

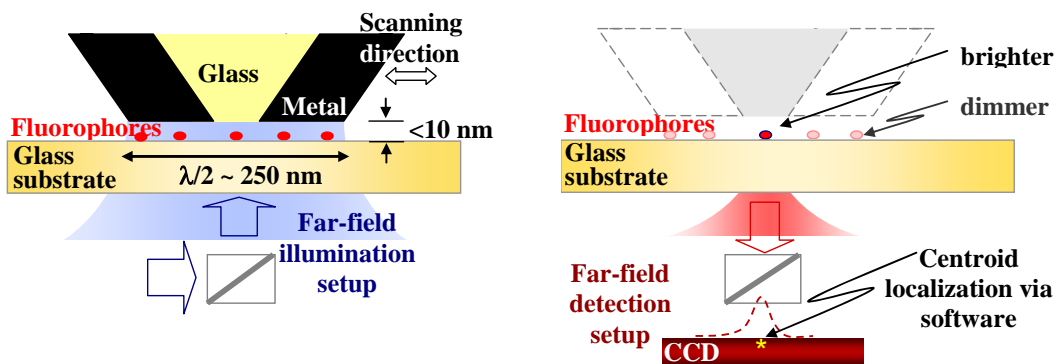
# BREAKING the DIFFRACTION BARRIER by SELECTIVE CONTROL OF NEAR- and FAR- FIELD OPTICAL MODES

## Concept, Metric and Bio-Applications

Andres La Rosa,<sup>1</sup> Tania Vu,<sup>2</sup> Scott Reed,<sup>3</sup> Peter Moeck,<sup>1</sup>  
Physics Department at PSU,<sup>1</sup> Oregon Health and Science University (OHSU),<sup>2</sup> Chemistry  
Department at PSU.<sup>3</sup>

### I. GOAL and OBJECTIVES

The development of a new *Integrated Near- and Far- Field Optics* (INFO) method for overcoming the diffraction-limited lateral-resolution that hampers conventional microscopy is proposed. It envisions the use of *a*) a metal-coated **near-field** probe, to optically isolate, one at a time, sparse fluorophore markers within a region of sub-wavelength dimension; and *b*) a **far-field** localization method for locating the selected fluorophore marker's position with nanometer precision. For its implementation, INFO turns into an advantage the non-radiative energy transfer that fluorophores experience when placed in the vicinity of a metal wall. Thus, fluorophores closer to the near-field probe's rim (see figure below) become dimmer, which, by contrast, helps to localize more clearly the fluorophore underneath the probe's aperture that is less affected by the metal. A **sequential repetition** of the process *a*) and *b*), in synchronization with the lateral **scanning** of the near-field probe, allows the localization of sparse fluorophores within a region of sub-wavelength size hence overcoming the diffraction barrier. We will investigate the flexibility of implementing INFO with low and high power laser system, as well as standard fluorophores (rhodamine), quantum dots, and photo-switchable fluorophores.



**Fig. 1** INFO working principle. **Left:** The propagating-mode emission of the fluorophores is hampered by nonradiative energy transfer to the metal, except for the one(s) underneath the near-field probe's aperture. **Right:** The position of the fluorophore that emits more efficiently is localized with nanometer precision with standard far-field localization methods. The lateral dimension of the near-field probe (metal coated tapered fiber) is drawn approximately on scale.

The **INFO detection system** *i*) avoids using the typically low photon-yield associated with near-field optical microscopy (only the probe and its associated position-control electronics are exploited here), and rather relies on *ii*) the higher-yield and more flexible far-field optical detection set ups (including a CCD camera with single photon

sensitivity) for localizing the fluorophores at millisecond rates. For **instrumentation calibration** purposes of the INFO, biopolymers (such as DNA), with metal particles and fluorophores attached at defined locations, will be used as **nano-scale rulers**; the determination of the particles' position and mutual separation is facilitated by the particles' different optical responses. INFO will be compared to current state of the art biological tools like electron microscopy and AFM.

Potential **INFO bioimaging applications** will be pursued through collaboration with OHSU. We will tag discrete protein receptors that are embedded on the extracellular surface of cellular membranes using quantum dot (QD) fluorophores. Using INFO, we seek imaging with sub-diffraction resolution the spatial distribution of these QD-tagged receptors in 'fixed' membranes that are adhered to a surface (this is in air, no aqueous solution), and subsequently extend this method for studies in live cell environments. Insight into the spatial distribution of specific receptor proteins at the level of single receptors in cellular membranes will yield insight into fundamental mechanisms of cellular signaling which are not easily studied with diffraction limited resolution tools.

This proposal also underscores the combination of talents for **inter-disciplinary participation** in nano-metrology instrumentation development at PSU, and enhancing **inter-institutional collaboration** between PSU and OHSU. From the metrology application point of view, the significance of implementing INFO lies in extending PSU's existent expertise in near-field metrology instrumentation into the realm of current developments in far-field Fluorescence nanoscopy. In the educational aspect, the collaboration with OHSU will help **training PSU students** in bio-imaging applications, which will have an immediate beneficial impact in their careers.

## **II. CURRENT OPTICAL METROLOGY DEVELOPMENTS UNDERLYING THIS PROPOSAL**

### **Breaking the diffraction barrier**

Conventional Optical microscopy would be the preferred tool for characterizing biological dynamic events with nanometer spatial resolution given its simple use, relatively low cost, and, quite important, non-invasive character. Unfortunately, diffraction effects prevent conventional optical microscopy from providing spatial lateral resolution better than  $\lambda/2$  (where  $\lambda \sim 500$  nm is the wavelength of the radiation used) as enunciated by Ernst Abbe in 1873.

In 1928, it was conjectured that the diffraction-limited lateral resolution was not fundamental, but rather an inherent constraint resulting from the (unavoidable at that time) use of lenses that had a working distance (WD) greater than a few wavelengths ( $WD > \lambda$ ; that is, "far-field.")<sup>1</sup> Synge argued that if a metallic pin-hole of sub-wavelength diameter were *i*) used as a photon collector, *ii*) brought into the proximity of the sample ( $WD < \lambda$ ; that is "near-field"), and *iii*) laterally scanned along the sample's surface, then a lateral resolution equal to the pin-hole aperture-size (*i.e.* sub-wavelength) could, in principle, be achieved. These ideas constituted the renaissance of Near-field Scanning Optical Microscopy (NSOM), which became the first optical technique to overcome the diffraction barrier, as demonstrated in 1984<sup>2</sup> and witnessing further instrumentation development during the 90's.<sup>3,4</sup> NSOM, however, has its own inherent limitations, one of them being the typically low-level signal available for analysis. The smaller the aperture

(aiming for a finer resolution), the more stringent the requirements on the NSOM's detection system.

### **Far-field Fluorescence Nanoscopy**

A plethora of far field-based alternative solutions for attaining sub-wavelength optical resolution have appeared lately (STED,<sup>5</sup> FIONA,<sup>6</sup> RESOLFT,<sup>7</sup> PALM<sup>8</sup>), altogether referred to as Far-field Fluorescence Nanoscopy.<sup>9</sup> They exploit basically the following principles to bypass Abbe's barrier:

*Diffraction does not prevent finding out the coordinates of a point-source with arbitrary precision if there is no other similar point-source within a  $\lambda/2$  distance.* ( I )

*Or,*

*Localization of individual fluorophores positioned within a distance much smaller than  $\lambda/2$  can be achieved by sequentially imaging them based on either different spectral response or selectively activating their "ON" and "OFF" states).* ( II )

Principle (I) exploits the fact that when particles are widely spaced from each other (separation distance greater than the wavelength), their diffraction-limited images may superimpose but the position of their corresponding centroids can be estimated with nanometer precision by fitting the experimental data (light intensity) to Gaussian functions.<sup>10</sup> Hence, a direct correlation can be made between the light point source and the center of its diffraction limited image. Nanometer-sized step motion of the centroid have been obtained with a CCD camera of sufficient dynamic range (able to distinguish fine difference in intensity levels.) The fitting method has successfully been used to track the motion of cargo-carrying myosin with 2 nm precision, helping to figure out that they undergo hand-over-hand motion.<sup>11</sup>

When several fluorophores (in their active state) are located inside a sub-wavelength-sized region, one way to distinguish them is by using an incident-beam having a doughnut-shape intensity lateral distribution, which causes all the fluorophores to quench except the one in the doughnut's center. In this stimulated emission depletion (STED)<sup>5</sup> method, it turns out, fluorescent spots of size far-below the diffraction are obtained. A disadvantage of the technique is the requirement of intense (picosecond) pulses (which tends to boost multiphoton-induced bleaching of the dye), in addition to the complex optical alignment required to obtain the doughnut-shape intensity. In this proposal, a much simpler approach for selectively quenching several surrounding fluorophores but leaving activated a single one is introduced.

## **III. AIMS**

### **III.1 To implement the "Integrated Near- and Far- Field Optics (INFO)" technique.**

*(La Rosa)*

The implementation will proceed along the working principle outlined in Fig. 1. In contrast to the STED technique (which is based entirely on far-field methods), the new INFO approach combines **a**) a typical near-field optics probe (see Fig. 2), used this time for selectively perturbing the emission of the fluorophores (instead of its more common NSOM's photon-collector role),<sup>12</sup> **b**) an epi-fluorescence far-field arrangement (IX-71

Olympus Epi-fluorescence Inverted Microscope) for both exciting the fluorophores (using a PLAN APO 100X objective lens, 1.45 NA; incident beam depicted in blue color in Fig. 1) and detecting the emission of the selected marker (scattered light depicted in red color) with a EM-CCD camera having single-photon sensitivity (Princeton Instrument/Acton.) , and *c*) a Gaussian function fitting algorithm to locate the centroid of the conjugate diffraction limited image (see right side of Fig. 2).



**Fig.2. Left:** Metal coated probe, with an aperture at the apex, fabricated at the PI's laboratory. **Right:** Image of spread fluorophore markers captured by our CCD camera. The arrow indicates the point-source chosen for the Gaussian function fitting algorithm (from preliminary work currently being performed at the PI's laboratory.)

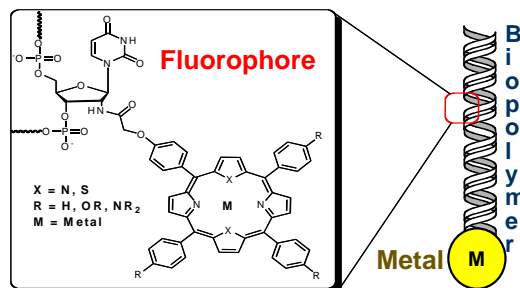
A key aspect in the technique is to carefully bring the probe into the proximity of the sample's surface,  $z \sim 10$  nm or less. This is achieved by *i*) using a piezotube actuator for fine sub-nanometer approaching steps, and then *ii*) by activating a shear-force feedback control, based on the response of a piezoelectric tuning fork to which the probe is attached, for maintaining such probe-sample separation very stable.<sup>13</sup> The lateral position of the probe is also maintained very accurately by using a piezo scanner stage equipped with capacitance sensor to overcome piezo hysteresis effects.

In the presence of the metal-coated tapered-fiber probe the efficiency of the propagating-mode emission of the fluorophores decreases due to the nonradiative energy transfer (NRET) to the probe's metallic rim. However the fluorophore at the center is much less affected since the NRET is strongly dependent on the fluorophore-metal distance.<sup>14</sup>

### III.2 Biopolymer Rulers and QD structural characterization (Reed, Moeck, La Rosa)

*Single molecules and nanoparticles attached to biopolymer strands will be used as nano-rulers to demonstrate nanometer localization of multiple single-particle within a subwavelength-sized region*

For the completion of this task, this proposal capitalizes on Dr. Reed's and Dr. Moeck's expertise in harnessing the use of gold nanoparticles (to alter the optical properties of adjacent chromophores, acting as antennae for absorbed and emitted light),<sup>15</sup> and structural characterization of



**Fig. 3 Nanorulers for INFO calibration.** Porphyrin fluorophores are attached using solid-phase phosphoramidite synthesis at defined locations along oligonucleotide chains, which are attached to metal nanoparticles.

semiconductor quantum dots (QD)<sup>16</sup> (which display size dependent chemical, optical, and catalytic properties.)<sup>17,18</sup> As implied from the discussion in Section II above, tuning the spectral response of optical tags opens an avenue for optical imaging beyond Abbe's barrier.

### **III.3 Imaging the Spatial Distribution of Membrane Receptor Proteins**

*(Vu, Reed, La Rosa)*

We will apply INFO to study the sub-diffractive distribution of receptor proteins in cellular membranes. To do this, we will tag discrete protein receptors that are embedded on the extracellular surface of cellular membranes using quantum dot (QD) fluorophores. We use a model ligand-receptor system that comprises QD-nerve growth factor-tyrosine kinase receptors; this is a system which Vu has past extensive experience in using QDs to target specific receptors.<sup>19,20</sup> The protein expression of these growth factor- tyrosine kinase receptors are implicated in a variety of disease states including neurodegeneration and neurogenesis and the capability to study with improved resolution the distribution and amount of receptors on cellular membranes would be invaluable to understanding the factors that regulate the distribution and response of this receptor signaling pathway. Using INFO, we will use QDs to tag NGF-Trk receptors in live cells and then we will fix these QD-tagged cells to preserve the QD-tags on cross-linked cellular membranes to retain structural integrity. We will apply INFO imaging to map with sub-diffractive resolution the spatial distribution of these QD-tagged Trk receptors in these 'fixed' membranes in air. If this method proves successful, we will extend it for studies in live cell (aqueous) environments. We will capitalize on previous applications of NSOM for imaging in aqueous environments.<sup>21</sup>

## **IV. BROADER IMPACT**

The broader impacts resulting from the proposed activity comprise promoting further interdisciplinary collaboration among members of the Portland Nanoscience and Nanotechnology Academy (PNNA) [www.pnna.groups.pdx.edu](http://www.pnna.groups.pdx.edu). The group consists of over 20 ONAMI researchers from the Departments of Biology, Chemistry, Electrical and Computer Engineering, Geology, Mechanical and Materials Engineering, and Physics at Portland State University (PSU), and the Biomedical Engineering Department at Oregon Health and Science University (OHSU). The PI and co-PIs participating in this proposal have played a key role in the organization of the PNNA. With a demonstrated track record implementing joined activities, the PNNA plans also to build a network of research equipment under the responsibility of smaller units but with coordinated accessibility to all PNNA members. The development of *Integrated Near- and Far- Field Optical* (INFO) microscopy will add to the list of shared nanometrology tools available to all PNNA members.

## **V. REFERENCES**

- 
- <sup>1</sup> E. H. Singe, "A suggested Method for extending Microscopic Resolution into the Ultra-Microscopic Regime", *Phil. Mag.* **6**, 356 (1928).
  - <sup>2</sup> D. W. Pohl, W. Denk, and M. Lanz, "Optical stethoscopy: Image recording with resolution  $\lambda/20$ ", *Appl. Phys. Lett.* **4**, 651 (1984).

- 
- <sup>3</sup> E. Betzig and R. J. Chichester, "Single Molecules Observed by Near-Field Scanning Optical Microscopy", *Science* **262**, pp. 1422-25 (1993).
- <sup>4</sup> Horia Metiu, Editor; Special Topic "Near-field microscopy and spectroscopy"; *The Journal of Chemical Physics*, **112**, Issue 18 (2000).
- <sup>5</sup> S. W. Hell and J. Wichmann, *Opt. Lett.* **19**, 780 (1994).
- <sup>6</sup> A. Yildiz, J. N. Forkey, S. A. McKinney, T. Ha, Y. E. Goldman, P. R. Selvin, "Myosin V Walks Hand-Over-Hand: Single Fluorophore Imaging with 1.5-nm Localization," *Science* **300**, 2061 (2003).
- <sup>7</sup> M. Hofmann, C. Eggeling, S. Jakobs, and S. W. Hell, "Breaking the diffraction barrier in fluorescence microscopy at low light intensities by using reversibly photoswitchable proteins," *PNAS* **102**, 1756 (2005).
- <sup>8</sup> E. Betzig et al., "Imaging Intracellular Fluorescent Proteins at Nanometer Resolution," *Science* **313**, 1642 (2006).
- <sup>9</sup> Stefan W. Hell, "Far-Field Optical Nanoscopy," *Science* **316**, 1153 (2007).
- <sup>10</sup> R. E. Thompson, D. R. Larson, and W. W. Webb, "Precise Nanometer Localization Analysis for Individual Fluorescent Probes," *Biophys. J.* **82**, 2775–2783 (2002).
- <sup>11</sup> A. Yildiz, J. N. Forkey, S. A. McKinney, T. Ha, Y. E. Goldman, P. R. Selvin, Myosin V Walks Hand-Over-Hand: Single Fluorophore Imaging with 1.5-nm Localization, *Science* **300**, 2061 (2003).
- <sup>12</sup> M. Ohtsu, K. Kobayashi, Motoichi Ohtsu, "Optical Near Fields: Introduction to Classical and Quantum Theories of Electromagnetic Phenomena at the Nanoscale," Springer-Verlag, (2004).
- <sup>13</sup> K. Karrai and R. D. Grober, in Near Field Optics, edited by M. A. Paesler and P. J. Moyer, SPIE Proceedings Series **2535** (SPIE Bellingham, WA, 1995), pp. 69-81 (1995).
- <sup>14</sup> P. Avouris and B. N. J. Persson, *J. Phys. Chem.* **88**, 837 (1984).
- <sup>15</sup> Walter W. Weare, S. M. Reed, Marvin G. Warner, and James E. Hutchison, "Improved Synthesis of Small (d core = 1.5nm) Phosphine-Stabilized Gold Nanoparticles." *J. Am. Chem. Soc.*, **122**, 12890-12891 (2000).
- <sup>16</sup> Allivasatos et al; *Nature* **382**, 609 (1996).
- <sup>17</sup> Manoj Nirmal and Louis Brus, "Luminescence Photophysics in Semiconductor Nanocrystals," *Acc. Chem. Res.* **32**, 407-414 (1999).
- <sup>18</sup> P. Moeck, O. Čertík, G. Upreti, B. Seipel, M. Harvey, W. Garrick, and P. Fraundorf, "Crystal structure visualizations in three dimensions with support from the open access Nano-Crystallography Database," *J. Mater. Educ.* **28**(1), 87 (2006). <http://crystallography.net>
- <sup>19</sup> Liu, H.Y. and T.Q. Vu, "Identification of quantum dot bioconjugates and cellular protein co-localization by hybrid gel blotting," *Nano Lett.* **7**, 1044 (2007).
- <sup>20</sup> Sundara Rajan, S. and T.Q. Vu, "Quantum Dots Monitor TrkA Receptor Dynamics in the Interior of Neural PC12 Cells," *Nano Letters*, **6**, 2049 (2006).
- <sup>21</sup> W. H. J. Rensena) and N. F. van Hulst, "Imaging soft samples in liquid with tuning fork based shear force microscopy," *Appl Phys. Lett.* **77**, 1557 (2000).