

Visible-light two-photon excitation for subtractive SAX imaging

Katelyn Miyasaki,^{1,2} Ryosuke Oketani,³ Toshiki Kubo,³ and Katsumasa Fujita³

¹*Department of Biomedical Engineering, Washington University in St. Louis, St. Louis, MO, USA*

²*Nakatani RIES: Research and International Experiences for Students Fellowship in Japan, Rice University, Houston, TX, USA*

³*Department of Applied Physics, Osaka University, Suita, Osaka, Japan*

Techniques for imaging beyond the diffraction limit, especially those that do not damage cells and can image multiple targets simultaneously, are particularly relevant to viewing biological processes. Since no perfect imaging technique exists, scientists continue to optimize imaging processes for different situations. Many of today's super-resolution imaging techniques rely upon nonlinear relationships in fluorescence emission. We are combining two techniques for super-resolution fluorescence imaging, subtractive saturated excitation (SAX) microscopy and visible-light two-photon excitation (2PE), in order to ascertain the level of detail that can be obtained. To this end, we developed an optical system to acquire scanning fluorescence images and obtained fluorescence curves for fluorescent proteins to confirm 2PE. Then, we imaged a number of different samples, including fluorescent beads and cells containing fluorescent protein tags. Using these images, we produced subtractive images in search of a nonlinear response that would confirm the effectiveness of subtractive SAX. We expect to be able to surpass the resolution achievable with either subtractive SAX or visible-light 2PE alone through combining them, thereby improving biological microscopy studies.

INTRODUCTION

- Many super-resolution imaging techniques rely upon nonlinear relationships in fluorescence emission
- Imaging biological systems has specific requirements that must be optimized for, e.g. imaging multiple targets and minimizing photodamage to samples

This project:

- Combine two techniques for super-resolution fluorescence imaging, subtractive saturated excitation (SSAX) microscopy and visible-light two-photon excitation (2PE)
- Develop optical system to obtain 2PE laser scanning images
- Image a variety of samples and produce SSAX images
- Evaluate resolution improvement of SSAX images

SUPER-RESOLUTION TECHNIQUES

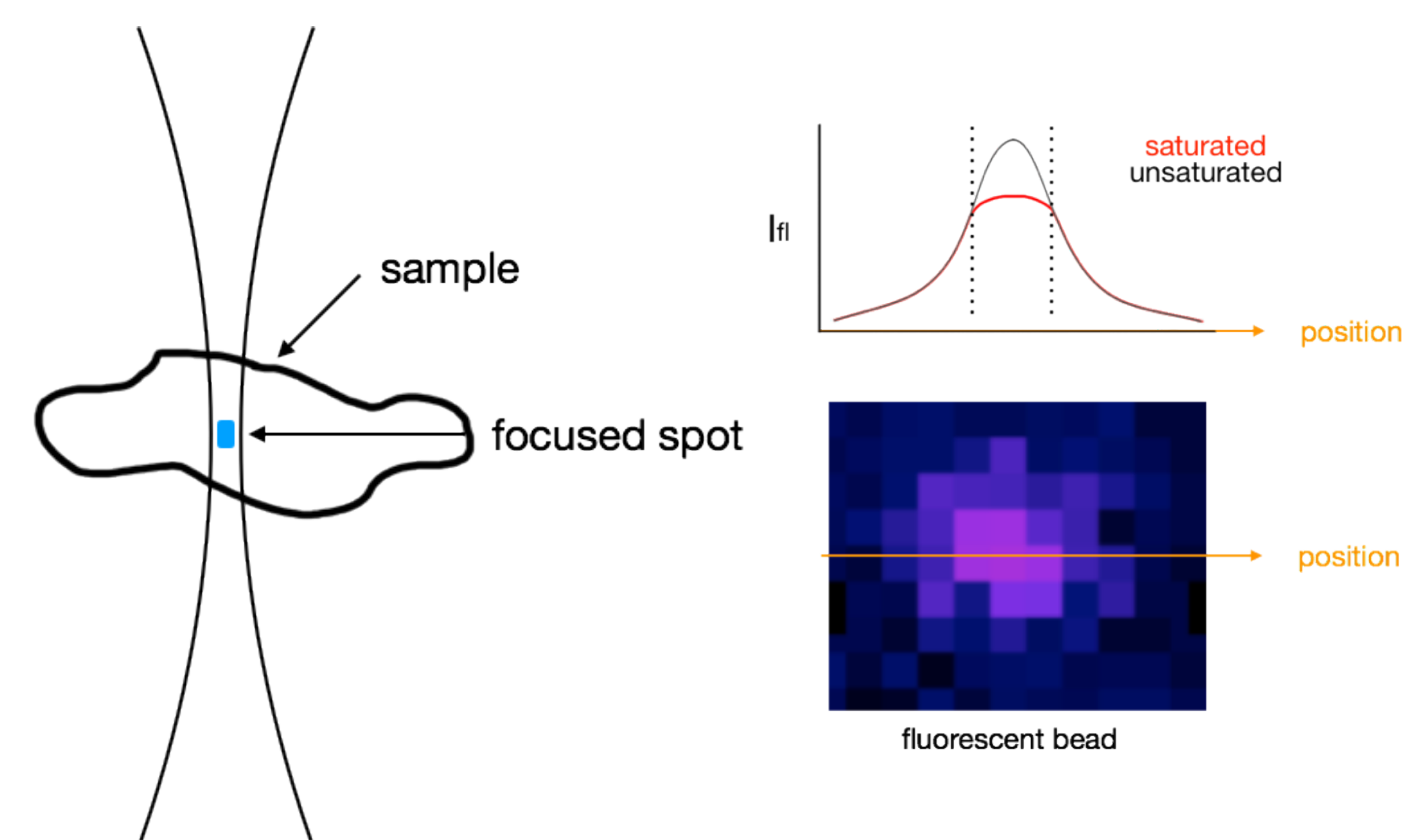


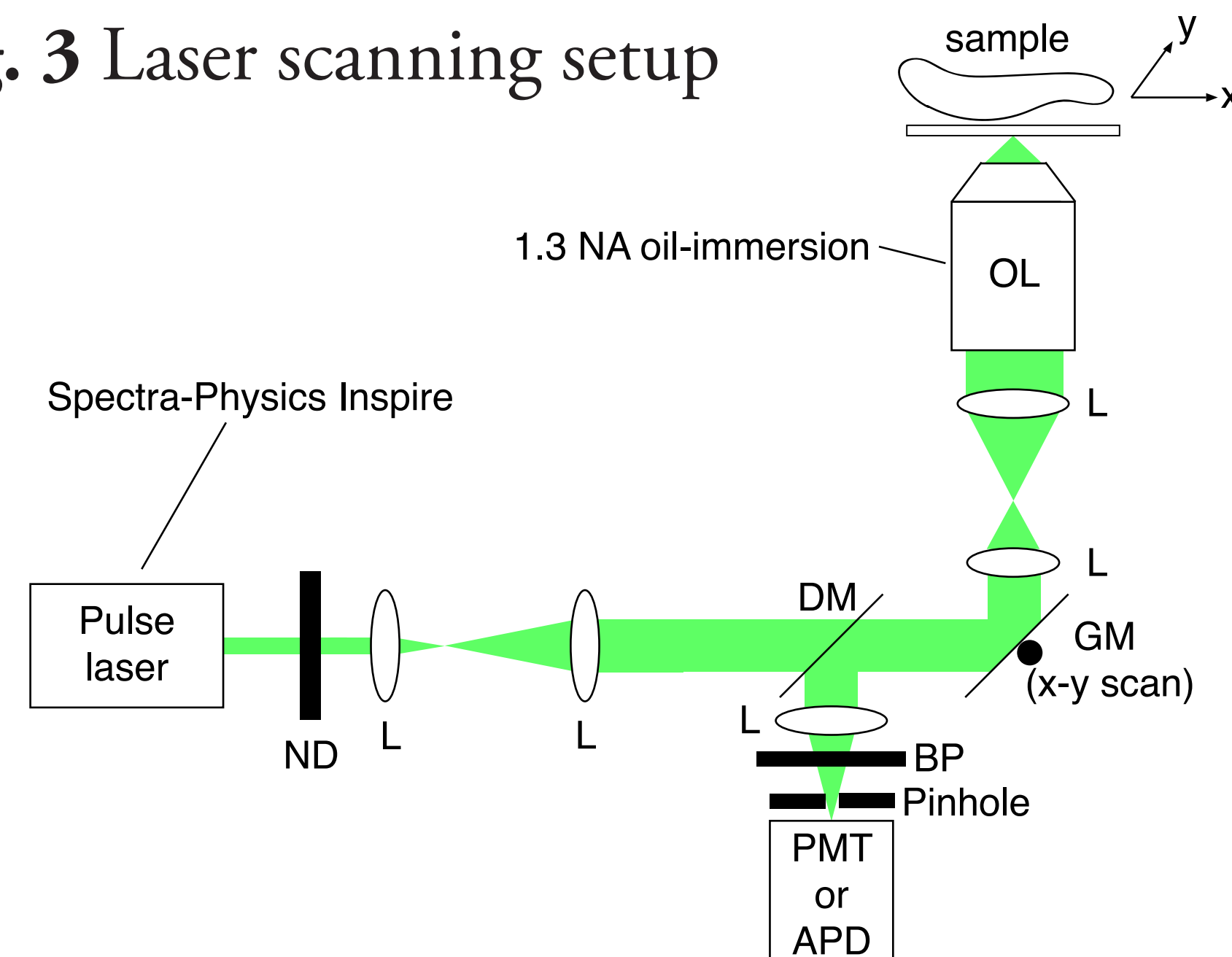
Fig. 1 2PE resolution improvement

Fig. 2 SSAX resolution improvement

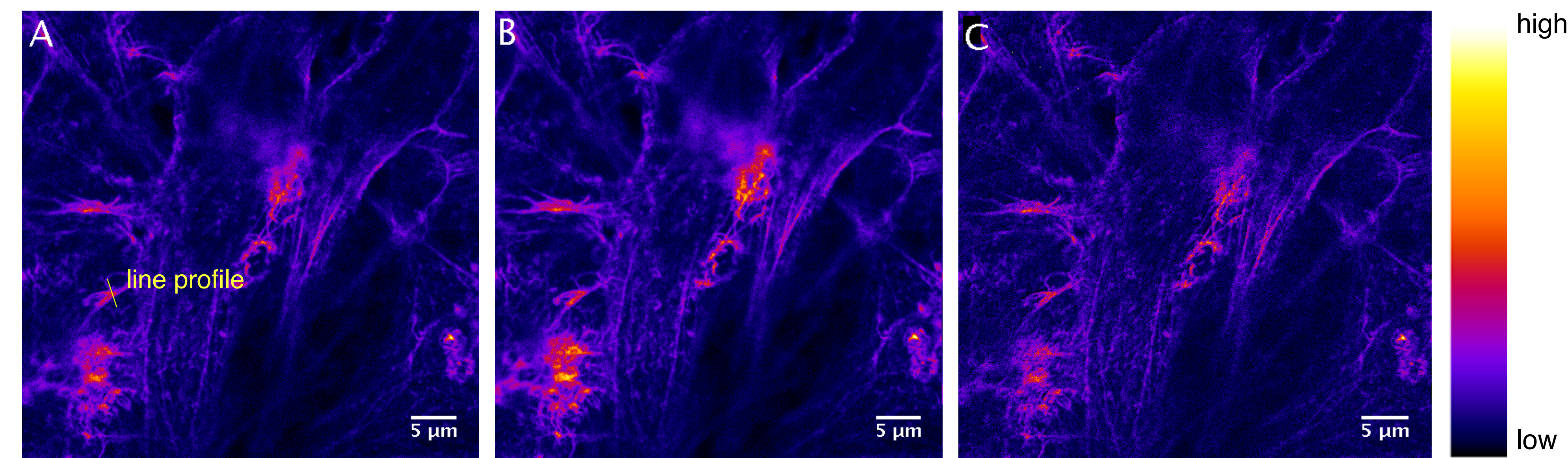
- 2PE limits area of fluorescence emittance
- Subtracting saturated curve from unsaturated curve results in narrower peak
- Result: Narrower signal and better resolution

OPTICAL SETUP

Fig. 3 Laser scanning setup



CELL IMAGING - DIFFERENTIATING PEAKS



Unsaturated, 0.48 mW

Saturated, 0.96 mW

SSAX

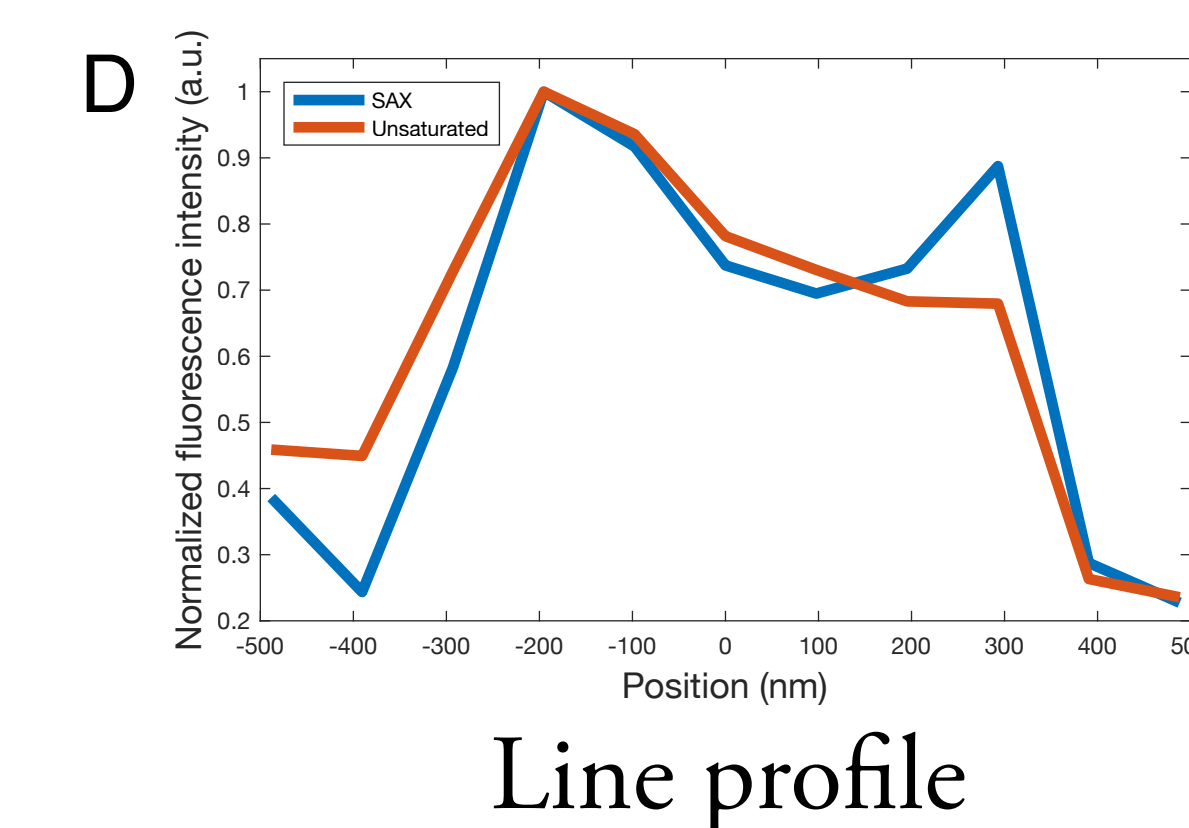
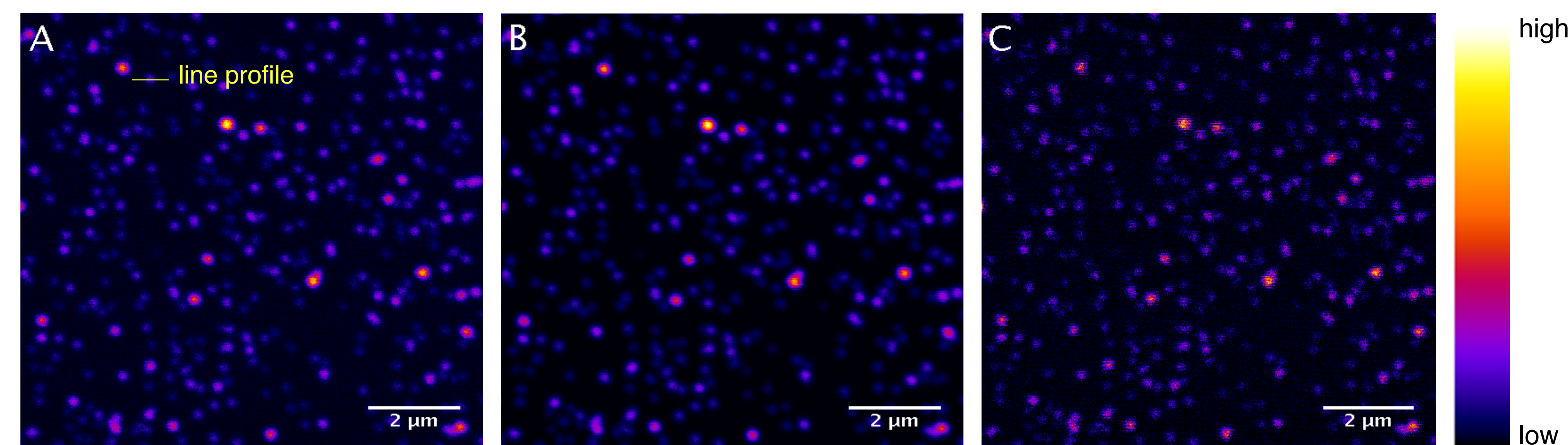


Fig. 4 Fixed HeLa cells; actin stained with ATTO Rho6G

- Excitation at 606 nm
- Resolution improvement visible from brightest parts of images
- Line profiles show that SSAX differentiates peaks better than the raw image

FLUORESCENT BEADS - REDUCING PEAK WIDTH



Unsaturated, 0.44 mW

Saturated, 1.01 mW

SSAX

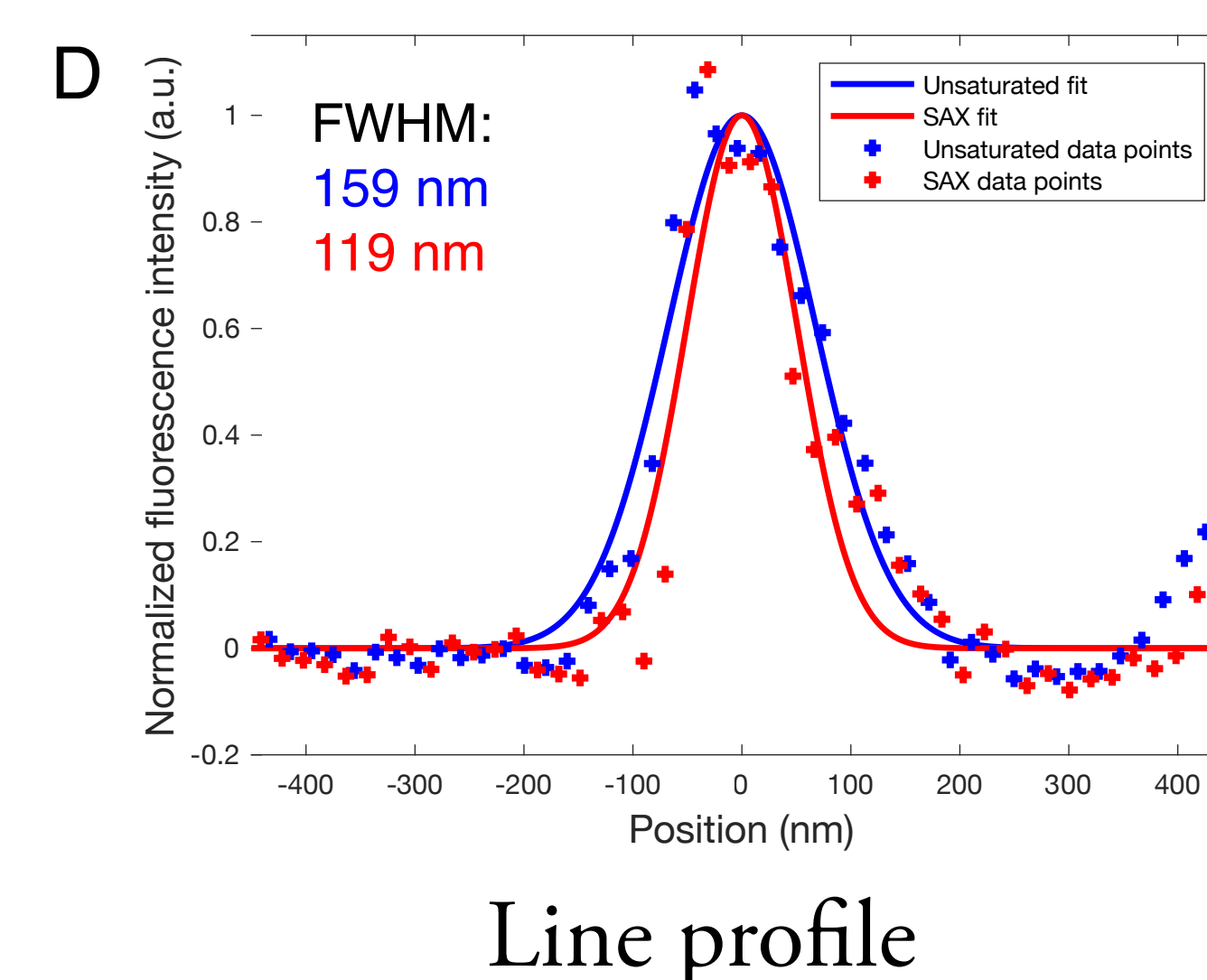


Fig. 5 100 nm fluorescent beads

- Line profiles allow quantification of resolution improvement
- Gaussian fit done on both line profiles
- Full width at half max (FWHM) calculated for both peaks
- FWHM is lower for the SSAX image, suggesting a resolution improvement

2ND ORDER FLUORESCENCE CURVE

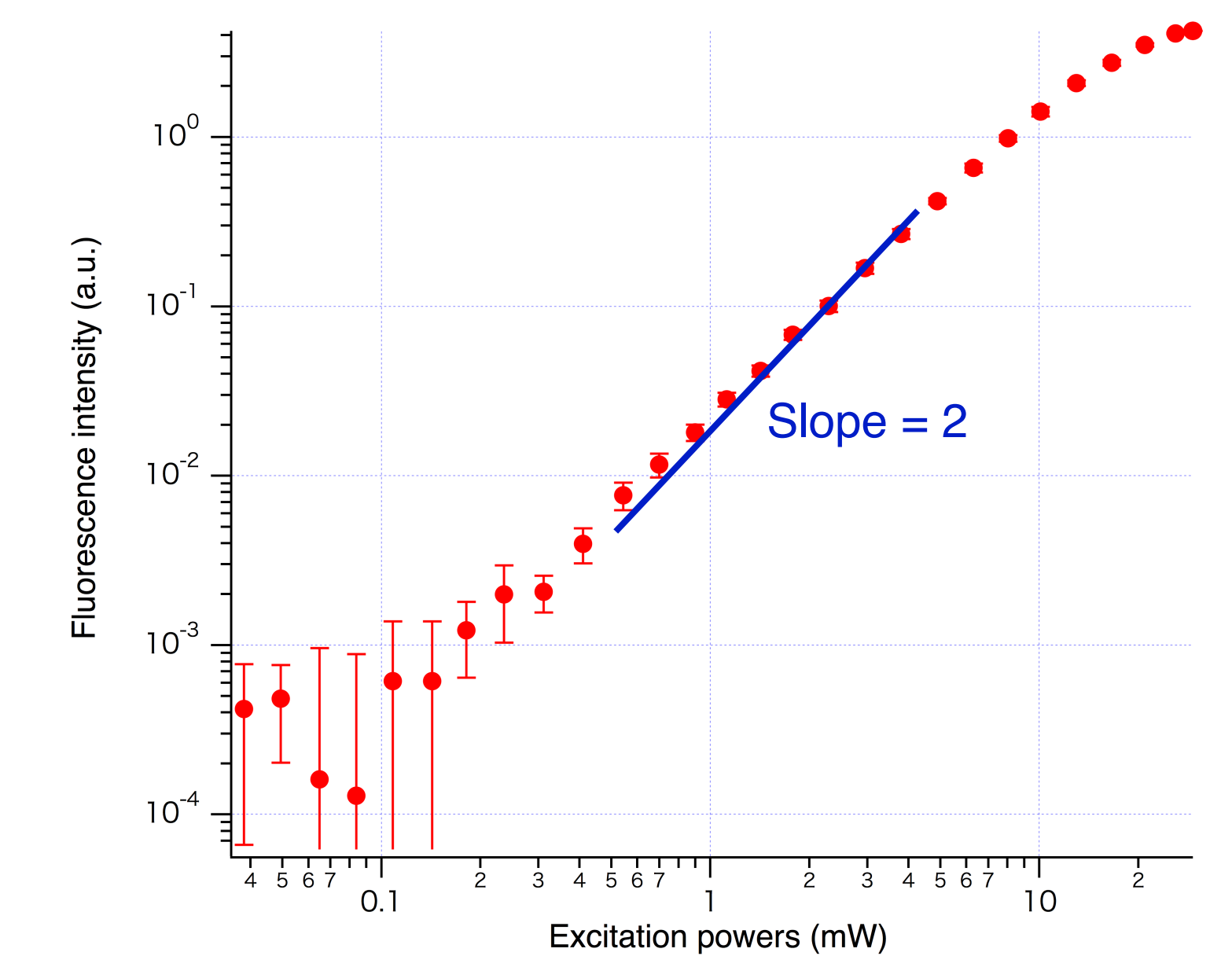


Fig. 6 Fluorescence curve for ATTO RhoG6 dye

- Slope of two implies second-order relationship
- Saturation evident
- Results support that cell images are visible 2PE

CONCLUSIONS & FUTURE WORK

- Combining visible-light 2PE and SSAX is possible and improves image resolution
- Future steps may include comparing theoretical and achieved resolutions, imaging fluorescent proteins, and imaging live cells

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