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Nanoscale Imaging

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I. Brief Overview of the Project and Significance

The goal of this project is to develop techniques to enable high-resolution imaging of fluorescent biological samples. A specific aim is to achieve an order of magnitude or better improvement in axial resolution relative to confocal microscopy to provide “sectioning” capability. The path we are pursuing is a combination of an interferometric technique and 4Pi microscopy. This combination provides confocal resolution in the lateral plane and an axial resolution that is around four times better than confocal axial resolution.

Bryn Davis, the graduate student funded by Gordon-CenSSIS for several years, graduated in 2006 and is now at the Beckman Institute at the University of Illinois, Urbana-Champaign. Mehmet Dogan, the currently funded student, is using 4Pi microscopy to study Shigella bacteria [1]. Together, Davis and Dogan have published papers on 4Pi-spectral self-interference fluorescence microscopy (SSFM) measurements and theoretical analysis [2-3].

II. State-of-the-Art

The state-of-the art in optical fluorescence microscopy can be roughly divided into three areas; diffraction limited confocal microscopy, interference localization, and optical point-spread function engineering [4], with the best results from combinations of these techniques. In the case of very sparse samples, an additional benefit of optical fluorescence microscopy is that localization becomes almost independent of resolution. For example, a stained single membrane layer provides a sparse sample in the axial direction. This is the type of sample that works the best for SSFM, which provides excellent localization measurement but low resolution. Recently, two new techniques, stochastic optical reconstruction microscopy (STORM) and photoactivatable localization microscopy (PALM), have been introduced. These two techniques exploit parallel localization of sparse emitters by estimating the center of the emission point-spread function of the emitters. Both techniques rely on the constraint that during a given integration time the system is sparsely emitting, fulfilling two criteria for sparse imaging – that the fluorescence emission originates from single molecules during the integration time of the detection system, and that the separation of molecules is larger than the resolution limit of the system.

It is necessary to consider fluorescence for these techniques to work. This is not a large limitation in cell biology, as fluorescence tags can be specifically attached to proteins, DNA, and

even produced by the cell itself. Secondly, the fluorescence can be manipulated which has made it possible to manipulate the size of the excited volume so as to get around Abbe's resolution limit on sound physical principles.

A. Diffraction Limited Microscopy

The diffraction limit is reached in confocal microscopy by using well-collimated laser light with a flat phase front for illumination and discriminating against out-of-focus light in collection with the use of a pinhole. Two-photon microscopy achieves a very high signal-to-noise ratio compared to confocal microscopy, since the excitation probability is proportional to the square of the intensity so that the out-of-focus light gets de-emphasized [5].

A large light collection angle improves the resolution, and a 4Pi microscope, which uses two opposing lenses to collect as much as possible of the full 4π solid angle, significantly improves the axial resolution when the collected light is coherently combined to yield close to optimal resolution. Here we review the experimental set-up of the 4Pi microscope that we are using.

Allowing the illumination wave-fronts to constructively interfere in the sample produces a main focal spot that is sharper in the z-direction by about 3-4 times (4Pi A). A similar improvement is obtained if the lenses add their collected fluorescence wave-fronts in a common point detector (4Pi B). Doing both together yields the best result and leads to a 5-7-fold improvement of resolution along z (4Pi C) [6]. Since a small part of the collection cones are missing, the resulting point spread function is not perfectly spherical, but has two weaker sidelobes in the axial direction which cause image artifacts. The images are restored via fast image processing. A diagram of the system is shown in Figure 1.

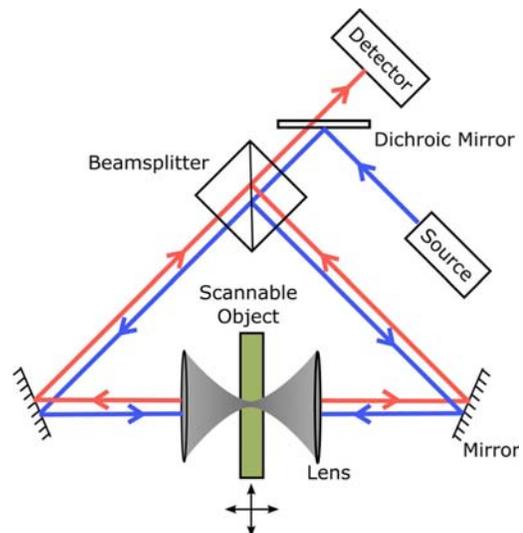


Figure 1. 4Pi microscope configuration. The fluorescent sample is coherently illuminated from two opposing objectives. The emitted light is coherently collected from the two opposing objectives and interfered at the detector.

B. Interference Localization Techniques

These measurements generally use a transparent thin film on a reflecting surface as a substrate to generate interference between light reflected or generated directly from the imaged object and the light reflected from the buried reflective interface.

Fluorescence intensity measurements

In a series of elegant experiments performed in the 1960s, the fluorescence decay of an organic dye embedded into a lipid layer was demonstrated to be an oscillatory function of its distance above a silver mirror [7]. This observation led to the idea that the position of a dye above a mirror can be determined from the amount of fluorescent light it emits including both emission and excitation interference [8-9]. In order to convert the intensity response to a height location, many different and well-known thin film thicknesses are necessary to calibrate the intensity response from the sample and account for fluorophore brightness and density.

Fluorescence color (spectral) measurements

Spectral self-interference fluorescence microscopy (SSFM), a technique we have developed [10-11], also determines the height of fluorescent molecules above the surface to sub-nm precision, but via analysis of the periodic intensity variations in the spectral domain. Analysis of the spectral fringes removes the need for many comparative measurements needed in intensity interference measurements. Furthermore, it is completely independent of the fluorophore density and makes it possible to resolve the axial position of spectrally non-overlapping fluorophores to nm precision.

White light color (spectral) measurements

Color changes caused by interference from a thin film can be used to measure nm thickness changes, even by the naked eye, when the film thicknesses and reflectivities are optimized [12]. For example, the interference technique is used to find a single layer of graphene on a surface by eye [13]. It is important to keep in mind that it is the averaged optical thickness that is measured; for example, a sparse film of DNA nucleotides registers as a fraction of the DNA sequence's actual physical extent. The combination of white light interference with fluorescent tags (SSFM) gives a wealth of information on DNA density and hybridization as well as conformational changes between single- and double-stranded DNA. We have utilized the combined methods to determine the effective hybridization of single-stranded to double-stranded DNA [14].

III. Technical Approach and Major Contributions

During the past year, we have made several advances that will be described below:

- The 4 Pi microscope is completed and in use.
- Analysis of the abilities and limitations of SSFM on planar surfaces and SSFM in 4Pi configuration has been published.
- The first measurements of the *Shigella Flexneri* bacterium have been accomplished.

A. Technical Approach

We have been working on integrating the SSFM technology with a 4Pi microscope to enable high lateral resolution while determining the position of sparse fluorescent emitters with nanometer precision. Although SSFM and 4Pi are implemented in very different fashions, they both rely on interference produced by two different paths to and from the object. The excitation and collection via the reflections from the SSFM mirror are very similar to the action of the second objective path in 4Pi microscopy. These methods have been described in detail in earlier reports.

The connection between a 4Pi system and a system with single objective over a mirror (as in SSFM) has also been noted in the literature [15]. While the operating principle behind 4Pi and SSFM is somewhat similar, there are several key differences:

Spectral Detection: SSFM relies on spectral detection to determine the axial position of the imaged fluorophore. 4Pi microscopes typically collect light in a window around a single central wavelength. Multi-wavelength 4Pi systems have been implemented [16] but their purpose is to simultaneously image a multiply-stained object, rather than to measure the spectrum from a single dye.

Scanning: SSFM scans in the lateral directions only as the axial resolution is provided by the spectral self-interference effect. By contrast, 4Pi microscopes scan in all three spatial dimensions when creating a three-dimensional image. However, it should be noted that a thick object may occupy an axial region greater than the depth of focus in SSFM. SSFM imaging of such an object may require some form of coarse axial scanning.

Unequal Path Lengths: SSFM relies on a difference between the two path lengths to produce wavelength-dependent interference and the characteristic spectral signatures. Equal optical path lengths are usually used in 4Pi microscopy so that both the excitation and detection patterns have a constructive peak at the twin lenses' focal point. 4Pi experiments with destructive interference have been conducted by other researchers who did not take advantage of the extra information [17]. If the path lengths in a 4Pi microscope differ by more than the fluorescent emission coherence length, the spectral fringes will wash out across the wavelengths for which the detector is sensitive, and no interference effects will be observed. Fine spectral resolution is employed in SSFM so that the fringes can be seen.

Foci Offset: The foci of the two objectives are matched in 4Pi microscopy, but this is not possible in SSFM. In SSFM the two optical paths have different lengths, but both pass through the same objective lens. This implies that both paths cannot be simultaneously focused to the same point.

Mirror Effects: In general, the mirror in SSFM will have a reflection coefficient of less than 1 and will introduce a phase shift. This reflection coefficient may also vary with incidence angle and thus modify the angular distribution of the reflected light. By contrast, a 4Pi system will have balanced power in both optical paths and (ideally) be free of phase shifts and angular distortions.

B. Accomplishments

Use of 4Pi SSFM to remove axial scanning for precise axial localization

The data shown in Figure 2 have features indicative of both 4Pi microscopy and SSFM. With the wavelength held fixed, the z profile has the characteristics of the 4Pi point-spread function (PSF).

Nanometer-scale precision can be achieved using non-spectral measurements (4Pi operation) but requires axial scanning to collect a z profile that can be fitted to the expected PSF. If z is held fixed, the spectral profile corresponds to the spectrum observed in SSFM when the fluorophore layer is at position z . As can be seen from Figure 2, the spectral profile changes significantly as z changes. It is this spectral sensitivity that gives SSFM the ability to localize structures from a single spectral measurement. That is, given spectral data from a single spatial point, the precise axial

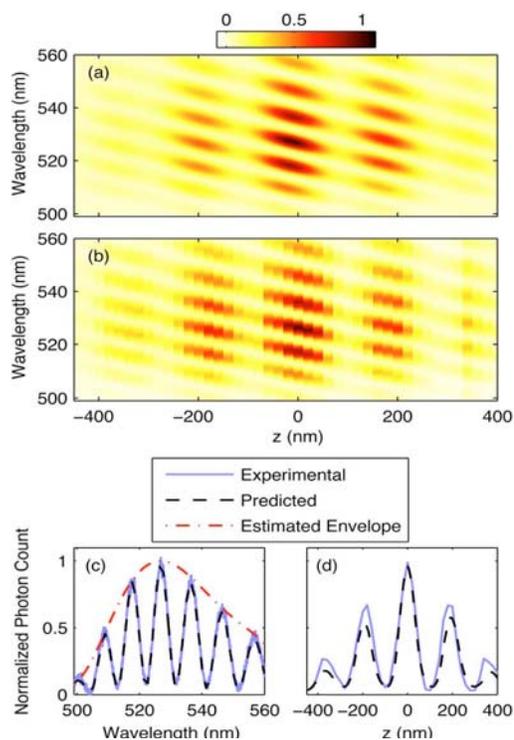


Figure 2. a) predicted and (b) measured axial spectral data from a 4Pi-SSM system when imaging a thin lateral fluorescent layer. Spectral profiles at $z=0$ and the estimated spectral envelope $s(\lambda)$ are shown in (c), as is a spatial profile at $\lambda=527$ nm in (d).

position of an emitter within the focal volume can be estimated—no axial scanning is required. In the 4Pi-SSFM instrument a much smaller lateral area contributes to the signal than in standard SSFM and thus the lateral resolution is improved [3].

Measurements of Shigella flexneri

Glucosylation is important to *Shigella* pathogenesis, since mutants in the glucosylation pathway are less virulent than the wild type. The two different types of sugars have different physical extent, and we are imaging these differences with the spectral 4Pi microscope.

We have used the 4Pi-SSFM system to probe the outer membrane of the gram-negative bacterium *Shigella flexneri*, a rod-shaped human pathogen that is typically 1.5-2 μm in diameter and 4-5 μm in length. We labeled the outer membrane of the bacteria with FM4-64 dye during the exponential phase growth. The labeled bacteria were harvested by centrifugation and fixed to a poly-L-lysine-coated glass cover slip. The mounting medium (0.1 M N-propyl gallate in 20 mM Tris-HCl, pH 8.0, 154 mM NaCl, 0.02% NaN_3 , 50% (v/v) glycerol) was added to the sample, which was then covered with a second glass cover slip. The outer membrane of the bacterium on the opposite side of the bacterium-glass contact was then scanned laterally within the common focus of the two objectives and self interference spectra were collected for 10 seconds for each lateral scan point. Each spectrum revealed an oscillatory profile whose period and phase depend on the average axial position of the probed volume. Using a simplified isotropic emission model for the emitters, we applied a fitting algorithm to find the axial position of each lateral point relative to the geometrical focus. As demonstrated in Figure 3, we generated a surface plot of the bacterial outer membrane that is a few microns away from the coverslip surface. Techniques like fluorescence interference-contrast (FLIC) microscopy that are utilized to provide membrane topography localize membrane-bound fluorophores close to solid supports. On the other hand, 4Pi-SSFM inherently does not rely on the presence of a support to determine axial position [3].

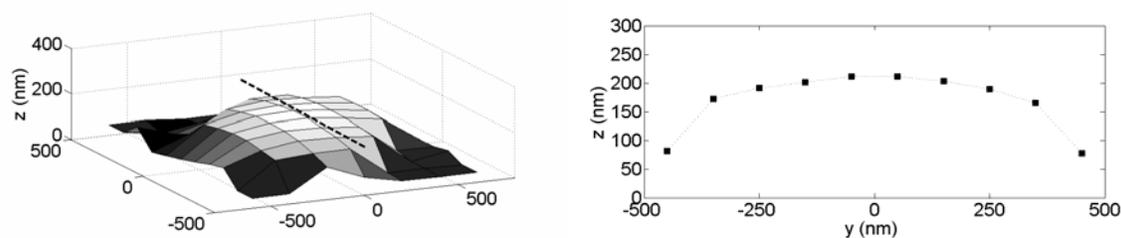


Figure 3. Left: Surface plot of the bacterial membrane probed by 4Pi-SSFM. Right: A line cut of the surface profile along the dashed line shown in left-hand image.

In addition to the membrane, the bacterial surface contains multiple biologically important structures, including a protective polysaccharide layer, surface protein receptors, and specialized secretion systems. These surface structures interact with molecules in the environment and, during bacterial infection of mammalian hosts, with components of the mammalian cell. The spatial positioning of these molecules is critical to these interactions. 4Pi-SSFM is promising as a tool for the analysis of the positioning of these structures *in vitro* and under biologically relevant conditions.

IV. Future Directions

The simplicity of the planar surface SSFM is well-suited for a subset of imaging (single layer determination). The spectral self-interference imaging methods have been further developed by non-CenSSIS students. Several promising approaches are being pursued in both fluorescence imaging and non-labeled dynamical imaging of binding events using spectral reflectance imaging biosensor.

4Pi-SSFM also provides excellent resolution which makes it suitable for cell imaging. We are continuing to pursue the Shigella system. In addition to the membrane, the bacterial surface contains multiple biologically important structures, including a protective polysaccharide layer, surface protein receptors, and specialized secretion systems. These surface structures interact with molecules in the environment and with components of the mammalian cell during bacterial infection of mammalian hosts. Spatial positioning of these molecules is critical to these interactions. 4Pi-SSFM is promising as a tool for the analysis of the positioning of these structures *in vitro* and under biologically relevant conditions.

V. Center Strategic Goals and Legacy

The impact of nano-bioimaging expertise in Boston is higher than most places in the world, given the large number of cutting-edge biomedical researchers in the area. However, there are no project linkages with the S-level at this time.

VI. References Cited

- [1] Dogan, M., Yalcin, A., Jain, S., Goldberg, B. B., Swan, A. K., Ünlü, M. S., and Goldberg, B. B., "Spectral Self-Interference Fluorescence Microscopy for Subcellular Imaging", *Journal of Special Topics In Quantum Electronics*, accepted Dec. 2007.
- [2] Davis, B. J., Dogan, M., Goldberg, B. B., Karl, W. C., Ünlü, M. S., Swan, A. K., "4Pi spectral self-interference microscopy," *Journal of the Optical Society of America A*, vol. 24, no. 12, pp. 3762-3771, Nov. 2007.
- [3] Davis, B. J., Swan, A. K., Ünlü, M. S., Karl, W. C., Goldberg, B. B., Schotland, J. C. and Carney, P. S., "Spectral self-interference microscopy for low-signal nanoscale axial imaging," *Journal of the Optical Society of America A*, vol. 24, no. 11, pp. 3587-3599, Oct. 2007.
- [4] The term "point-spread Engineering" is borrowed from Stefan Hell.
- [5] So, P. T. C., Dong, C. Y., Masters, B. R., and Berland, K. M., "Two-photon excitation fluorescence microscopy," *Ann. Rev. Biomed. Eng.*, vol. 2, pp. 399-429, 2000.
- [6] Hell, S. W. and Stelzer, E. H. K., "Properties of a 4Pi-confocal fluorescence microscope", *J. Opt. Soc. Am. A*, vol. 9, pp. 2159-2166, 1992.
- [7] Drexhage, K. H., "Interaction of Light with Monomolecular Dye Layers", *Prog. Opt.*, vol. 12, p. 163, 1974.
- [8] A. Lambacher and P. Fromherz, "Fluorescence interference-contrast microscopy on oxidized silicon using a monomolecular dye layer," *Appl. Phys. A-Mater.*, vol. 63, pp. 207-216, 1996.
- [9] Braun, D., and Fromherz, P., "Fluorescence interferometry of neuronal cell adhesion on microstructured silicon," *Phys. Rev. Lett.*, vol. 81, pp. 5241-5244, 1998.

- [10] Swan, A.K., Moiseev, L., Cantor, C. R., Davis, B., Ippolito, S. B., “Towards nanometer-scale resolution in fluorescence microscopy using spectral self-interference,” *Journal of Special Topics In Quantum Electronics*, vol. 9, pp. 294-300, 2003.
- [11] Moiseev, L., Cantor, C., Aksun, R. I., Dogan, M., Goldberg, B. B., Swan, A. K., and Ünlü, M. S., “Spectral self-interference fluorescence microscopy” *J. Appl. Phys.*, vol. 96, pp. 5311-5315, 2004.
- [12] Jenison, R, Yang, S, Haeberli, A, and Polisky, B, “Interference-based detection of nucleic acid targets on optically coated silicon” *Nature Biotechnology*, vol. 19, no. 1, p. 62, 2001.
- [13] <http://ultra.bu.edu/research.asp> and Ferrari, A.C., Meyer, J.C., Scardaci, V., et al, “Raman spectrum of graphene and graphene layers”, *Phys.Rev.Lett.*, vol. 97, article 187401, 2006.
- [14] Moiseev, Lev, Ünlü, M. Selim, Swan, Anna K., Goldberg, Bennett B., and Cantor, Charles R., “DNA Conformation on Surfaces Measured by Fluorescence Self-Interference”, *Proceedings of the National Academy of Science*, vol. 103, no. 21, pp. 2623-2628, February 2006.
- [15] van de Nes, A. S. Billy, L., Pereira, S. F., and Braat, J. J. M., “Calculation of the vectorial field distribution in a stratified focal region of a high numerical aperture imaging system,” *Optics Express*, vol. 12, no. 7, pp. 1281–1293, Apr. 2004.
- [16] Kano, H. Jakobs, S., Nagorni, M. and Hell, S. W., “Dual-color 4Pi-confocal microscopy with 3D-resolution in the 100 nm range,” *Ultramicroscopy*, vol. 90, no. 2, pp. 207–213 Feb. 2002.
- [17] Hell, S. W. and Nagorni, M., “4Pi confocal microscopy with alternate interference,” *Optics Letters*, vol. 23, no. 20, pp. 1567-1569, 1998.

VII. Documentation

A. Publications Acknowledging NSF Support

1. Davis, B. J., Dogan, M., Goldberg, B. B., Karl, W. C., Ünlü, M. S., Swan, A. K., “4Pi spectral self-interference microscopy,” *Journal of the Optical Society of America A*, vol. 24, no. 12, pp. 3762-3771, Nov. 2007.
2. Davis, B. J., Swan, A. K., Ünlü, M. S., Karl, W. C., Goldberg, B. B., Schotland, J. C. and Carney, P. S., “Spectral self-interference microscopy for low-signal nanoscale axial imaging,” *Journal of the Optical Society of America A*, vol. 24, no. 11, pp. 3587-3599, Oct. 2007.
3. Dogan, M., Swan, A. K., Ünlü, M. S. and Goldberg, B. B., “Nanometer Scale Axial Localization of Fluorescent Emitters for Cellular Imaging,” *Proceedings of IEEE Lasers and Electro-Optics Society 2007 Annual Meeting*, Lake Buena Vista FL, pp. 151-152, Oct. 2007.
4. Dogan, M., Dröge, P., Swan, A. K., Ünlü, M. S. and Goldberg, B. B., "Probing DNA-Protein Interactions on Surfaces Using Spectral Self-interference Fluorescence Microscopy," *Bulletin of the American Physical Society Meeting*, Denver CO, Mar. 2007.

B. Relevant RICC Posters

1. Dogan, M., Moiseev, L., Ippolito, S. B., Swan, A. K., Goldberg, B. B. and Ünlü, M. S., “Spectral Self-interference Fluorescence Microscopy in 4Pi Mode”, presented at the Gordon-CenSSIS Research & Industrial Collaboration Conference (RICC), Boston MA, Oct. 2007.