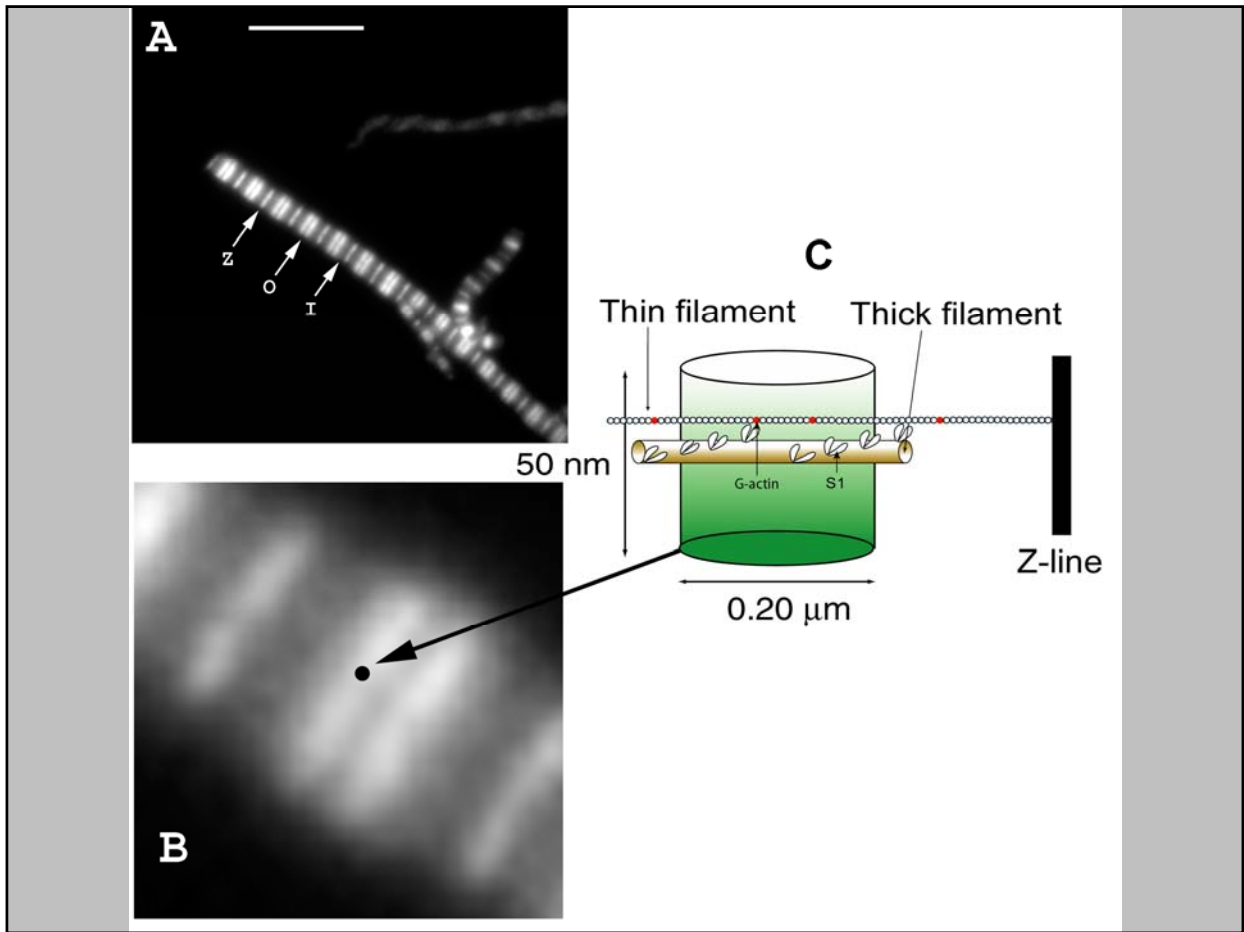








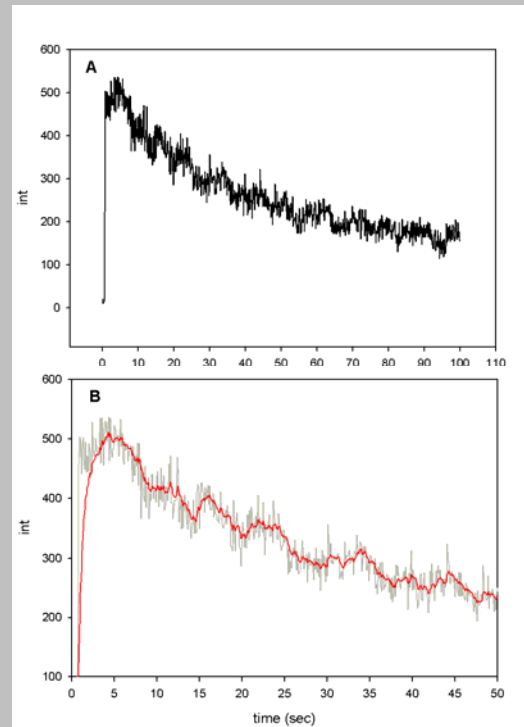




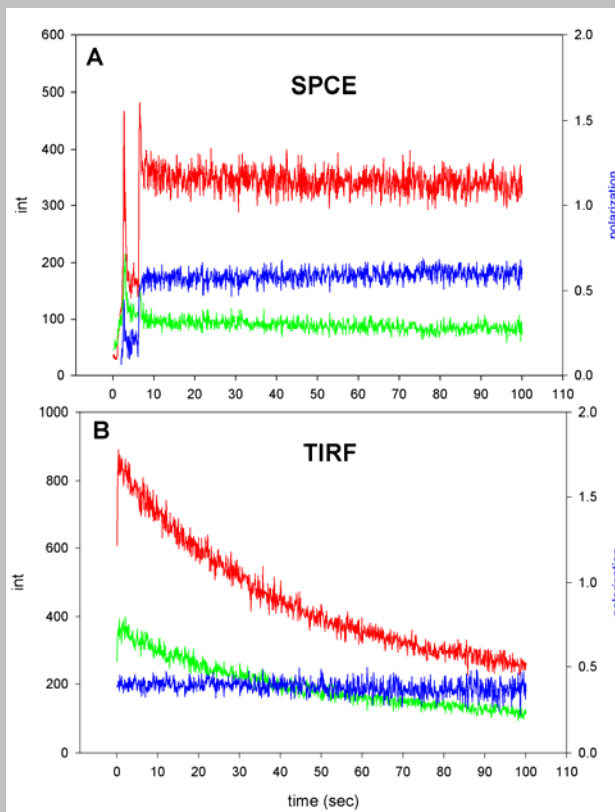
Muscle, cntd

Confocal SPCE signal from rigor myofibril. The data was collected from the overlap zone. Myofibril labeled with 0.1 μM rhodamine-phalloidin on gold coverslips. The exciting light was perpendicular to the plane of coverslip (p-polarization).

- A:** Fluorescence intensity.
- B:** intensity on expanded scale, gray-original signal, red-low pass filtered.

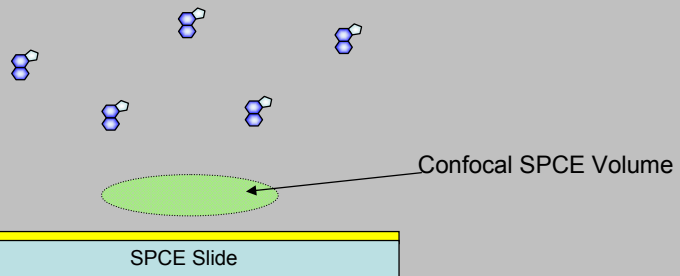
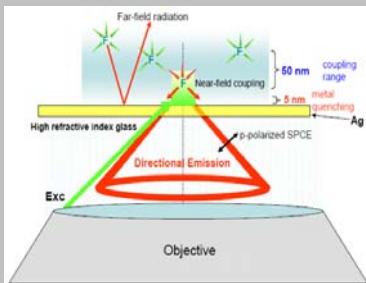


Photostability



???

FCS with Surface Plasmons



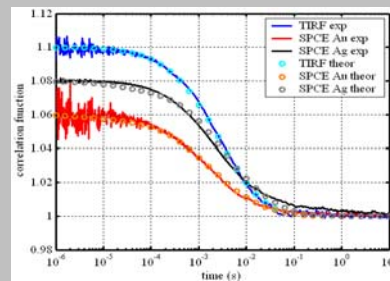
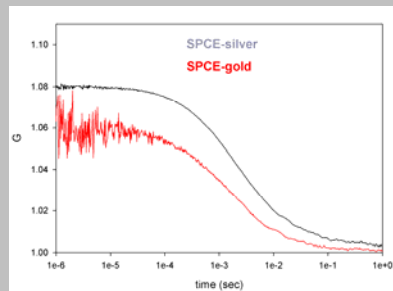
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$$G(t) = 1 + \frac{1}{2N} \frac{\sum_{nm} \frac{d_n d_m}{d_n + d_m} A_n A_m R_{nm}(t)}{\sum_{nm} \frac{d_n d_m}{d_n + d_m} A_n A_m}$$

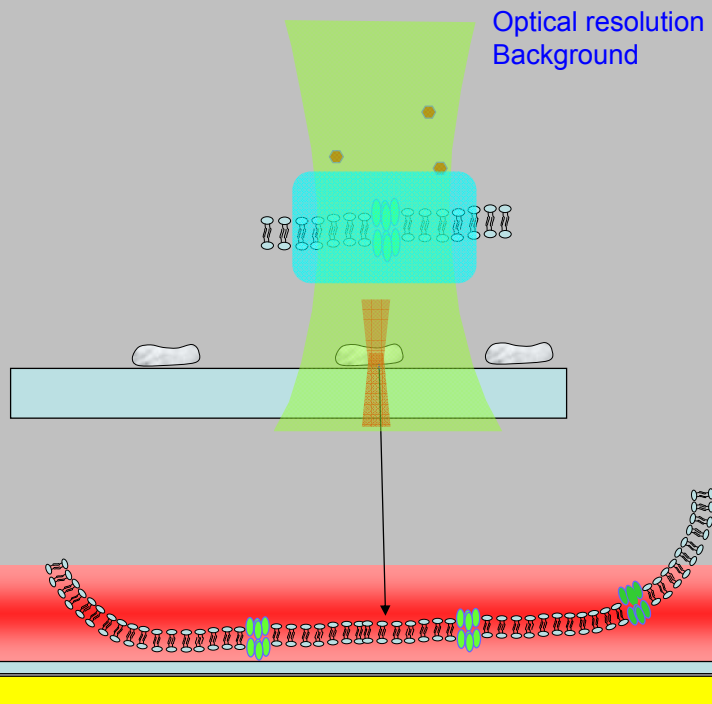
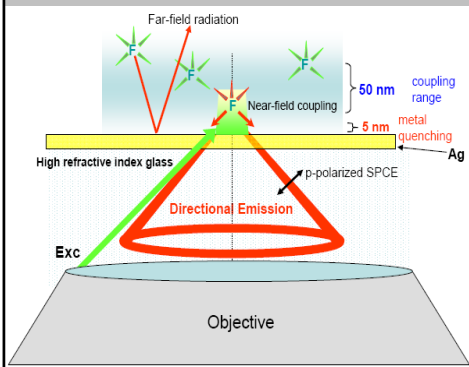
$$R_m(t) = \left(1 + \frac{Dt}{\sigma^2}\right)^{-1} \left(\left(1 - \frac{2Dt}{d_n^2}\right) \operatorname{erfc}\left(\sqrt{\frac{Dt}{d_n^2}}\right) \exp\left(\frac{Dt}{d_n^2}\right) + \sqrt{\frac{4Dt}{d_n^2}}\right)$$

$$R_{nm}(t) = \left(1 + \frac{Dt}{\sigma^2}\right)^{-1} \left(\frac{d_m}{d_m - d_n} \operatorname{erfc}\left(\sqrt{\frac{Dt}{d_m^2}}\right) \exp\left(\frac{Dt}{d_m^2}\right) + \frac{d_n}{d_n - d_m} \operatorname{erfc}\left(\sqrt{\frac{Dt}{d_n^2}}\right) \exp\left(\frac{Dt}{d_n^2}\right) \right)$$



Fluorescence Correlation Spectroscopy in Surface Plasmon Coupled Emission Microscopy. (2006) Borejdo, J., N. Calander, Z. Gryczynski, and I. Gryczynski. *Optic Express*. 14. (17), 7878-7888.

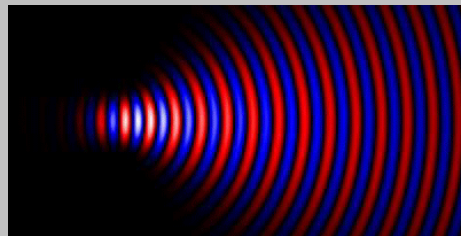
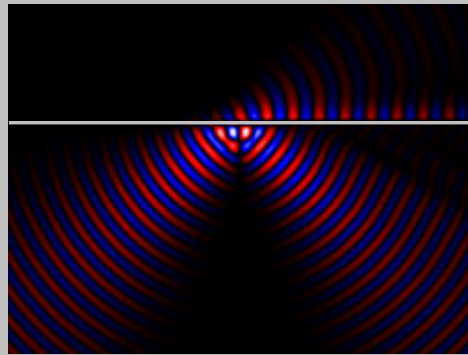
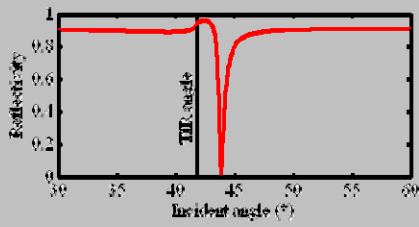
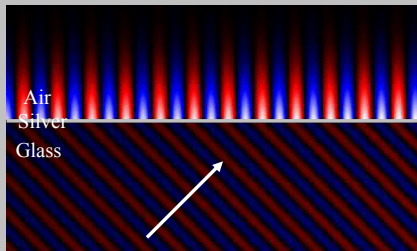
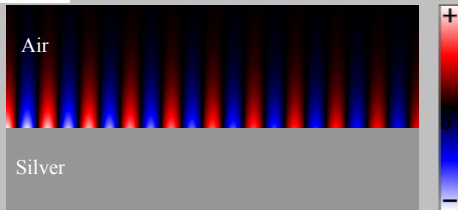
Where Surface Confined Fluorescence Could Be Used Visualizing Membrane Processes



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Travelling surface plasmons



N. Calander. *Focused optical beams at planar structures by an imaginary shift in position.* Journal of the Optical Society of America A 24 (9), 2513-2515, (2007).

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Gaussian beam in terms of the sum of plane waves with an imaginary shift in position

$$\iint_{4\pi} d\Omega_k e^{ik \cdot r} = 2\pi \frac{e^{ikr}}{ikr} - 2\pi \frac{e^{-ikr}}{ikr} = 4\pi \frac{\sin(kr)}{kr}$$

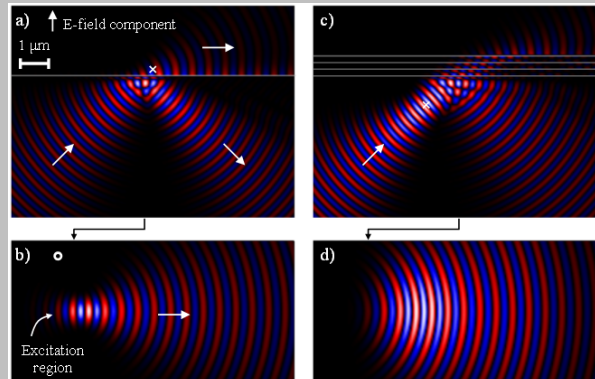
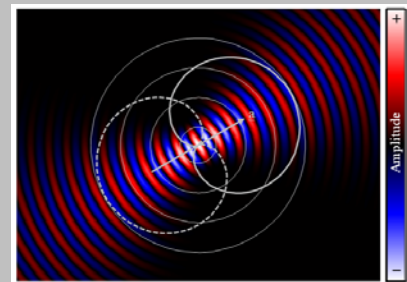
$$\sqrt{(\mathbf{r} - i\mathbf{a}/k) \cdot (\mathbf{r} - i\mathbf{a}/k)}$$

$$\iint_{4\pi} d\Omega_k e^{ik \cdot r} e^{i\mathbf{k} \cdot \mathbf{a}} = \iint_{4\pi} d\Omega_k e^{ik(r - i\mathbf{a}/k)}$$

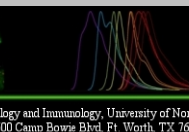
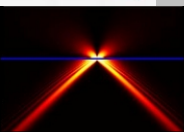
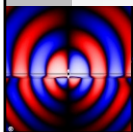
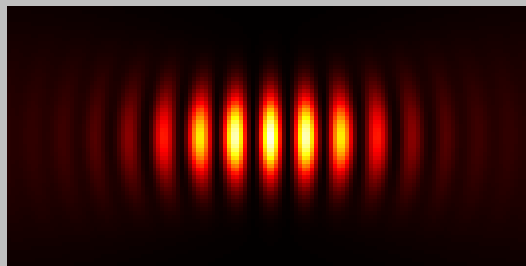
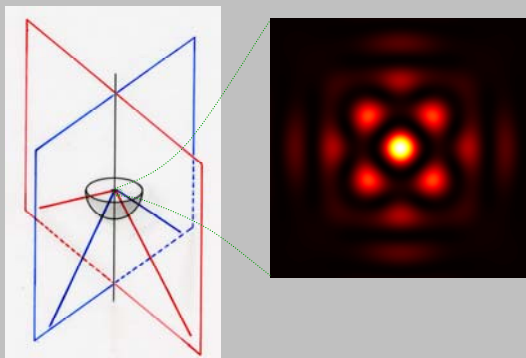
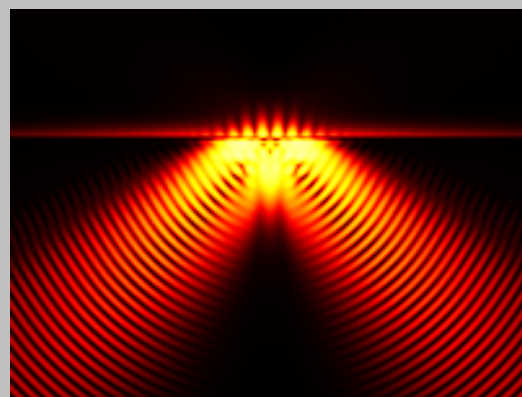
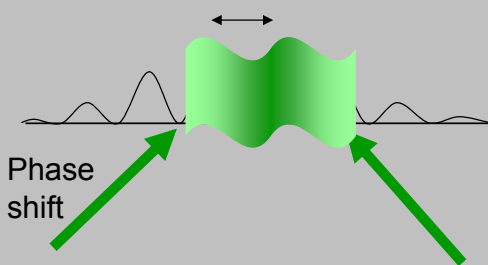
$$\iint_{4\pi} d\Omega_k e^{ik \cdot r} e^{i\mathbf{k} \cdot \mathbf{a}} = 2\pi \frac{e^{ikr}}{ikr} e^{i\mathbf{k} \cdot \mathbf{a}} + 2\pi \frac{e^{-ikr}}{ikr} e^{-i\mathbf{k} \cdot \mathbf{a}} + O\left(\frac{1}{r^2}\right)$$

$$\iint_{4\pi} d\Omega_k e^{ik \cdot r} w(\mathbf{k}) = 2\pi \frac{e^{ikr}}{ikr} w(\hat{\mathbf{r}}) + 2\pi \frac{e^{-ikr}}{ikr} w(-\hat{\mathbf{r}}) + O\left(\frac{1}{r^2}\right)$$

N. Calander. *Focused optical beams at planar structures by an imaginary shift in position*. Journal of the Optical Society of America A **24** (9), 2513-2515, (2007).



Interference of two planar surface plasmons



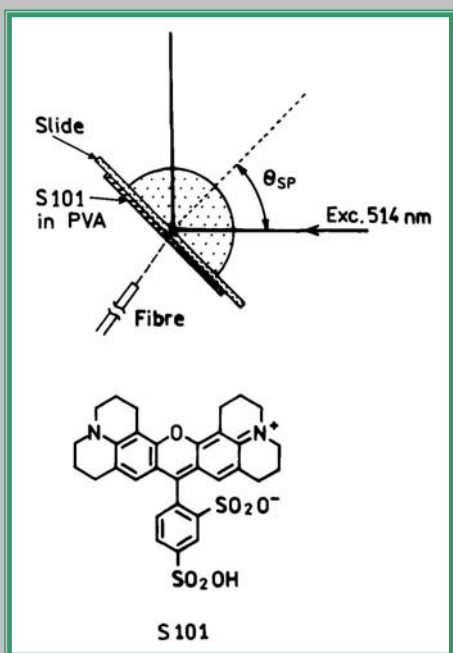
Another Interesting Property of SPCE

Waveguide Assisted SPCE

SPR – Salomon and Tolin

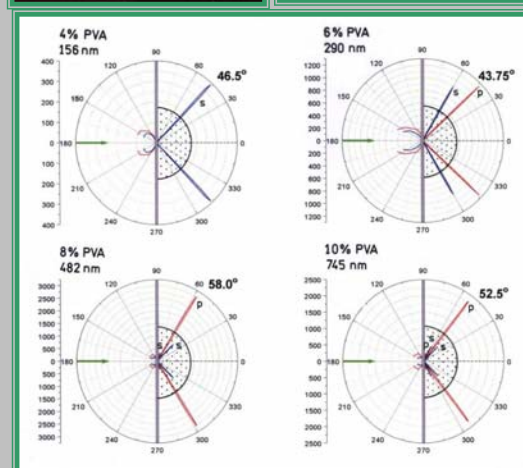
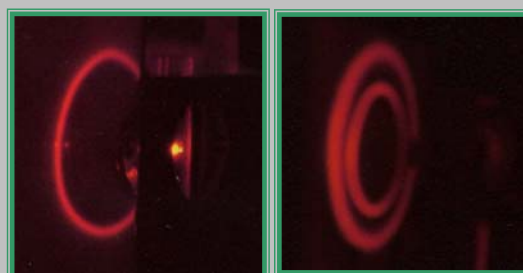
SPCE from Thick and Thin PVA Films

Effects of Film Thickness on the Angular Distribution of SPCE

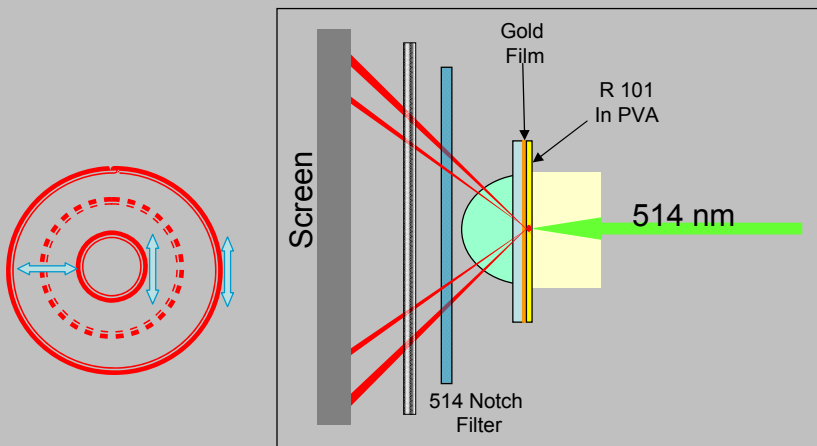


150 nm thick PVA

290 nm thick PVA



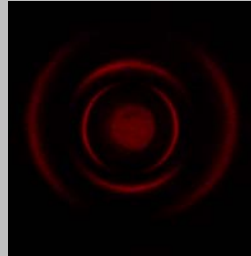
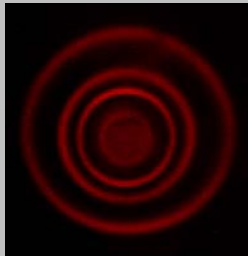
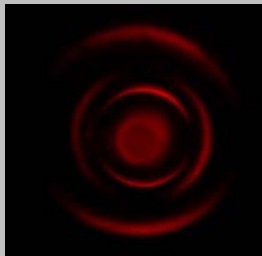
p & s Ring Polarization



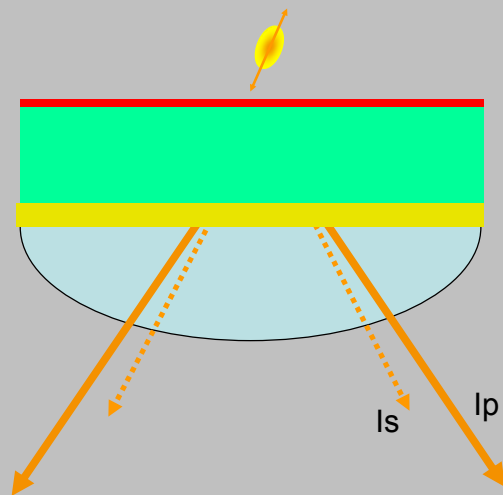
Vertical

None

Horizontal



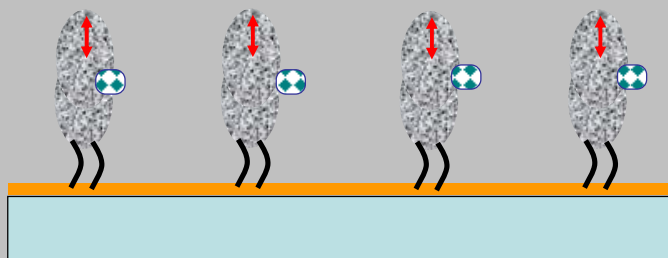
New Concept for Measuring Conformational Changes



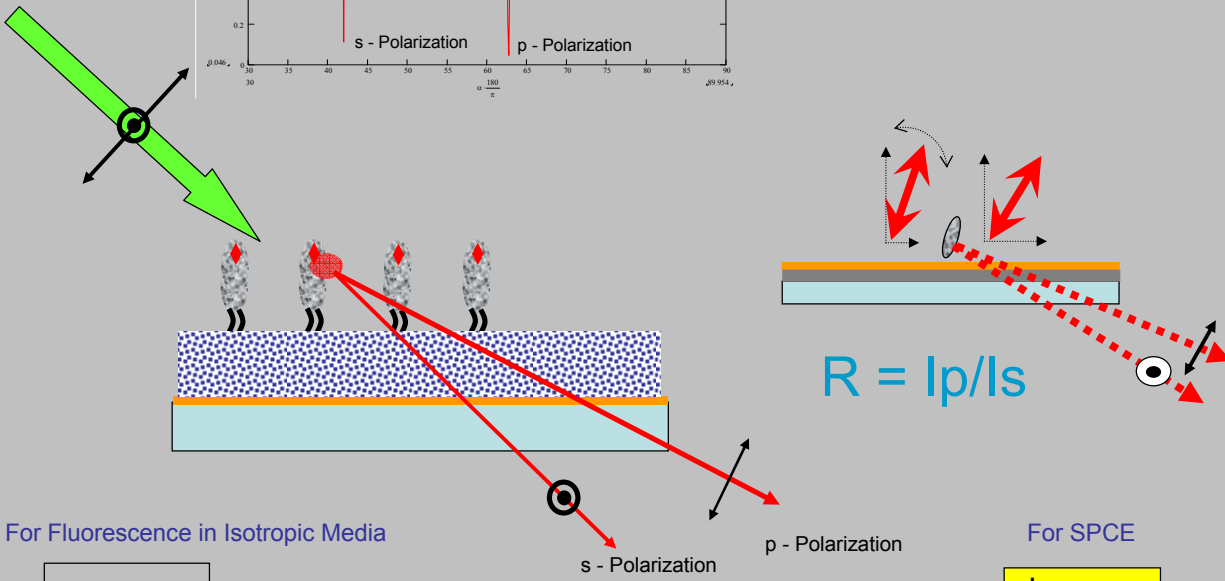
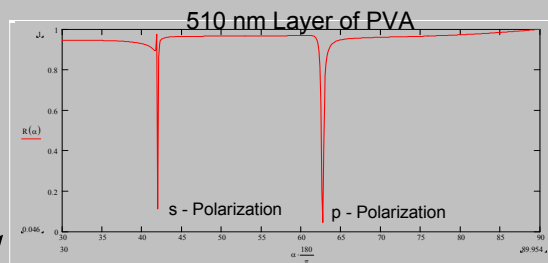
$$R = l_p/l_s$$

Studying Conformational Dynamics

Process that involve Conformational Change



Utilizing the Waveguide Technology



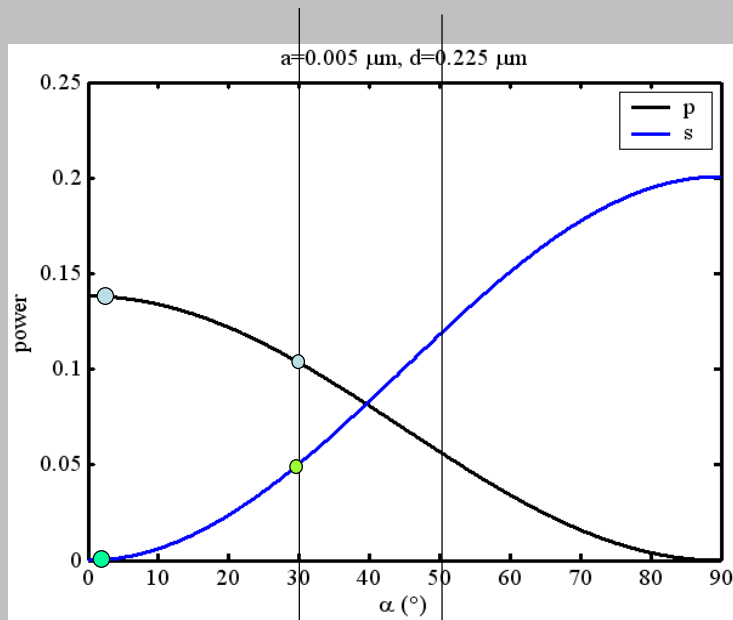
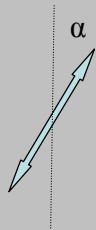
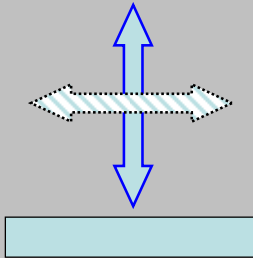
For Fluorescence in Isotropic Media

$$\frac{I_{\parallel}}{I_{\perp}} < 3$$

For SPCE

$$\frac{I_{\parallel}}{I_{\perp}} > 100$$

Theoretical Calculation

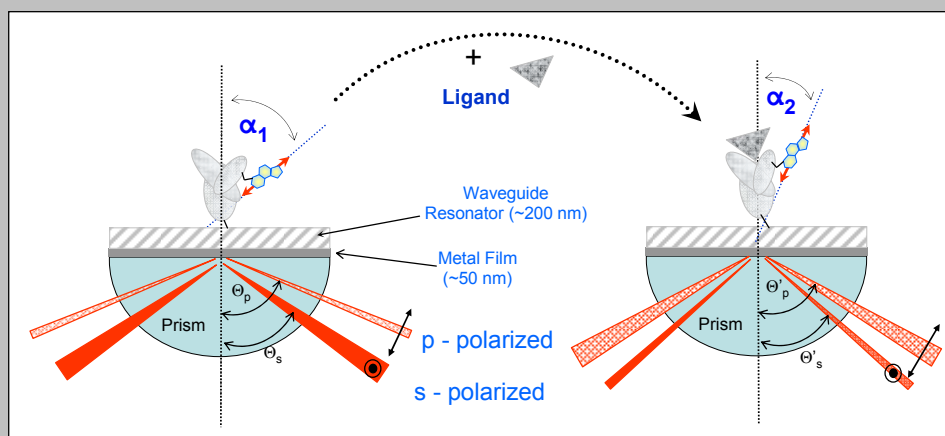


Power flow of p-polarized (black) and s-polarized light versus dipole angle α .

SPCE Microscope - Tool For Direct Detection of Conformational Changes

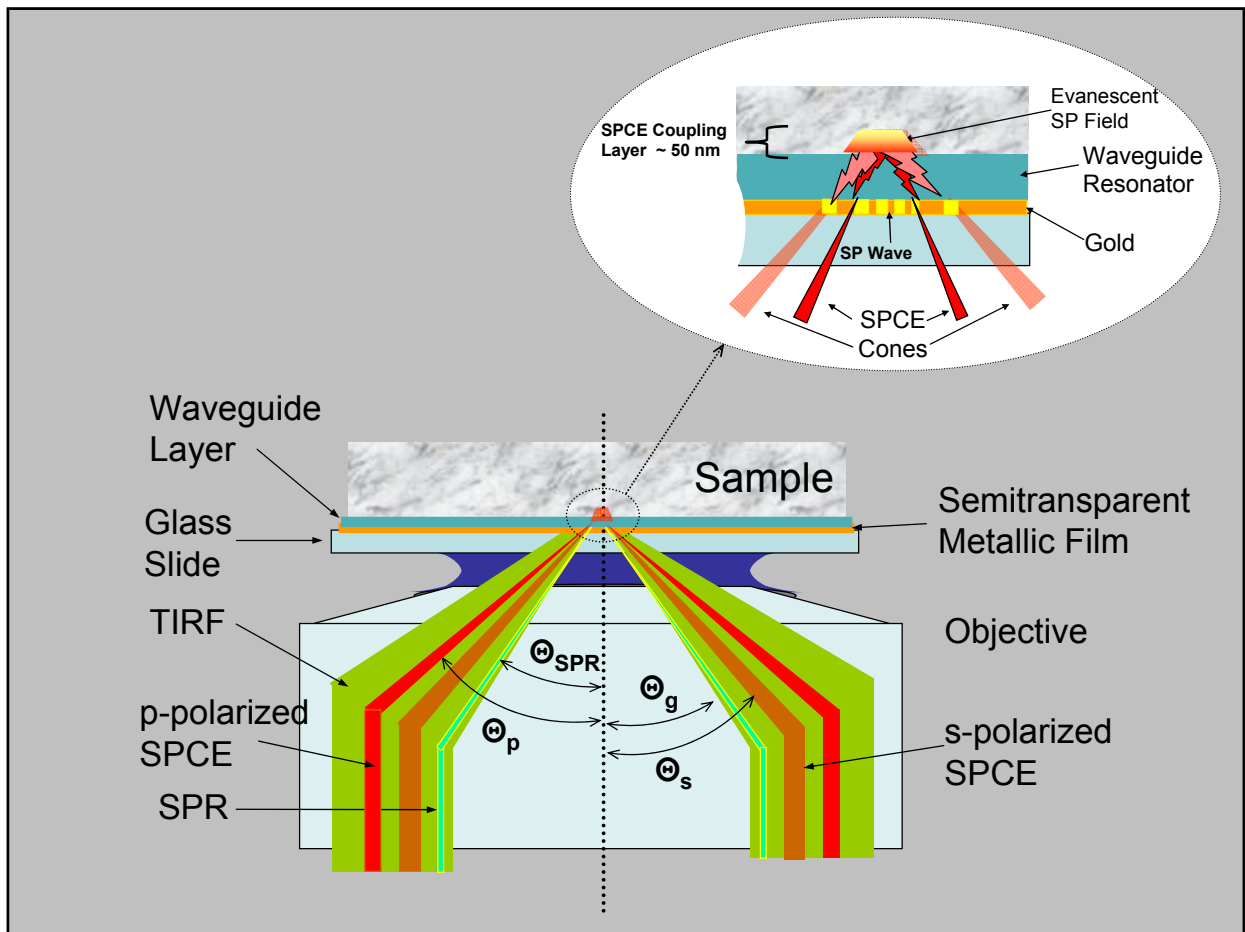


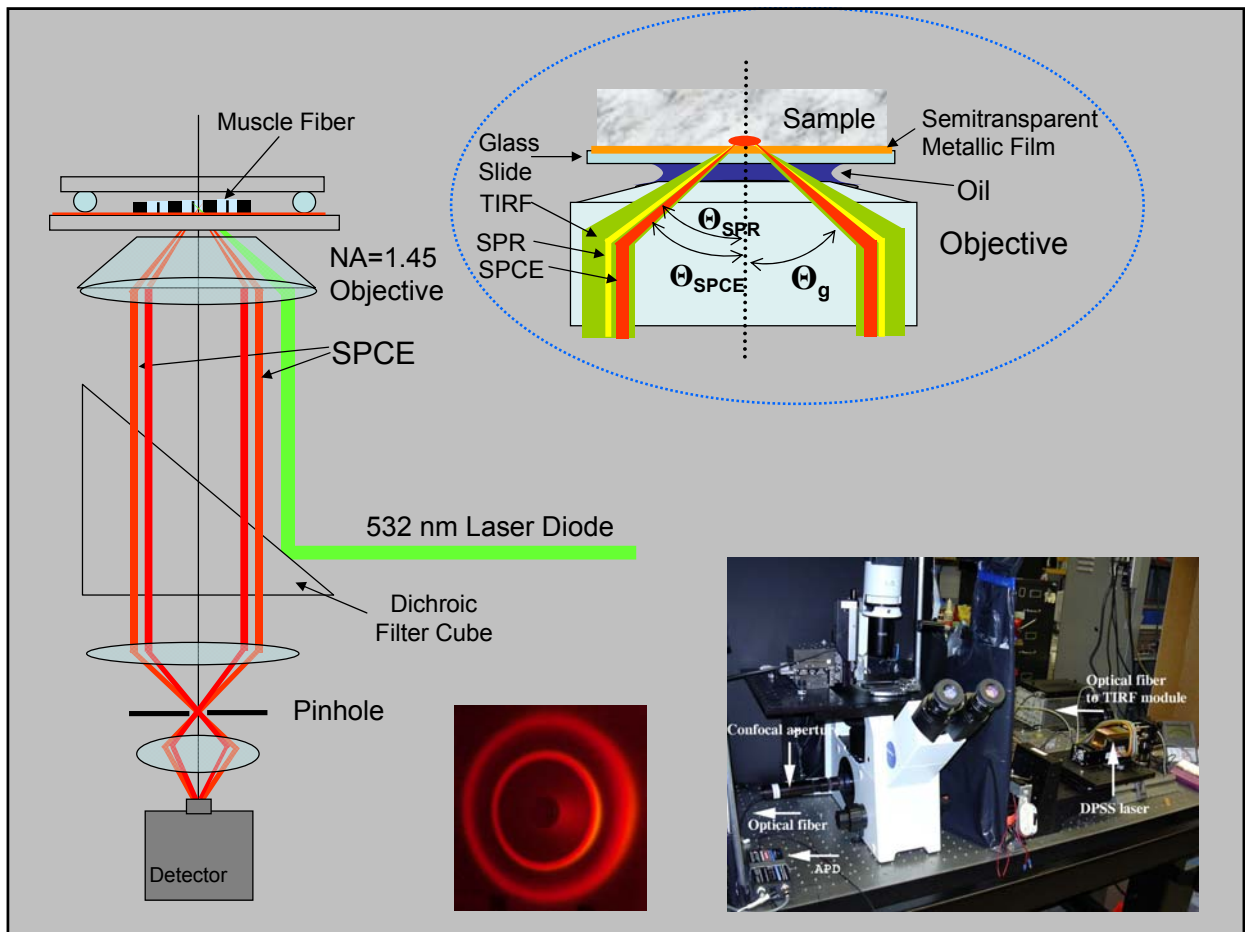
Conformational Change Upon Ligand Binding



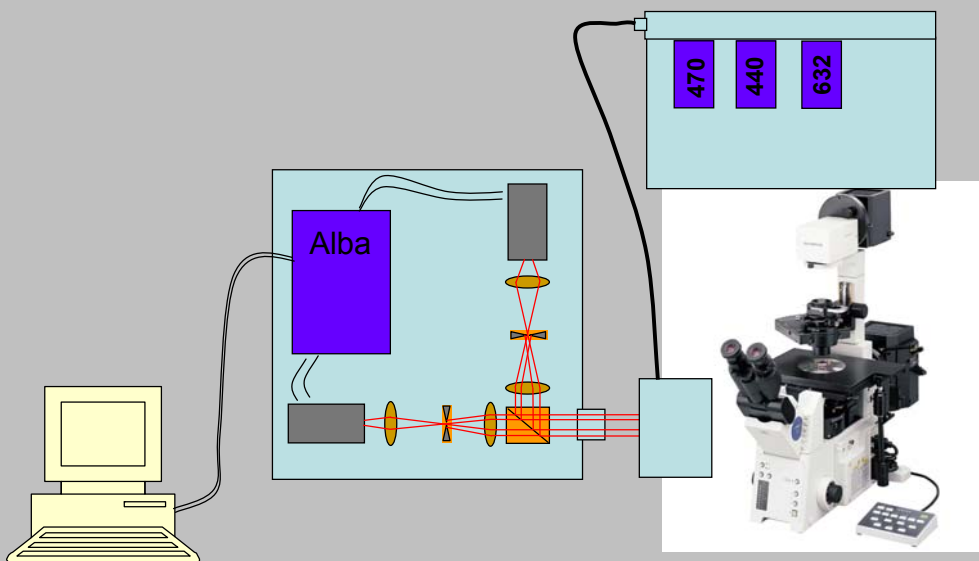
A schematic description of conformational change occurring upon ligand binding. We will detect bounding (mass change), conformational change (reorientation of the binding side), and by time-resolved measurement change in the protein mobility (if induced by binding).





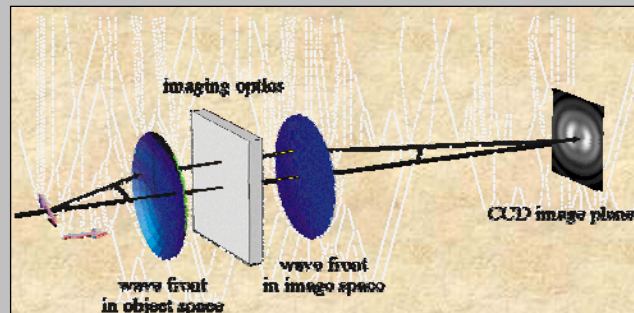
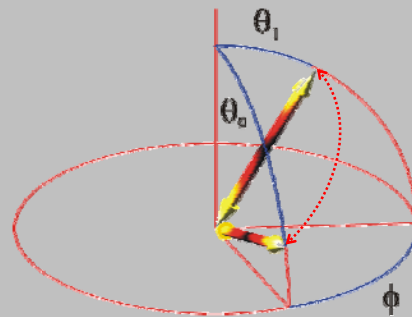
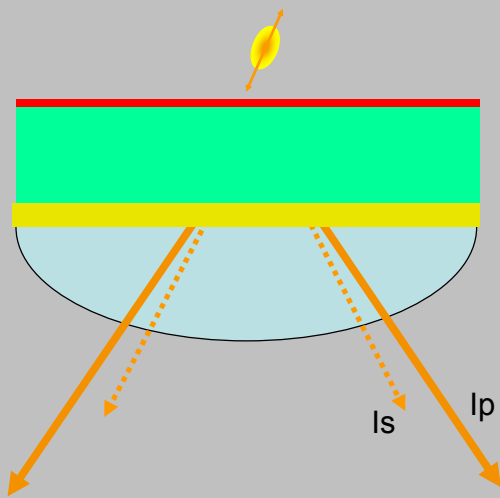


How to Separate Two Same Color Rings?



Detecting Different Motions – in plane and out-of plane

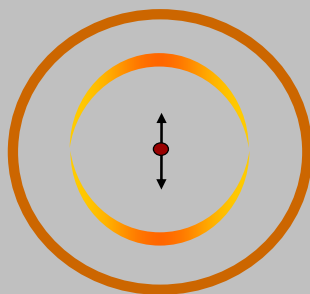
Collaboration with: Joerg Enderline, Gottingen; John Schetz, Pharmacology, UNTHSC; Jim Harper, UNH.



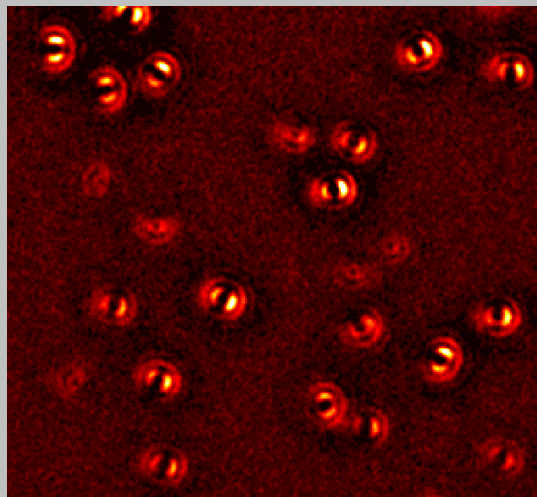
SPCE From Single Horizontal Dipole
What about in plane rotations?



Molecular Motion in Azimuthal Angle



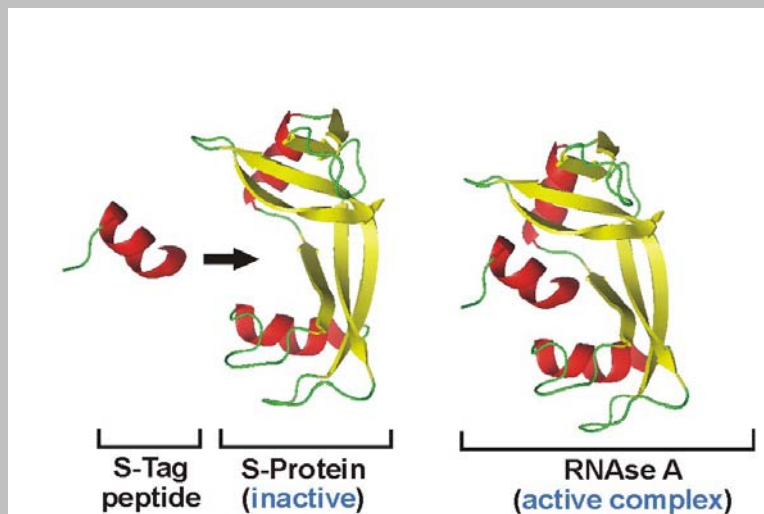
Rotational diffusion of molecules



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A Model System

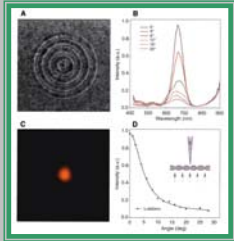


Interactions between protein pairs in bovine pancreatic ribonuclease A (RNase A). The structure of the crystalline protein is rendered as cartoon depicting the secondary structural elements with helices colored red, sheets colored yellow and random coils colored green. A) Cleavage of the N-terminal 15 amino acids of RNase A produces what is called an S-Tag peptide and an enzymatically inactive protein called S-protein. B) The high affinity binding of the S-Tag peptide to the inactive S-Protein forms a bimolecular complex with RNase activity.

Plasmonic Technology and Practical Applications

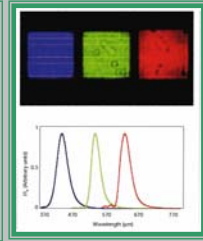
Anomalous Transmission of Light Through Silver Films

Beam-like transmission through concentric silver rings



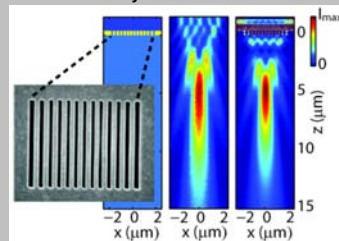
Lezec et al. 2002

Optical filters using thick silver films with holes



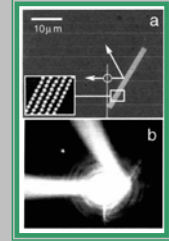
Degiron, Barnes, 2003

Planar Lenses Based on Nanoscale Slit Arrays in a Metallic Film



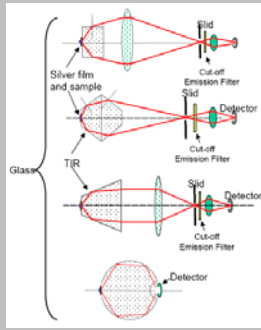
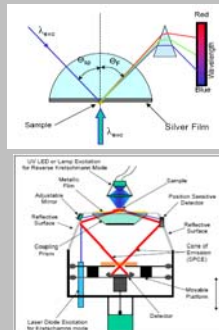
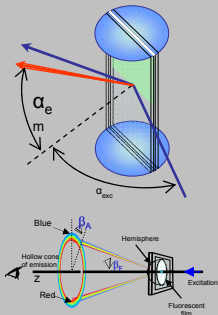
Verslegers et al. 2009

Plasmon mirror based on a line of silver dots

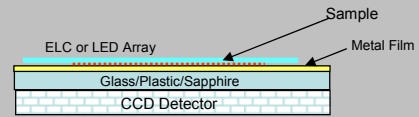
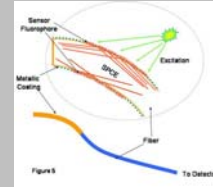


Krenn et al. 2003

Practical Applications of SPCE



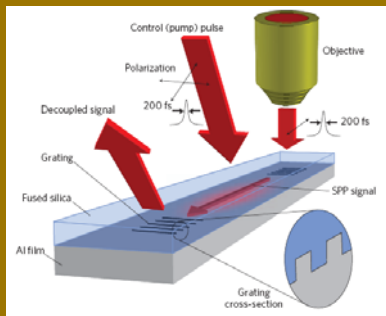
Fiber Optics Based Detection



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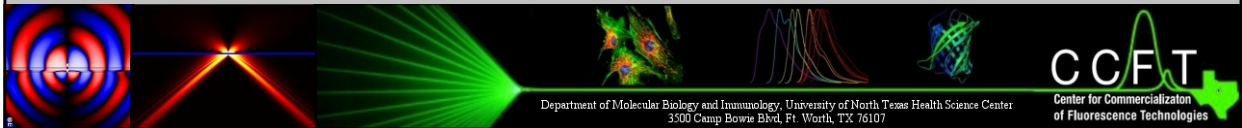
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New Directions



nature
photonics

Kevin F. MacDonald^{1*}, Zsolt L. Sámson¹, Mark I. Stockman² and Nikolay I. Zheludev¹

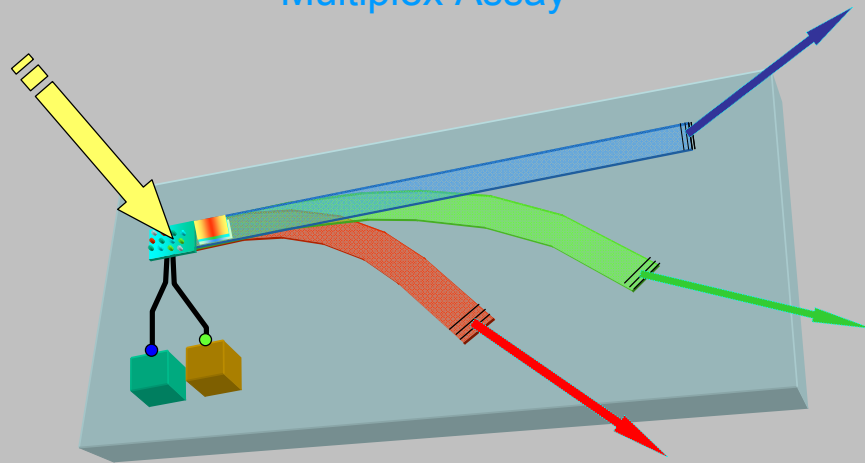


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New Directions

Multiplex Assay



The footer banner contains several scientific images and logos. From left to right: a circular interference pattern with red and blue concentric rings; a red laser beam intersecting a blue laser beam; a green laser beam illuminating a surface; a microscopic image of cells; a graph with multiple colored curves; a 3D molecular structure; and the CCFT logo, which includes the text "CCFT" and "Center for Commercialization of Fluorescence Technologies" next to a green outline of the state of Texas.

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Sashank Bharil
Pabak Sakar
Prasad Yala Maticola

New after submission

Example of
Example of
Practical Application
Practical Application

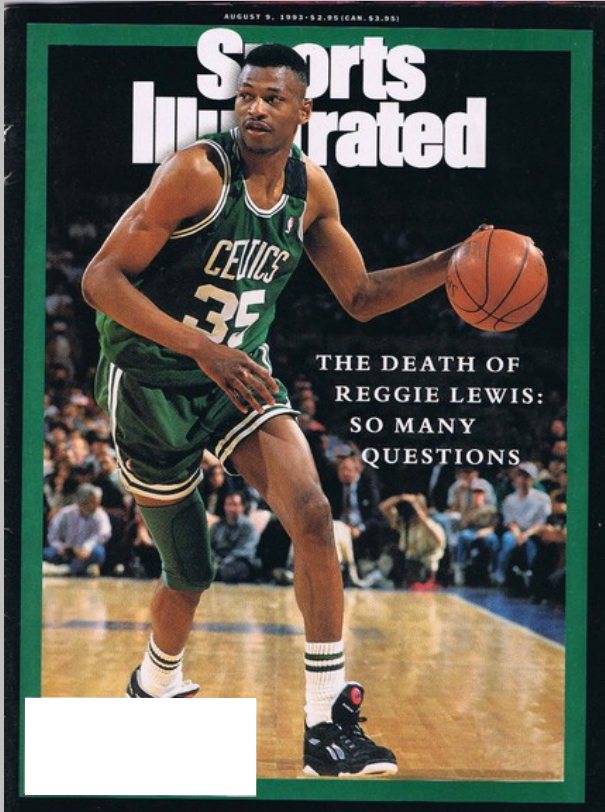
Familial Hypertrophic Cardiomyopathy

**NEW DEVELOPMENTS SINCE SUBMISSION
IN SEPT 2008:**

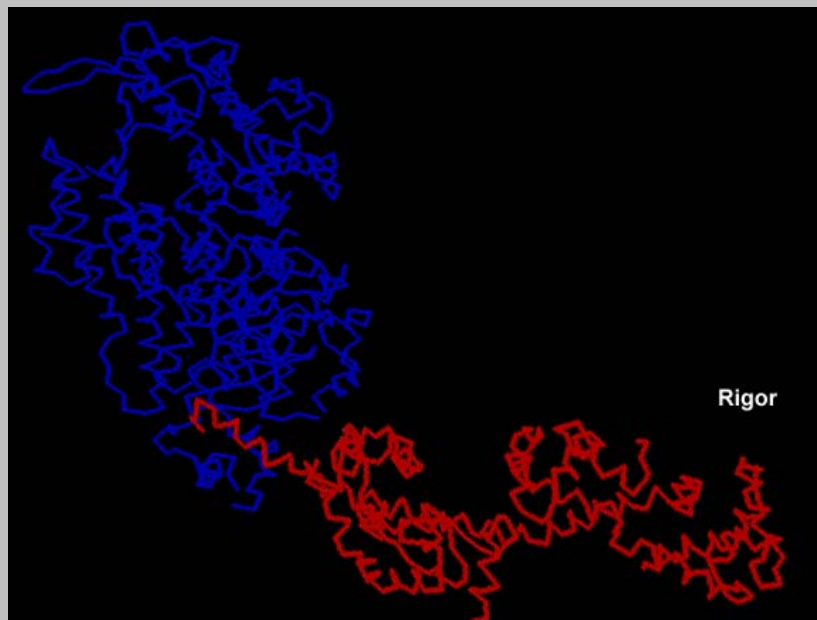
**Application of Single Molecule Detection to
diagnosis of Familial Hypertrophic Cardiomyopathy**

We are studying human heart disease -- Familial Hypertrophic Cardiomyopathy (a disease resulting from inability of a heart to adequately pump blood thus leading to premature fatigue, hypertrophy, cardiac failure and sudden death).

Best known example:
Reggie Lewis

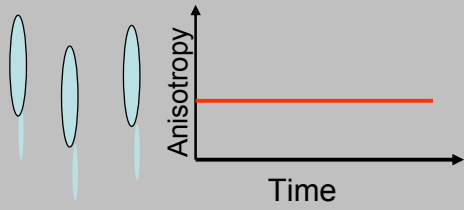


Familial Hypertrophic Cardiomyopathy is a molecular disease that originates from mutations in genes that encode for the major contractile proteins of the heart, mainly myosin. We hypothesize that myosin from sick heart has a different kinetics than myosin from healthy heart.

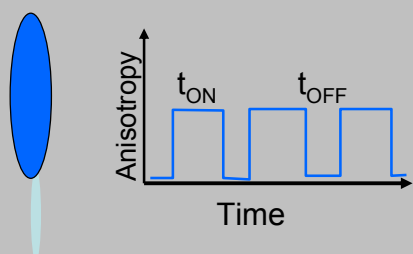
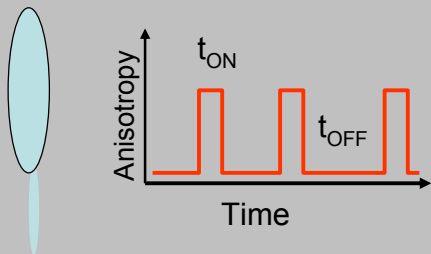
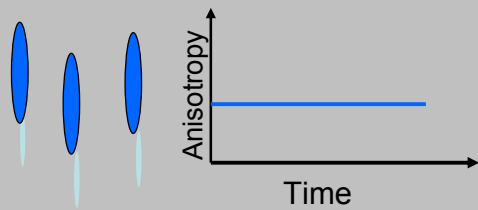


Since FHC is dominant heterozygous disease, it is expected that 50% of molecules are mutated and 50% are not. Therefore to determine whether a particular heart is suffering from the disease it is crucial to focus on **one** molecule to avoid averaging.

Fast (healthy) Molecule

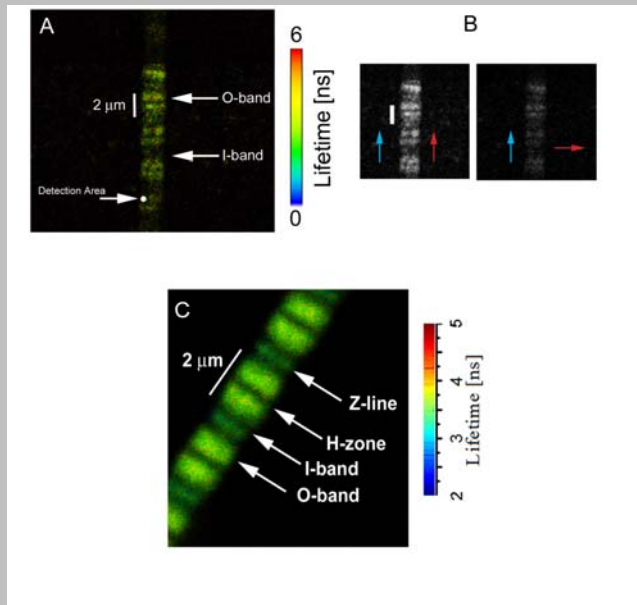


Slow (sick) Molecule



Objective 1: measure t_{OFF} and t_{ON} during isometric contraction of muscle.

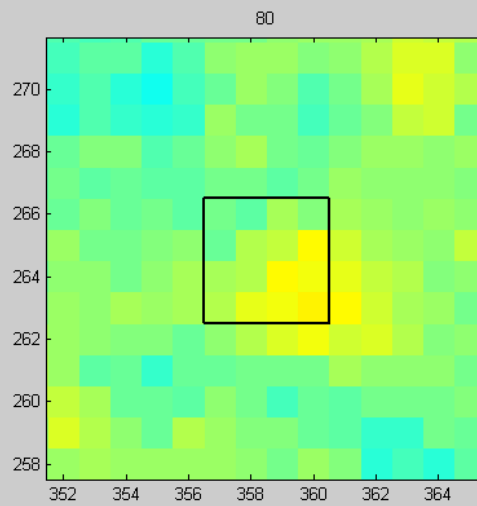
A. Myofibril was irrigated with extremely diluted dye (0.01 nM Alexa488). The corresponding polarized intensity images are shown in **B**. The various bands are best identified with the aid of image of myofibrils more heavily labeled with phalloidin (10 nM RP) (**C**).



Photobleaching of actin molecule in muscle.

New since submission

Single molecule
delivers ~5,000
photons/sec.



Here is a typical signal from muscle. There was a significant change in the number of collected photons at 6.5 s (indicated by the white arrow). The total photon rate during 4-6.5 s interval was 9,200 photons/s. This corresponds to ~two molecules. The total photon rate during 6.5-10 s was 5,300 photons/s. This corresponds to a single fluorophore.

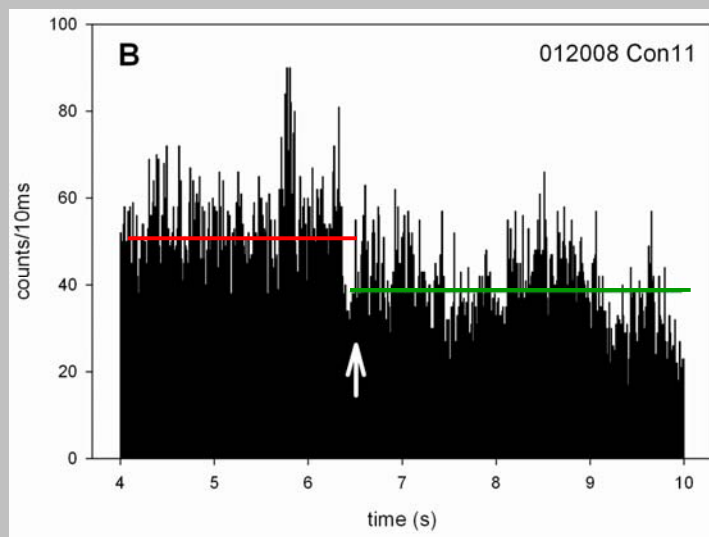
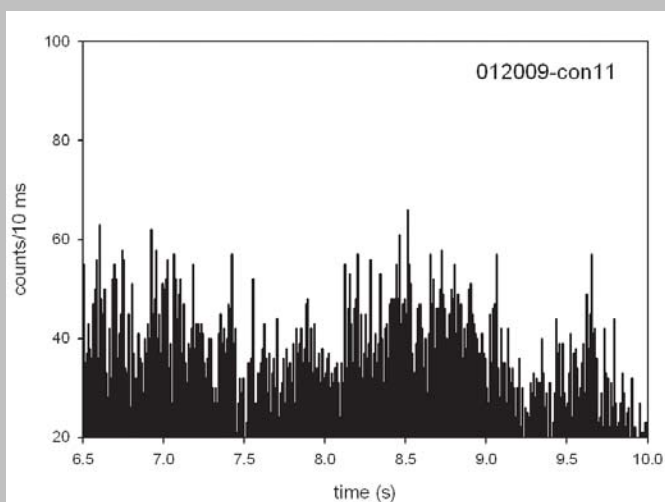
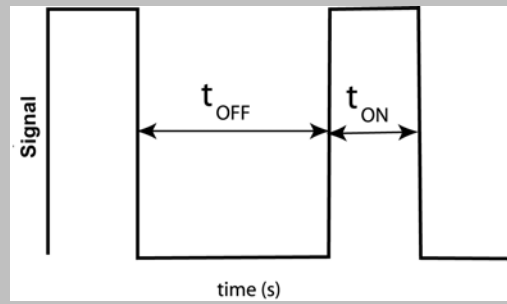
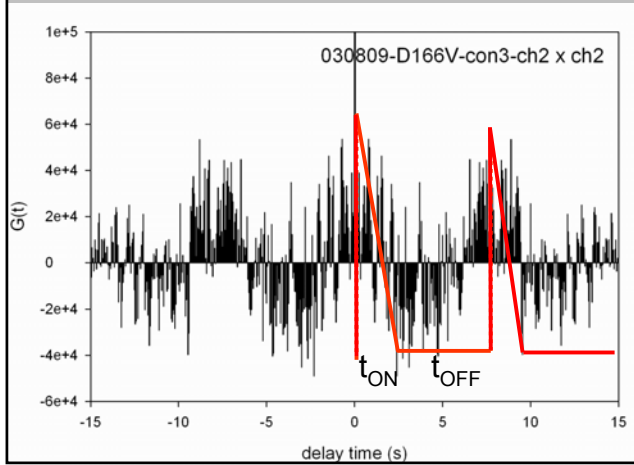
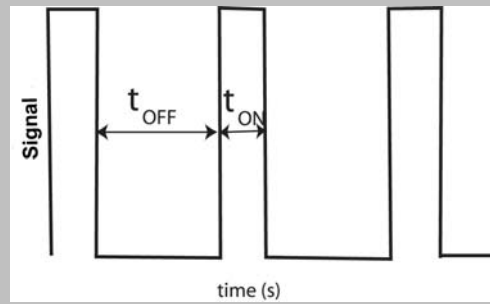
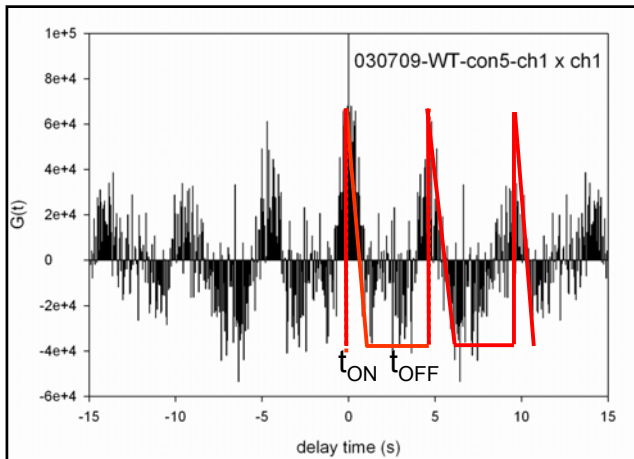


Fig. 4

A typical signal from single molecule of a heart ventricle. The total photon rate during 6.5-10 s was 5,300 photons/s.





Conclusions

FHC changes kinetics of actomyosin interaction. In healthy heart the cycle time ($t_{ON}+t_{OFF}$) is 3.5 sec. This decreases to 8.3 sec in sick heart.

