Temporal Focusing-Based Multiphoton Microscopy and Microprocessing

Shean-Jen Chen（陳顯禎）
Department of Engineering Science
Center for Micro/Nano Sceience and Technology (CMNST)
National Cheng Kung University (NCKU), Tainan 701, Taiwan

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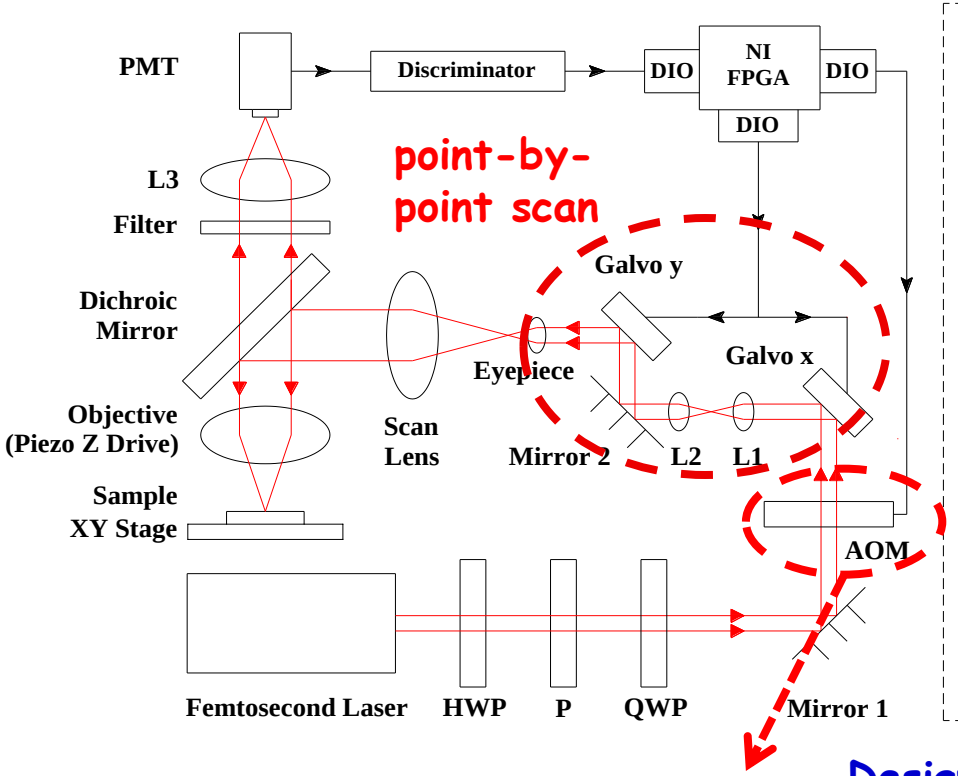
Adaptive Photonics Lab, NCKU

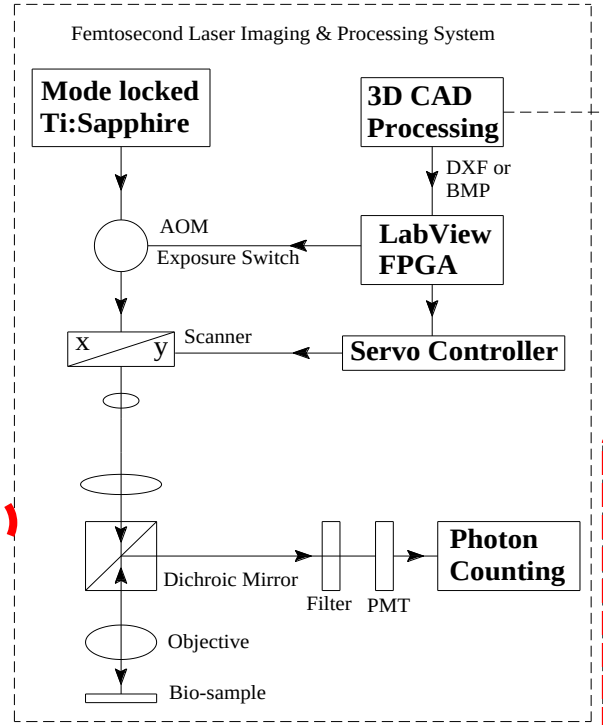
- Plasmonic Biosensing & Molecular Imaging

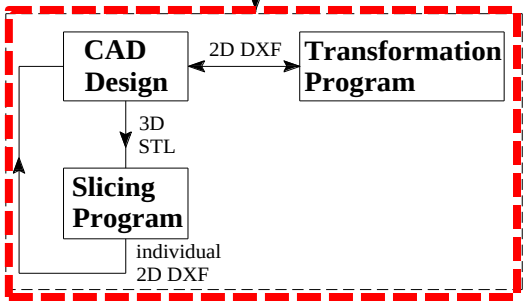
- Ultrafast Laser Microscopy & Microprocessing

Conventional Femtosecond Laser System

- **Molecular Imaging**: Two-photon excited fluorescence (TPEF) imaging, second harmonic generation (SHG) imaging, FLIM.
- **Microprocessing**: Microfabrication, nanosurgery, nanomachining.



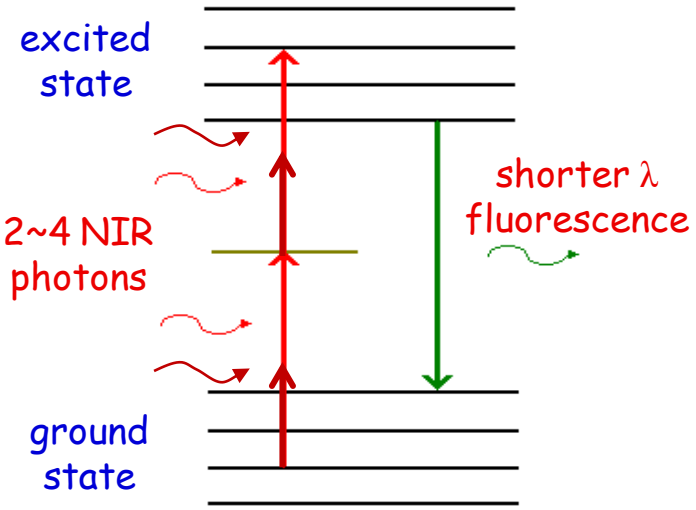




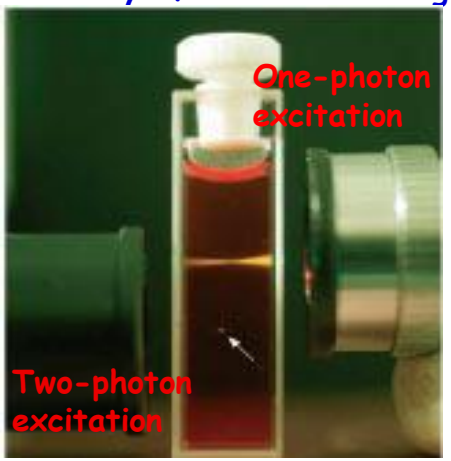
Rapid on/off switching of the laser and pulse selection

Designing 3D freeform structures: To transform 3D models into 2D processing patterns, and the program convert the 3D model into sequential 2D DXF files.

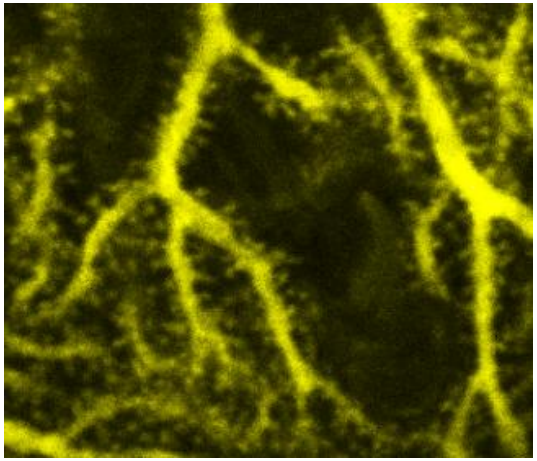
Biomedical Nonlinear Optical Microscopy



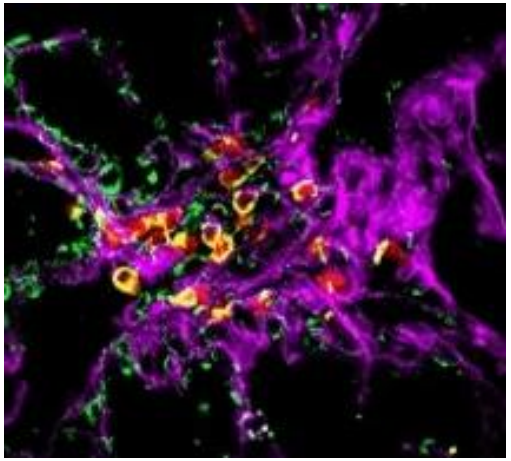
MRC Laboratory of Molecular Biology, UK



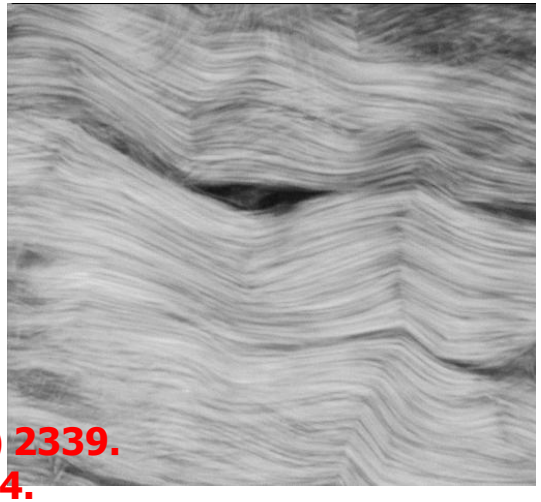
Dendritic Spine w 2PEF



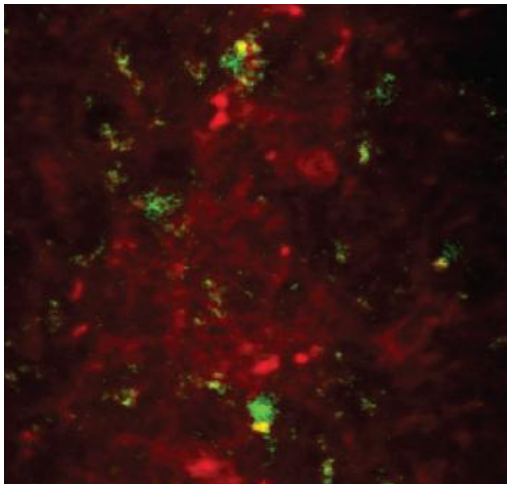
Mutli-color 2PEF



Tendon Collagens w SHG



Rat Brain w 4PEF + THG



Y.-C. Hsu *et al., J. Hypertension* 29 (2011) 2339.
K. Tilbury *et al.,* Biophys. J. 106 (2014) 354.
L.-C. Chung *et al., Biomed. Opt. Express* 5 (2014) 3427.

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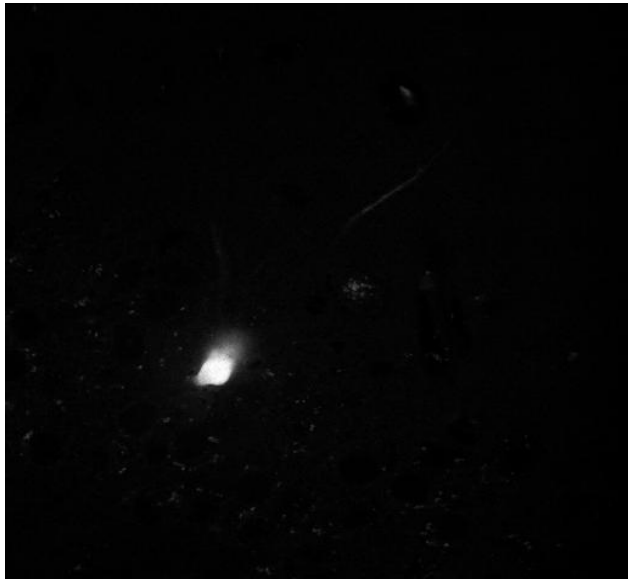
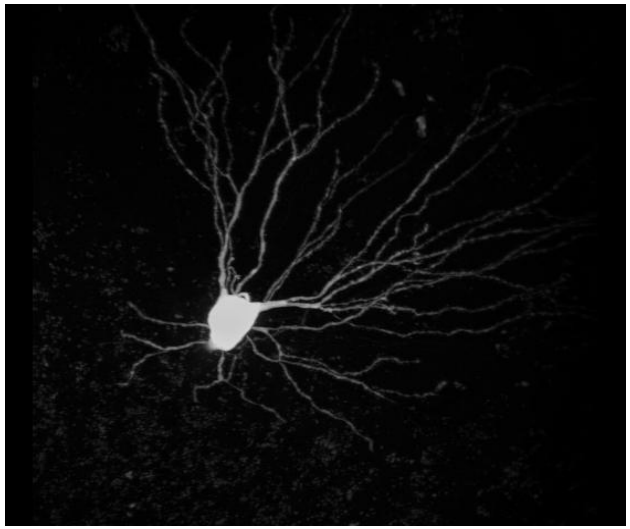
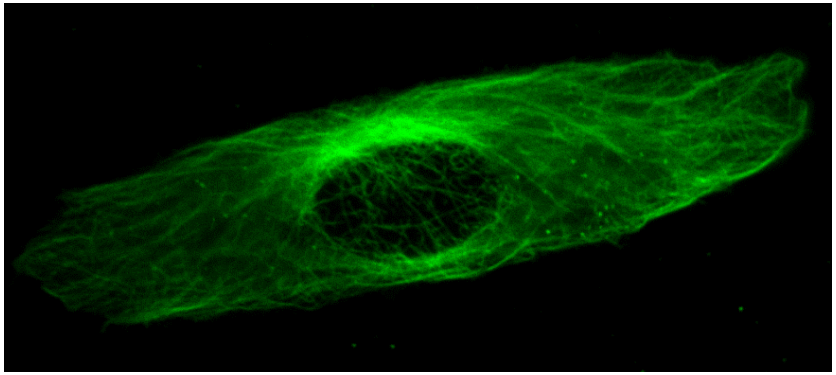
3D Reconstruction Imaging

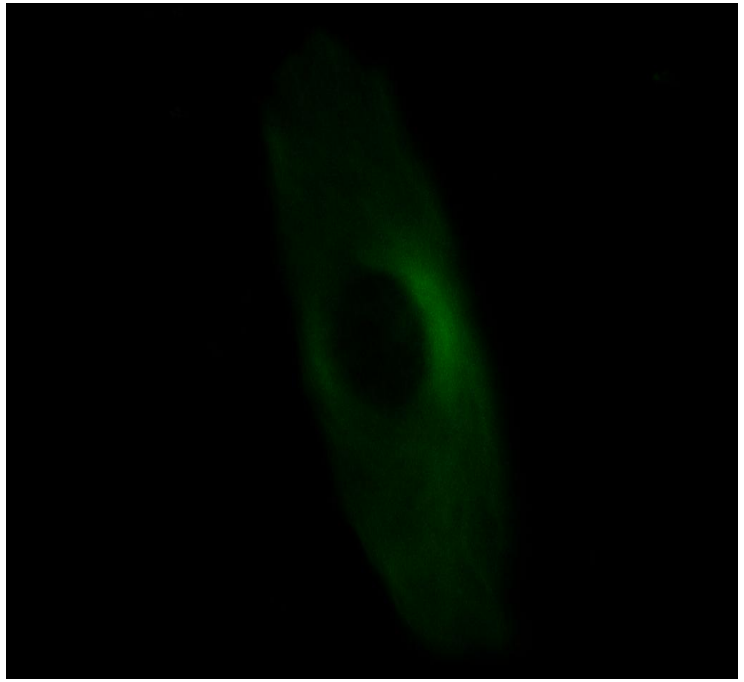
Neuron w Lucifer Yellow





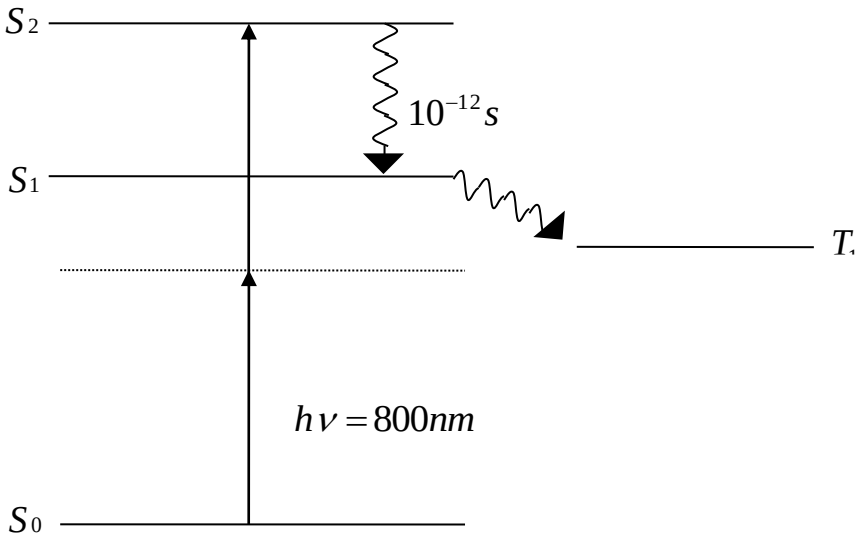
Cytoskeleton Tubulin w Alex 488



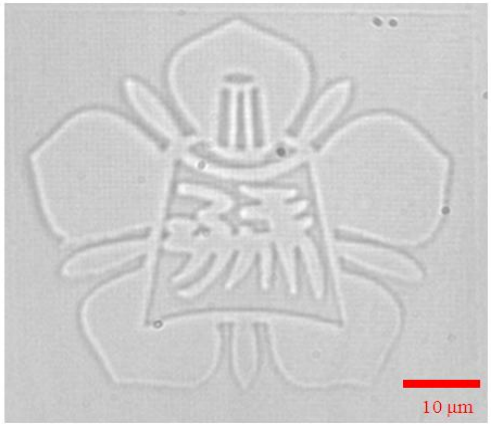


Multiphoton Fabrication of 3D Microstructures

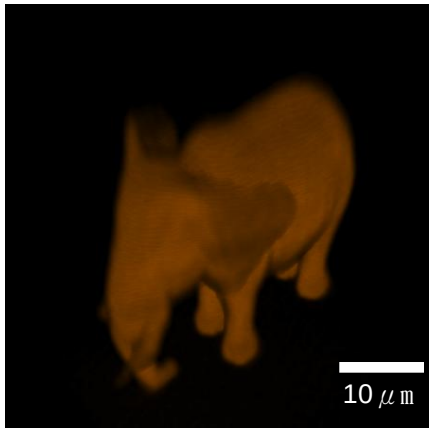
- Rose Bengal as Photoinitiator for Two-photon Polymerization & Crosslinking



NCKU Emblem



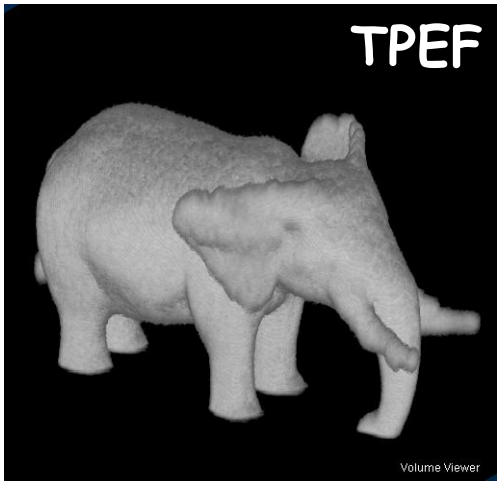
Micro-elephant



- Two-photon absorption cross section

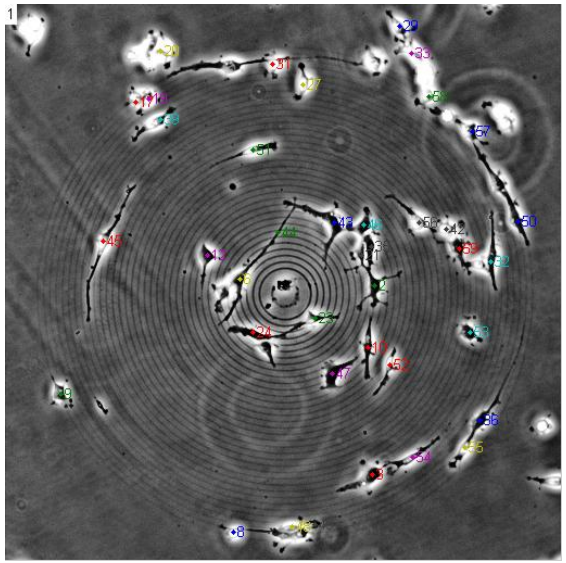
$$\delta\eta_2 \propto \lambda\tau\langle F(t)\rangle$$

Excitation wavelength: to the maximum value of relative two-photon absorption (TPA) of RB at 715 nm.

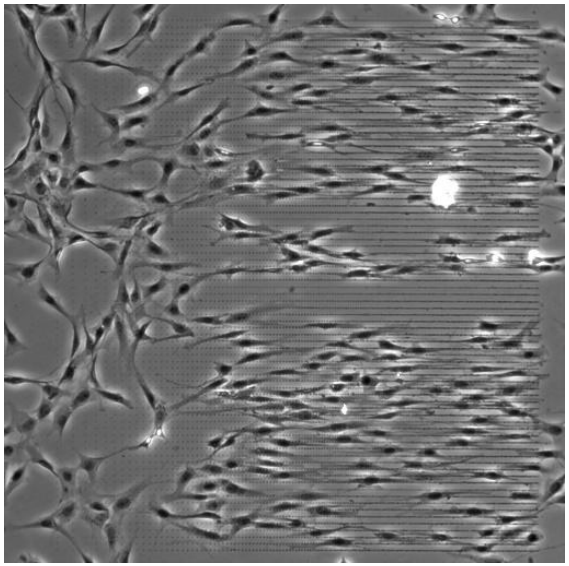


Extracellular Matrix (ECM) Biopolymer

- Living Cells on Concentric Laminin Gradient & Fibronectin Gradient via Two-photon Crosslinking



Concentric laminin gradient with 800 x 800 microns



Fibronectin gradient with a dynamic range of nearly 40 fold in concentration

The morphological and cytoskeletal responses of 3T3 fibroblasts were investigated, where the cell morphology and actin cytoskeleton became increasingly elongated and aligned with the direction of the gradient at increasing concentration.

X. Chen, *et al.*, *Cell. Mol. Bioengn.* 5 (2012) 307.
V. Ajeti, *et al.*, *Opt. Express* 21 (2013) 25346.

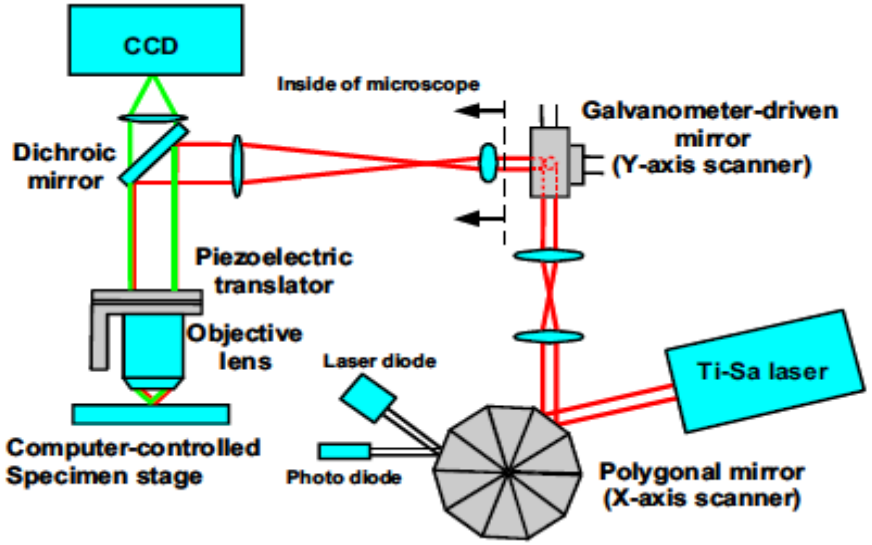
Current Challenges in Biomedical Nonlinear Optical Microscopy

- ✓ Providing Fast Sectioning Images for Real-time Applications
  - -> Temporal Focusing-Based Widefield Microscopy
- ✓ Breaking Diffraction Limit for Super-resolution Imaging
  - -> NSIM (Nonlinear Structured Illumination Microscopy), PALM (Photoactivated Localization Microscopy), STORM (Stochastic Optical Reconstruction Microscopy), STED (Stimulated Emission Depletion), …
- ✓ Imaging Thick Tissues for Deeper Information
  - -> Adaptive Optics System
- ✓ Multifunctional (or Selecting) Capability (ex. All Optical Histology)
  - -> Multiphoton-induced Laser Ablation

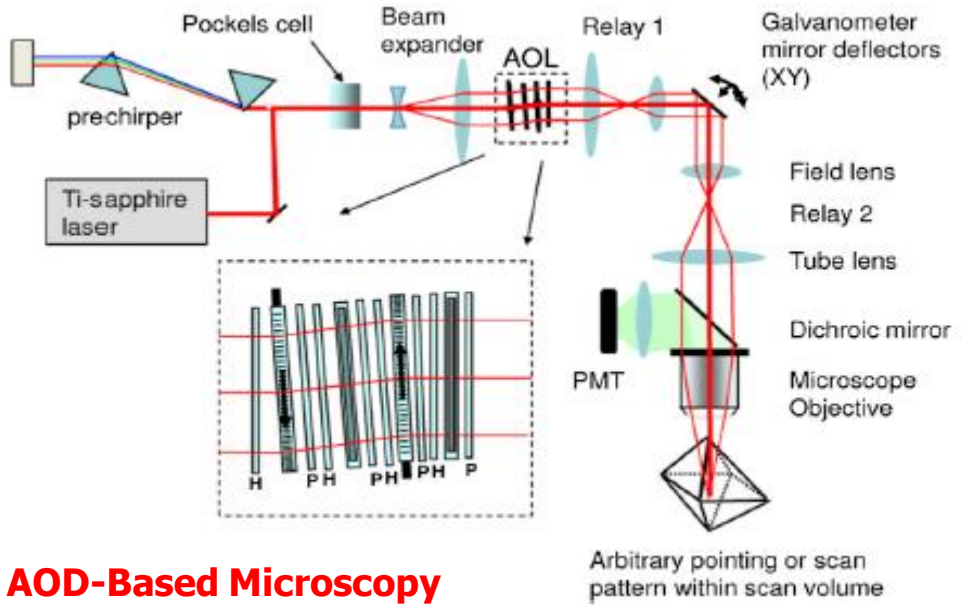
Outlines

- Motivation & Principle: Temporal Focusing-Based Multiphoton Microscopy and Microprocessing
- High-speed 3D Sectioning Images (over 100 Hz)
- To Head Super-resolution Microscopy
- To Improve Deep Imaging with Adaptive Optics
- Fast Multiphoton Microfabrication (3D Lithography)
- High-throughput Multiphoton-induced Laser Ablation
- Summary

High-Throughput Nonlinear Optical Microscopy

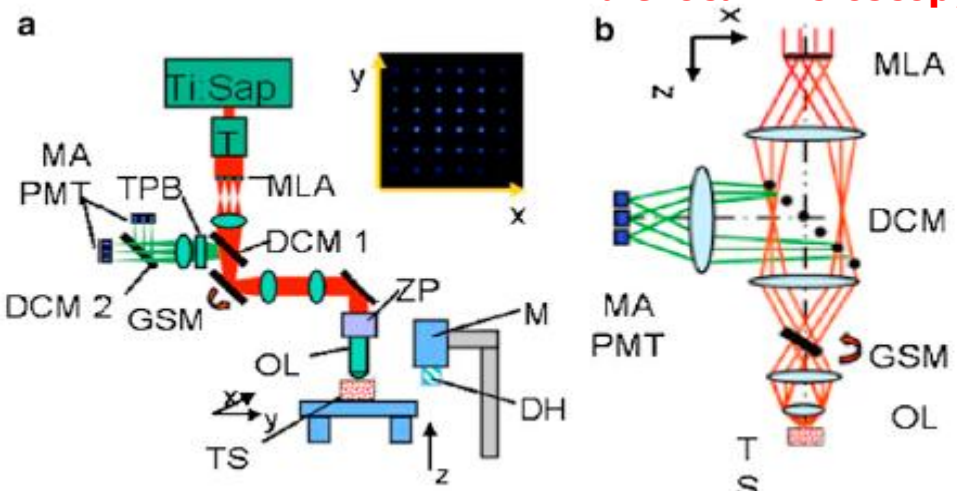


Polygonal Scanner-Based Microscopy

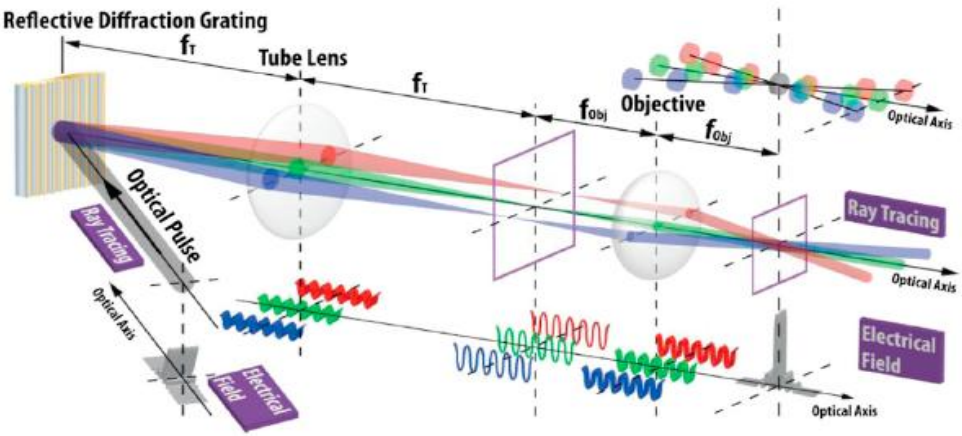


AOD-Based Microscopy

Multifocal Microscopy



Temporal Focusing-Based Microscopy



P. T. C. So *et al., Biophys. J.* 105 (2013) 2641.

Adaptive Photonics Lab, NCKU

High-Throughput Nonlinear Optical Microscopy

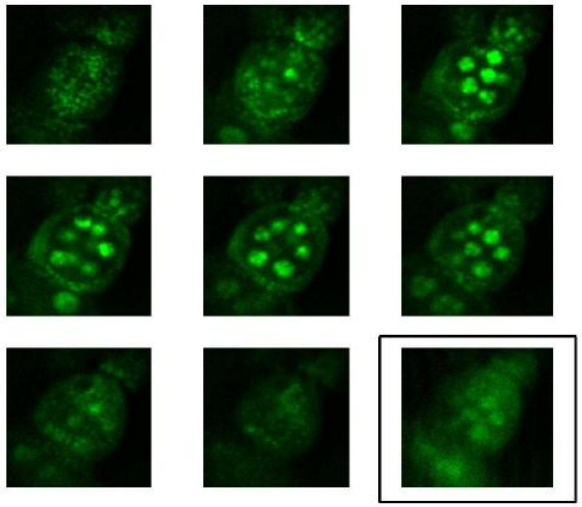
Function	Pros	Cons
Polygonal mirrors	>10,000 scan lines/s. Progression of scan is necessarily sequential.	User unable to determine where to point the scanner. Requires separate sensing device. Microscope must be synchronized to the scanner.
Resonance scanners	Faster than galvanometric scanners. Essentially galvanometric scanner without feedback. Operates at a constant frequency after settling.	User unable to determine where to point the scanner. Requires separate sensing device. Microscope must be synchronized to the scanner.
Acousto-optic deflector	Fast response (tens of microseconds). Arbitrary/random access of sample. Speed comparable to resonance or polygonal scanners in some instances. Possible to also do axial scanning to a slight degree.	Dispersion leading to broader pulse. Requires precompensation device. Chromatic aberrations, especially for short pulses or large diffraction angles.
Multiple foci	Data extracted from multiple regions simultaneously. Dwell time and signal/noise preserved. Possible for configuring beamlets on-the-fly when using acousto-optical deflectors. Spatial or temporal separation and use of multianode photomultiplier tubes can help reduce scattering and increase imaging depth.	Critical to maintain evenness of illumination over all beamlets. May suffer from inefficient use of excitation beam. May require careful alignment. Poor imaging depth achieved due to scattering (but addressable).
Temporal focusing	Wide-field excitation. Optical sectioning due to the two-photon absorption. Possible to achieve submicron axial resolution. Very high frame rates. Excitation is less sensitive to tissue scattering. Axial scanning through optical means.	Imaging depth still limited by scattering of signal. Relatively poor axial resolution, unless a more complex setup is used. Low excitation efficiency.

P. T. C. So *et al., Biophys. J.* 105 (2013) 2641.

Motivation

- Serial scanning microscopy → Not fast enough
- Widefield microscopy → No depth-resolved ability
- Widefield multiphoton microscopy base on spatiotemporal focusing

  → Less laser power, lower frame rate

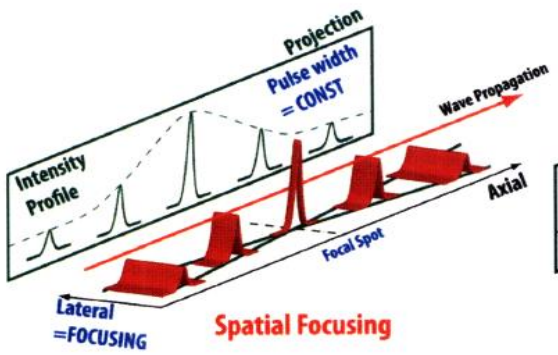


D. Oron *et al.*, *Opt. Express* 13 (2005) 1468.

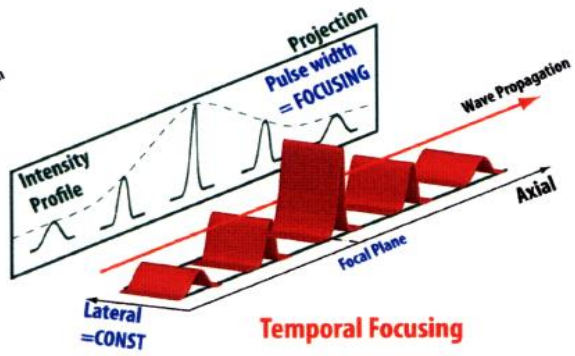
Goal: To develop a state-of-the-art femtosecond laser system with fast 3D molecular imaging and microprocessing for bio-research.

Principle: Spatial and Temporal Focusing

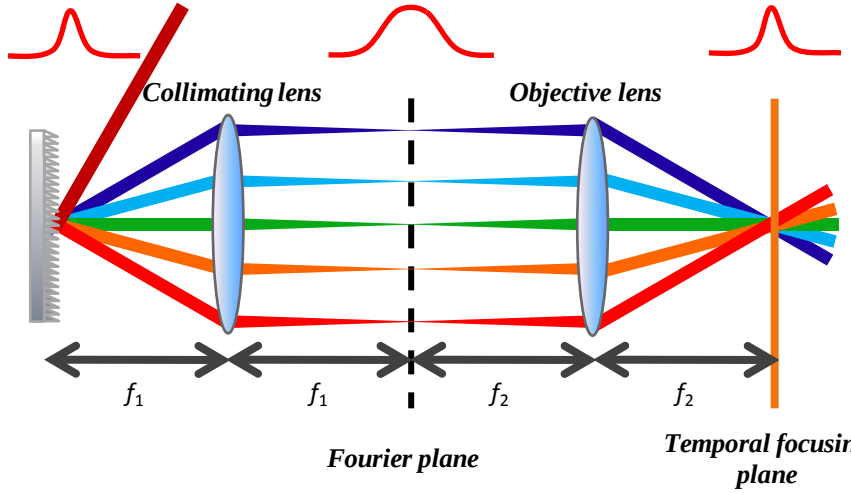
- Spatial focusing: The pulse width remains unchanged, and the lateral beam size is focused.
- Temporal focusing: The pulse width is focused, and the lateral beam size remains unchanged.



Spatial Focusing



Temporal Focusing



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- **High-speed 3D Sectioning Images (over 100 Hz)**
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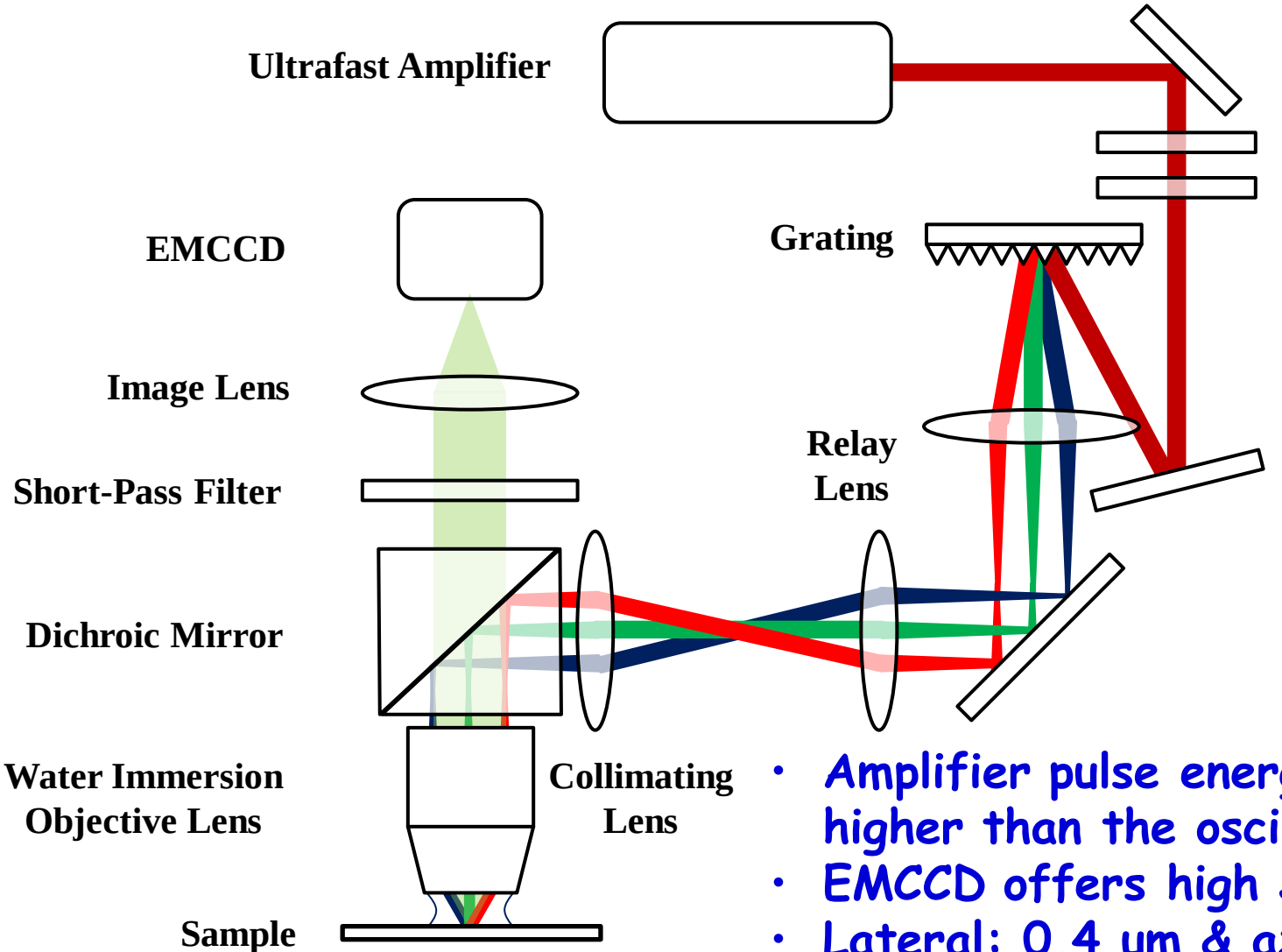
- Fast Multiphoton Microfabrication (3D Lithography)
- High-throughput Multiphoton-induced Laser Ablation
- Summary

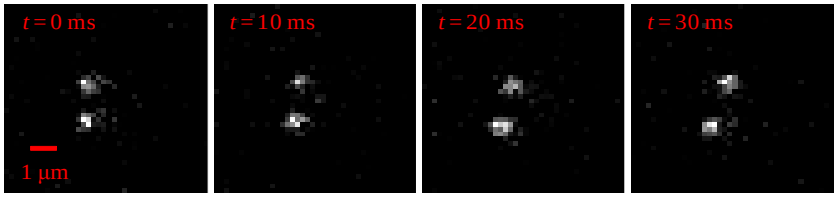
System Setup



- Amplifier pulse energy is 8000 times higher than the oscillator
- EMCCD offers high SNR and frame rate
- Lateral: 0.4 μm & axial: 3 μm

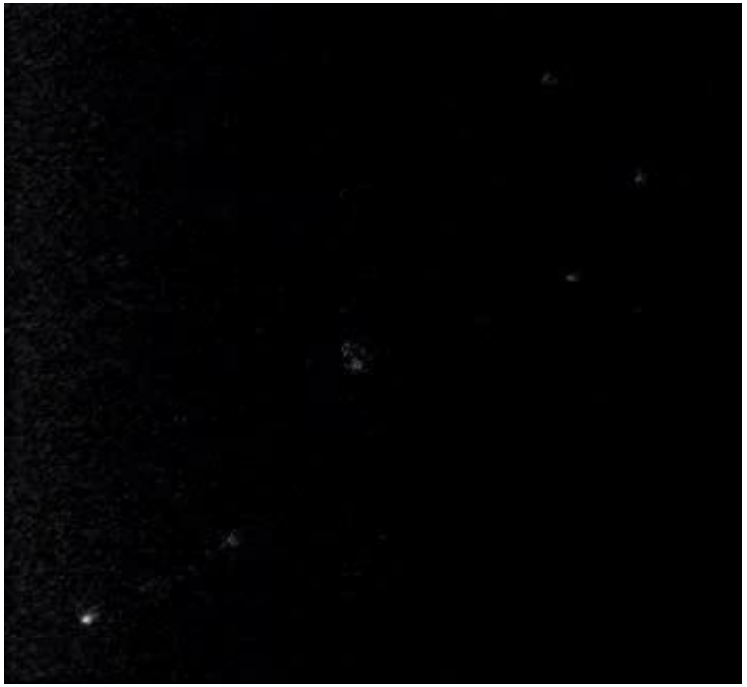
Brownian Motion of Micro-Beads

- Frame rate: 100 Hz
- Field of veiw: 50 x 50 μm^2



1 μm beads

0.5 μm beads



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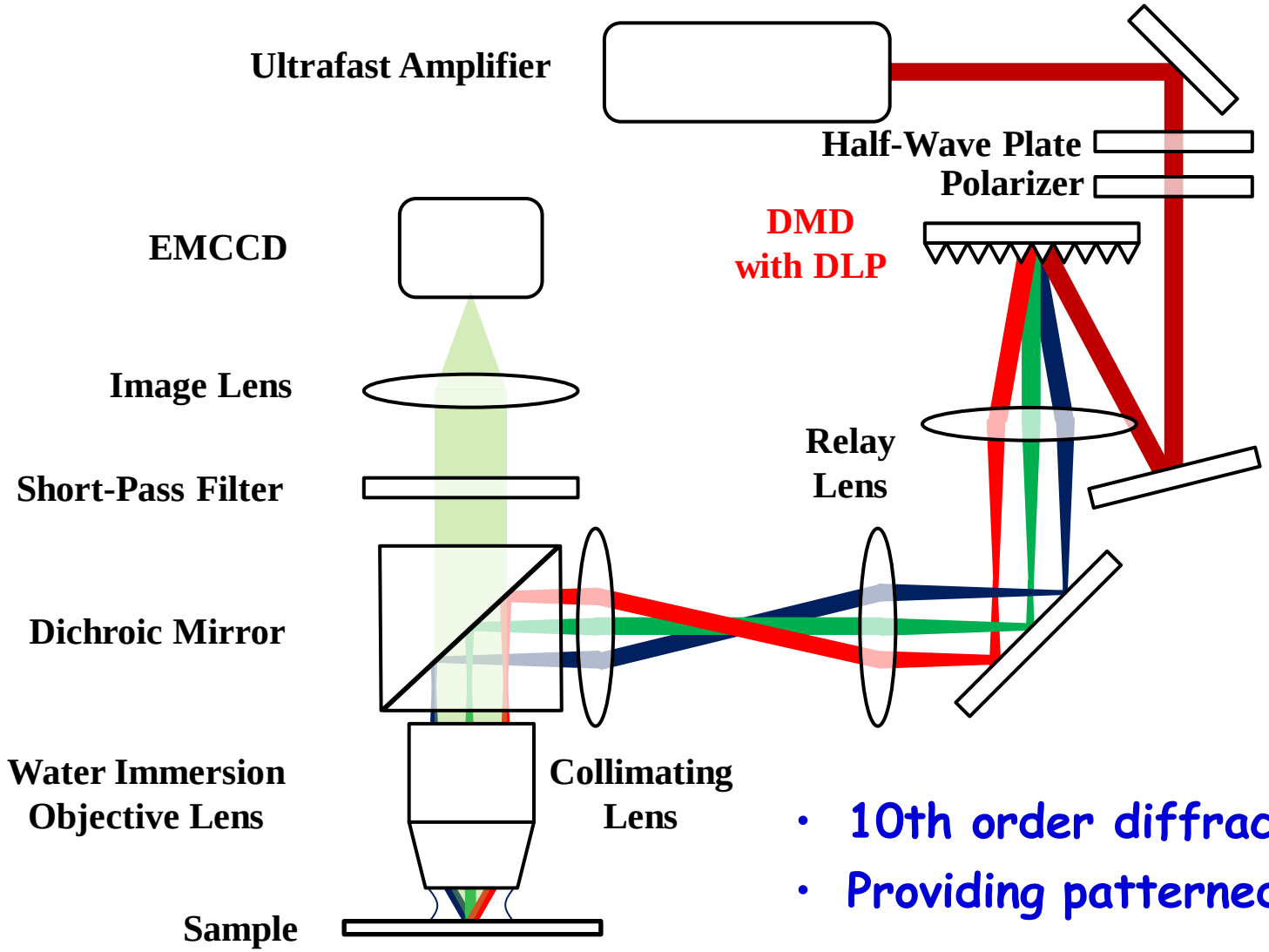
Super-resolution Microscopy

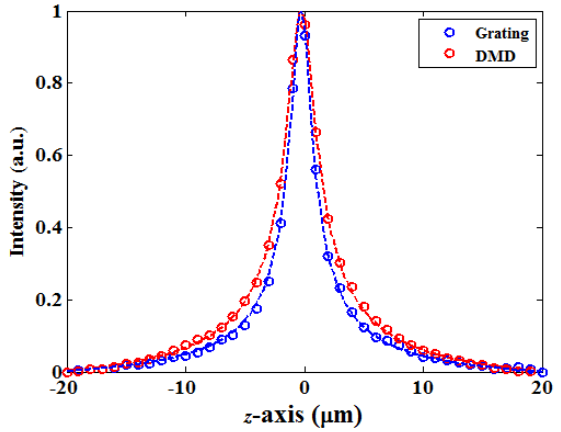
Table 1 Fluorescence-based imaging methods with their theoretical and practical resolutions.

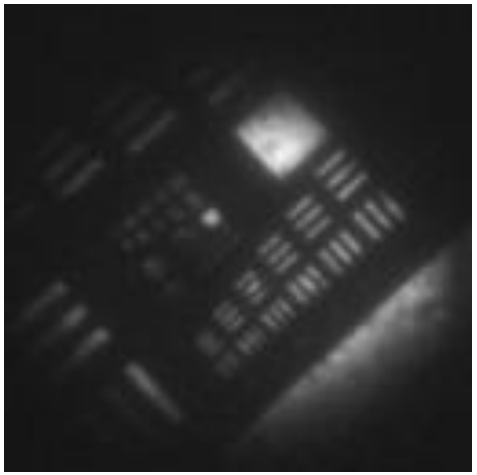
Imaging method[a]	Resolution equation[b]	Parameters[c]	Laser intensities[d]	Practical resolutions[e]
Conventional far-field microscopy (Abbé's diffraction law) (wide-field and scanning)	$d=\frac{\lambda}{2n\ \sin\alpha}$ (1a) $d=\frac{2\lambda}{(n\ \sin\alpha)^2}$ (1b)	d=conventional lateral (a) and axial (b) resolution limit λ=wavelength of light n=refractive index of imaging medium α=half of angular aperture of objective $n\ sin\alpha$=numerical aperture (NA) of objective	1–100 mW/cm² (wide-field) 1–100 kW/cm² (scanning)	Lateral: ~200–300 nm Axial: ~500–700 nm
Photo-activated localization microscopy (PALM) (wide-field)	$x=\frac{d}{\sqrt{N}}$ (2)	x=resolution limit d=conventional resolution limit N=number of detected photons	1–10 kW/cm²	Lateral: ~20–50 nm Axial: ~10–70 nm
Stimulated emission depletion (STED) (scanning)	$x=\frac{d}{\sqrt{1+\frac{I}{I_s}}}$ (3)	x=resolution limit d=conventional resolution limit I=light intensity of depletion laser I_{sat}=saturation intensity of depletion laser	0.1–1 GW/cm²	Lateral: ~70–90 nm Axial: ~100–200 nm
Stochastic Optical Reconstruction Microscopy (STORM)				
Super-resolution optical fluctuation imaging (SOFI) (wide-field and scanning)	$x=\frac{d}{\sqrt{k}}$ (4)	x=resolution limit d=conventional resolution limit k=order of temporal cumulant calculated from fluctuations	0.1–1 kW/cm²	Lateral: ~100–120 nm Axial: ~350–400 nm
Saturated structured illumination Microscopy (SSIM) (wide-field and scanning)	$x=\frac{d}{1+(1+m)}$ (5)	x=resolution limit d=conventional resolution limit m=number of higher harmonics	1–10 kW/cm²	Lateral: ~50–100 nm Axial: ~125–250 nm

K. Weisshart *et al., Adv. Opt. Technol.* 2 (2013) 211.

Temporal Focusing via Digital Micromirror Device







- 10th order diffraction i.e. 520 lines/μm
- Providing patterned illumination (NSIM)

J.-N. Yih *et al., Opt. Lett.* 39 (2014) 3134.

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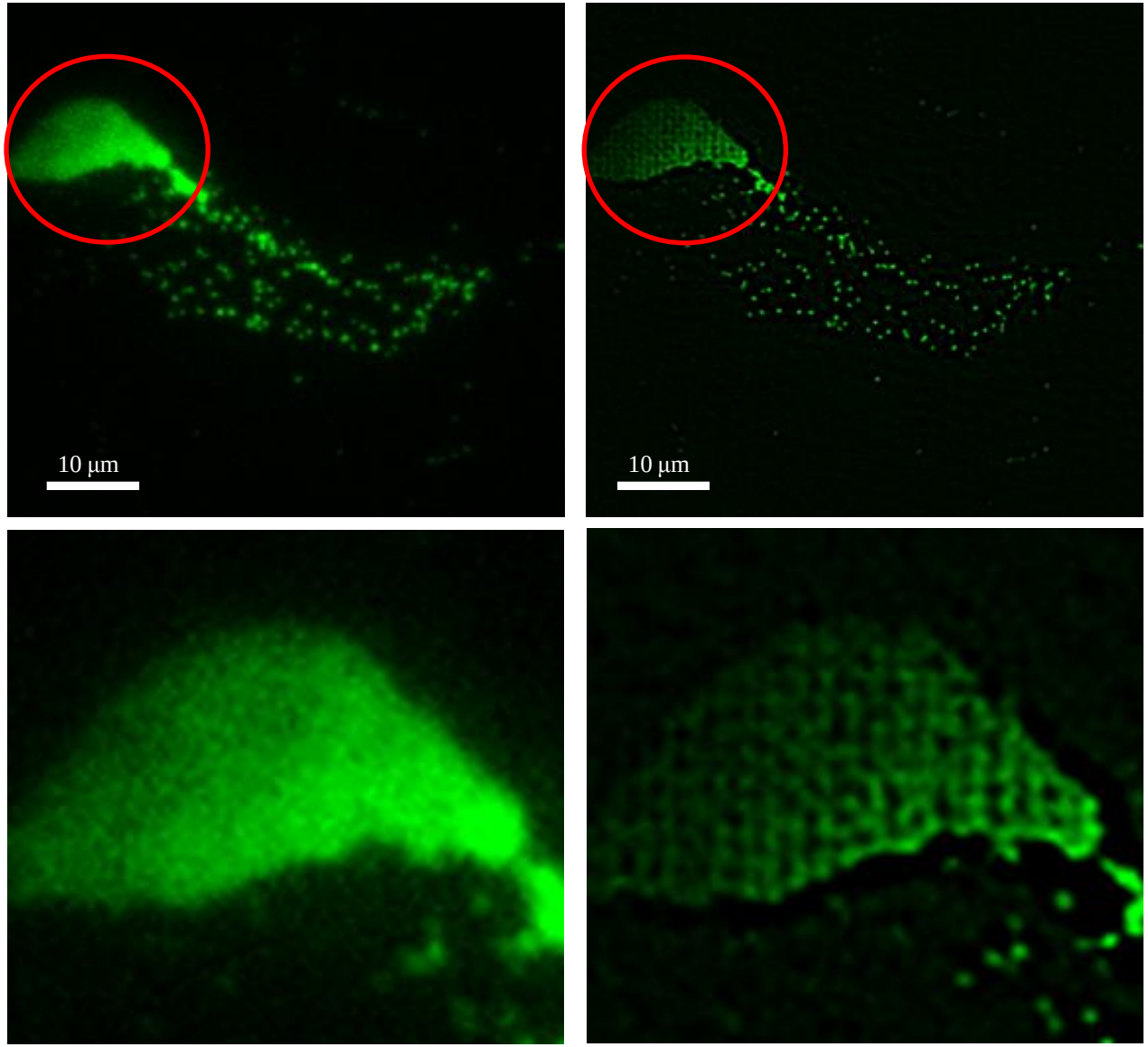
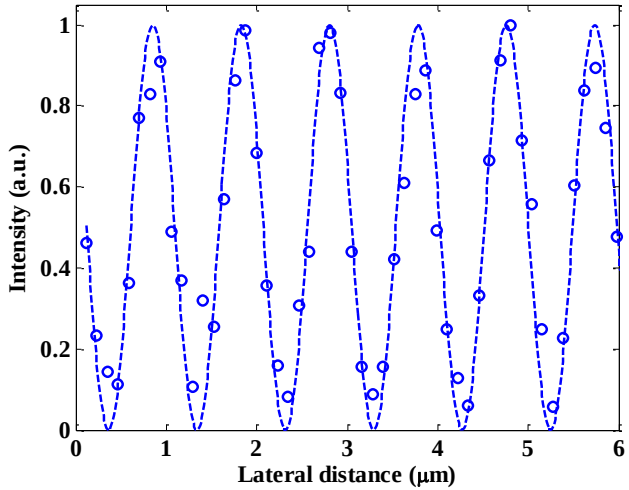
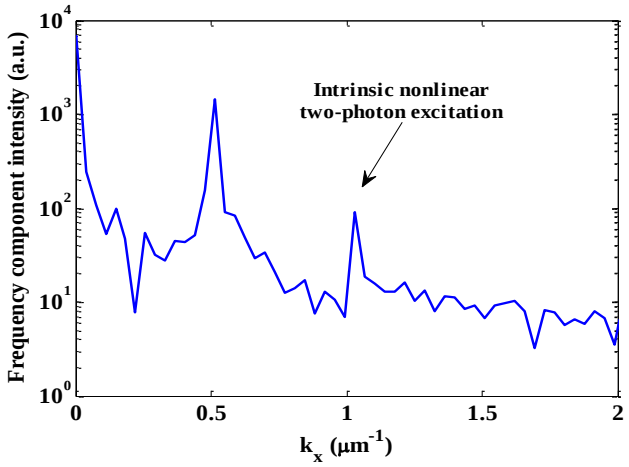
Nonlinear Structured Illumination Microscopy (NSIM)





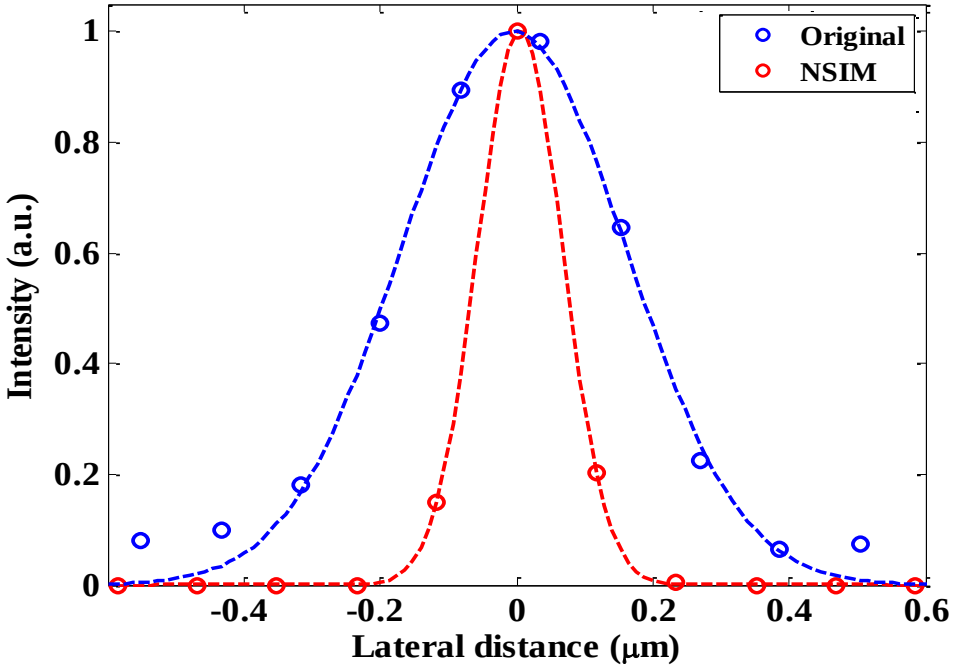


L.-C. Chung *et al., Biomed. Opt. Express* 5 (2014) 2526.

Adaptive Photonics Lab, NCKU

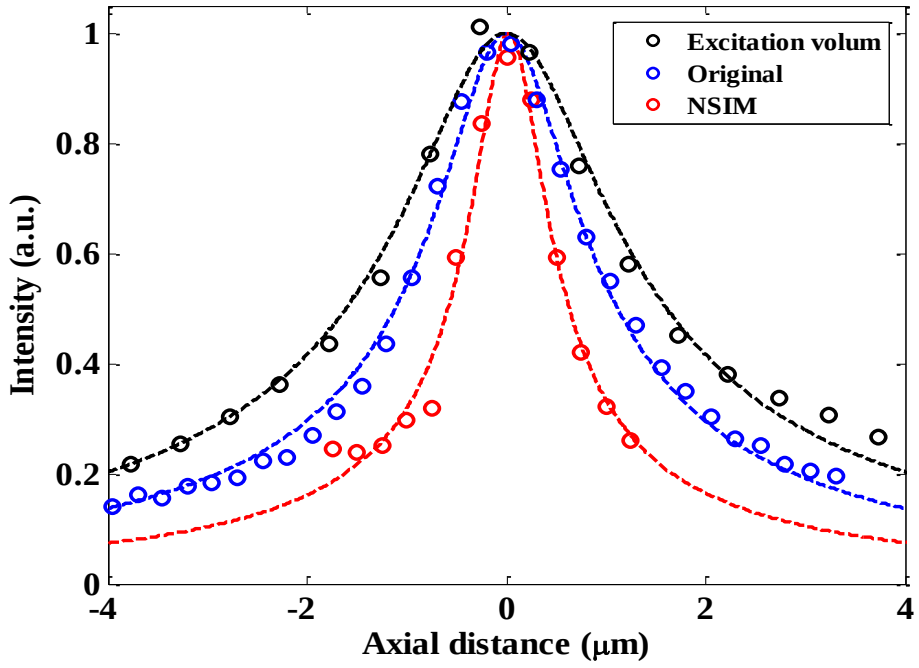
Lateral & Axial Spatial Resolutions

Lateral resolution



$FWHM_{Original}$ = 397 nm
$FWHM_{NSIM}$ = 168 nm

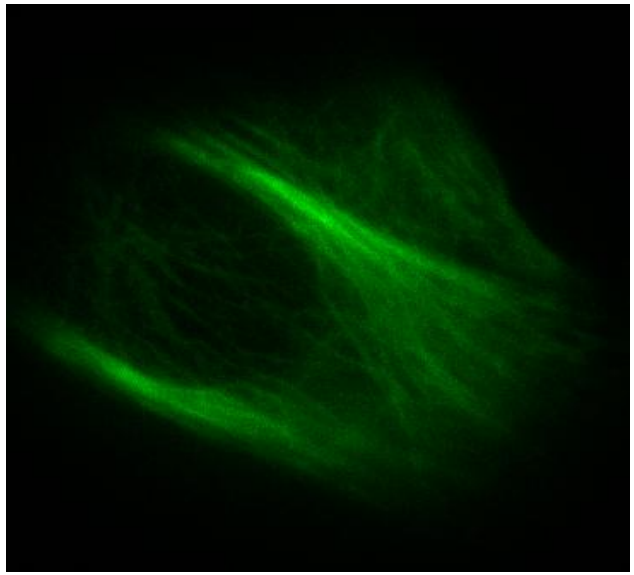
Axial resolution

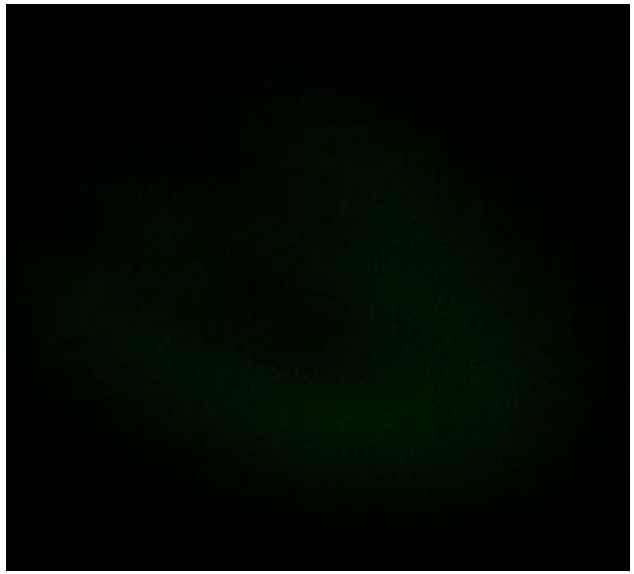


$FWHM_{Excitation}$ = 3.1 μm
$FWHM_{Original}$ = 2.3 μm
$FWHM_{NSIM}$ = 1.2 μm

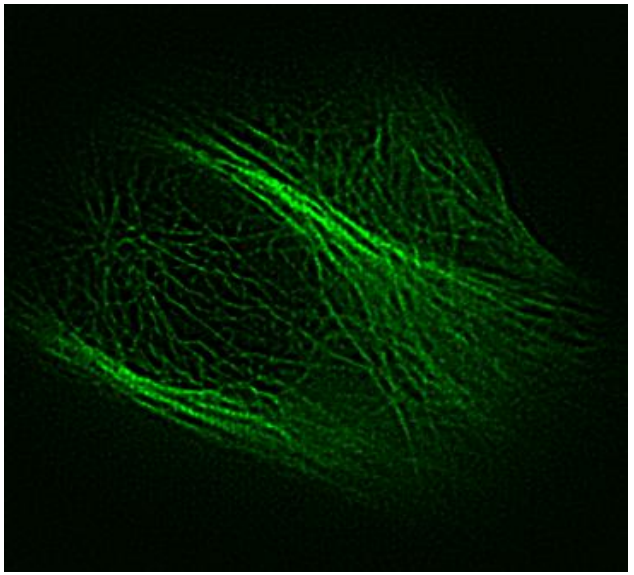
Cytoskeleton Image with NSIM

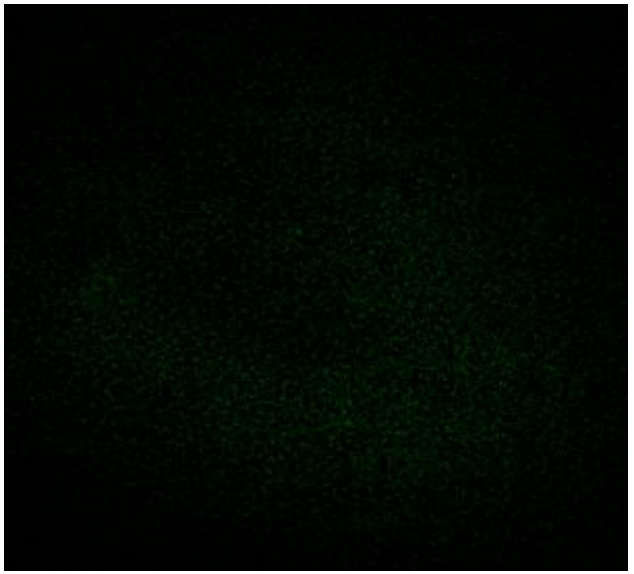
w/o NSIM



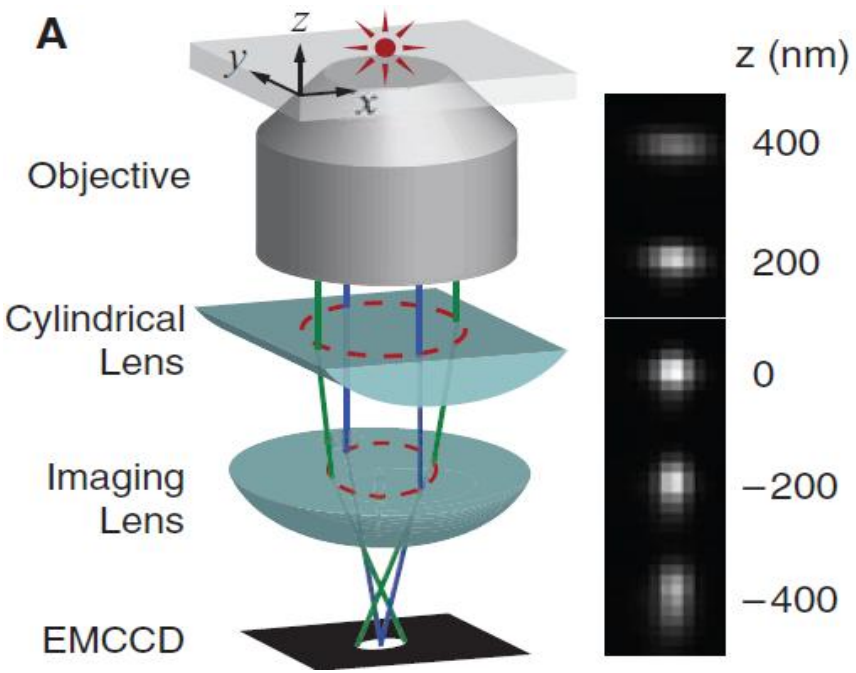


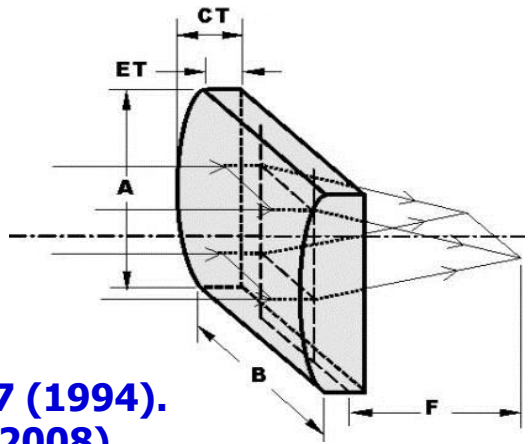
with NSIM

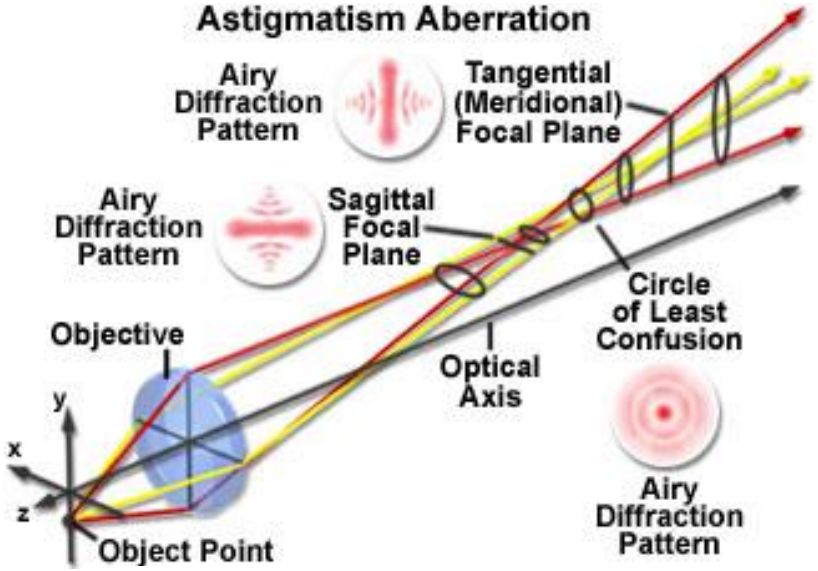


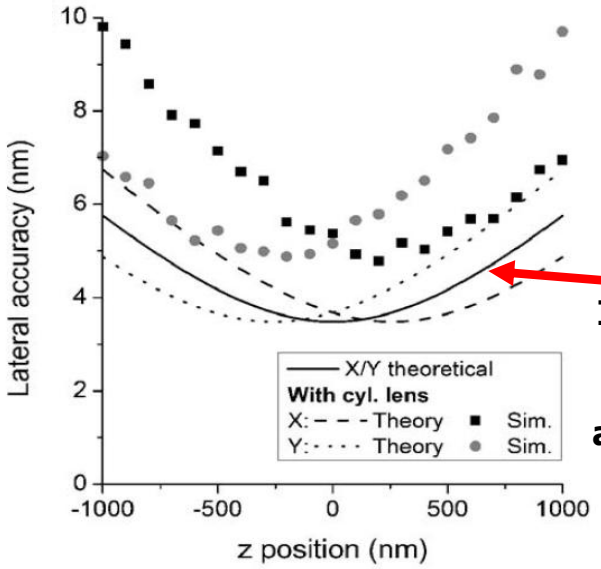


z-Axis Super Resolution via Astigmatism Configuration





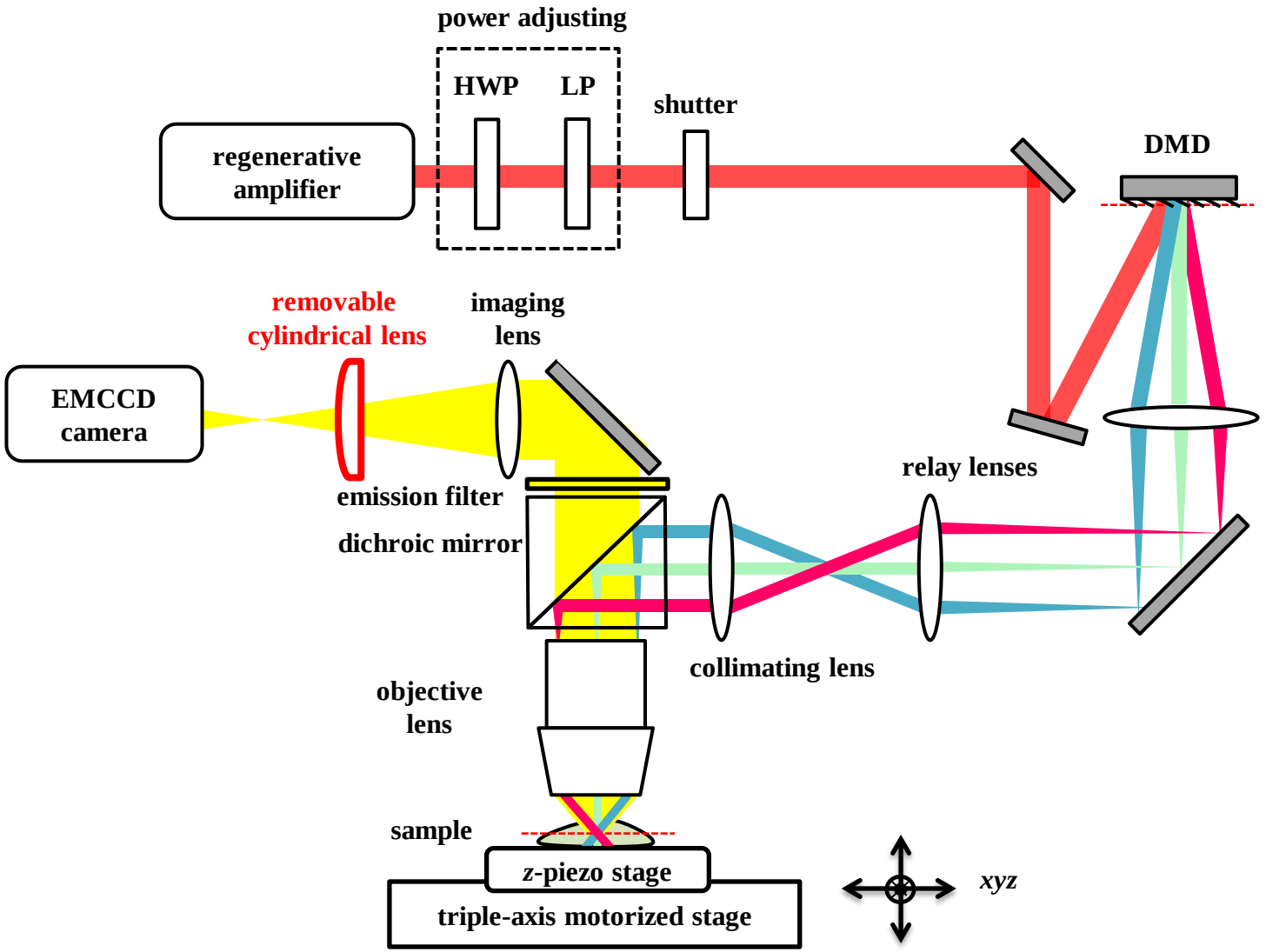




Identify the everything without astigmatism

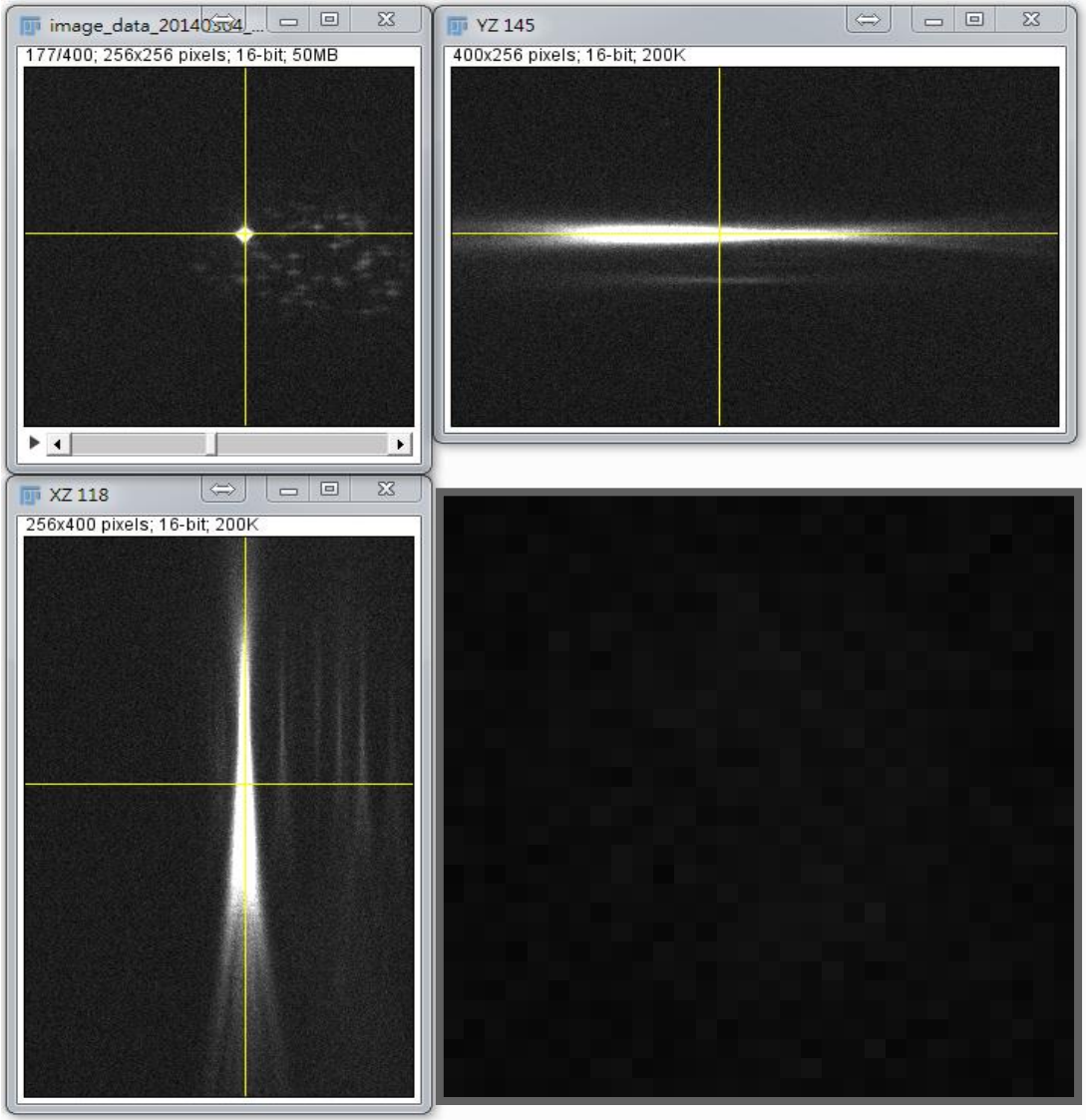
Biophys. J. 67 (1994).
Science 319 (2008).
Appl. Phys. Lett. 90 (2007).

TFMPEM with Astigmatism Imaging

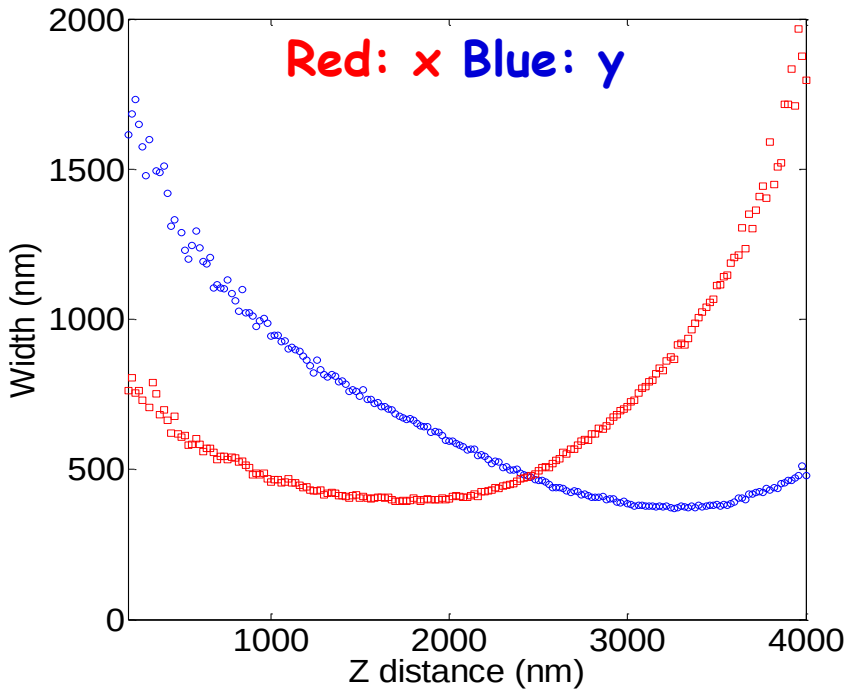


C.-H. Lien *et al.*, *Opt. Express* 22 (2014) 27290.

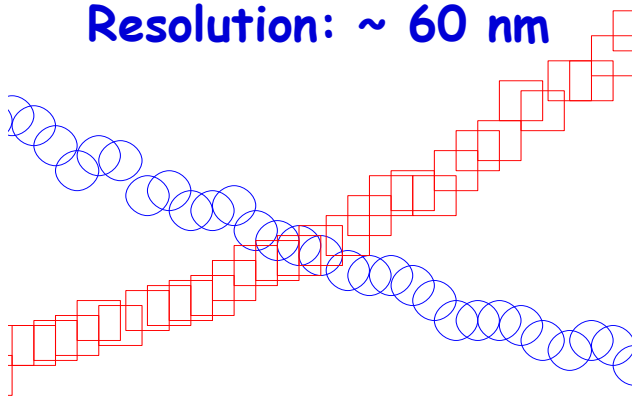
Inducing Astigmatism to z-axis Localized Optical Section



z step: 20 nm



Resolution: ~ 60 nm

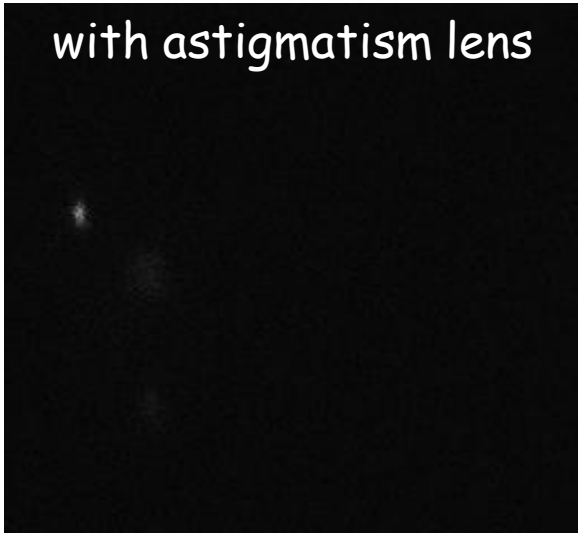


Adaptive Photonics Lab, NCKU

Brownian Motion of Fluorospheres

$$D = \frac{\kappa_B T}{3\pi\eta d} \qquad \Delta d_{rms} = \sqrt{6D(\Delta t)}$$

100 frames/sec at focal plane



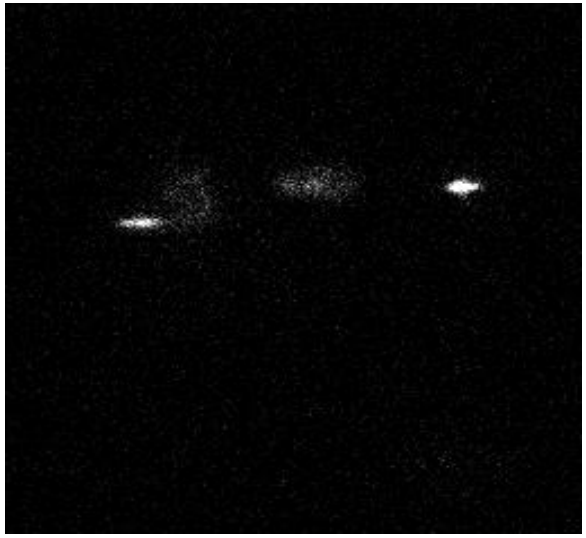
- 500 nm beads in water

Δd_{rms} ≈ 227 nm

- Beads in 55wt% glycerol with astigmatism lens

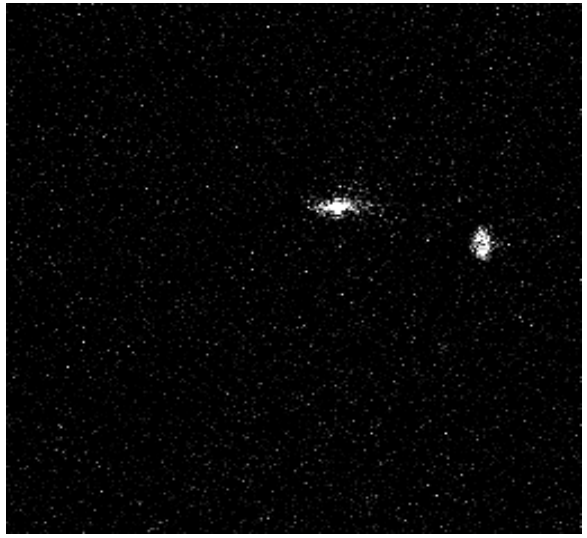
500 nm

Δd_{rms} ≈ 78 nm



200 nm

Δd_{rms} ≈ 123 nm

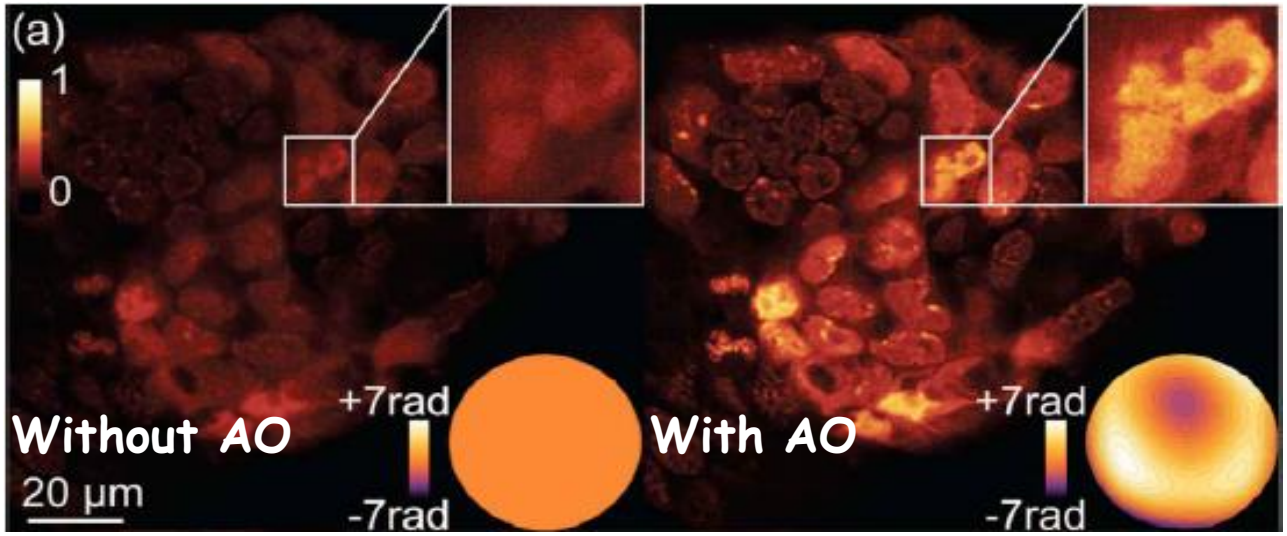


Outlines

- Motivation & Principle: Temporal Focusing-Based Multiphoton Microscopy and Microprocessing
- High-speed 3D Sectioning Images (over 100 Hz)
- To Head Super-resolution Microscopy
- **To Improve Deep Imaging with Adaptive Optics**
- Fast Multiphoton Microfabrication (3D Lithography)
- High-throughput Multiphoton-induced Laser Ablation
- Summary

Why Adaptive Optics?

- ✓ Image quality is seriously affected by external disturbances such as optical aberrations and environmental turbulence.
- ✓ Applications in astronomy, laser weapon, industry machining, microscopy, and free space optical communication.



Binary star image taken by Hale Telescope in Palomar Observatory located in San Diego County, California

Mouse embryo taken by TIFFM

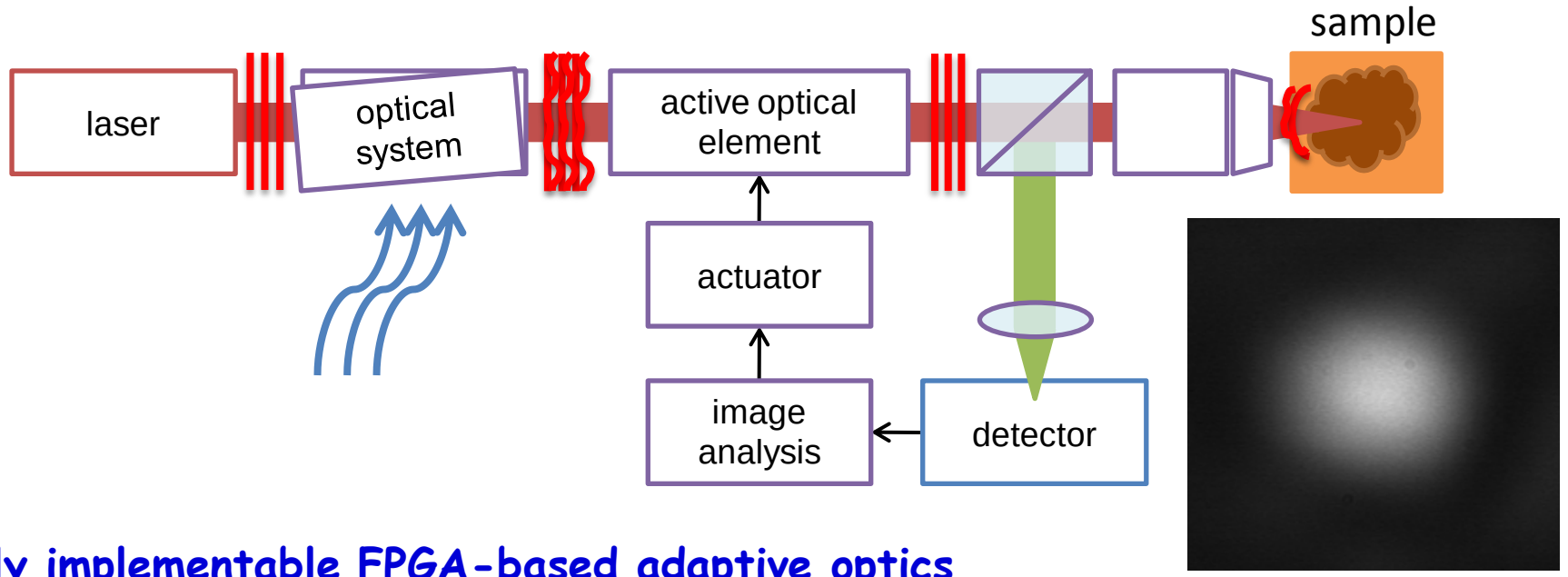
D. Débarre *et al.*, *Opt. Lett.* 34 (2009) 2495.

http://www.astro.caltech.edu/

CALTECH ASTRONOMY

What Adaptive Optics System (AOS)?

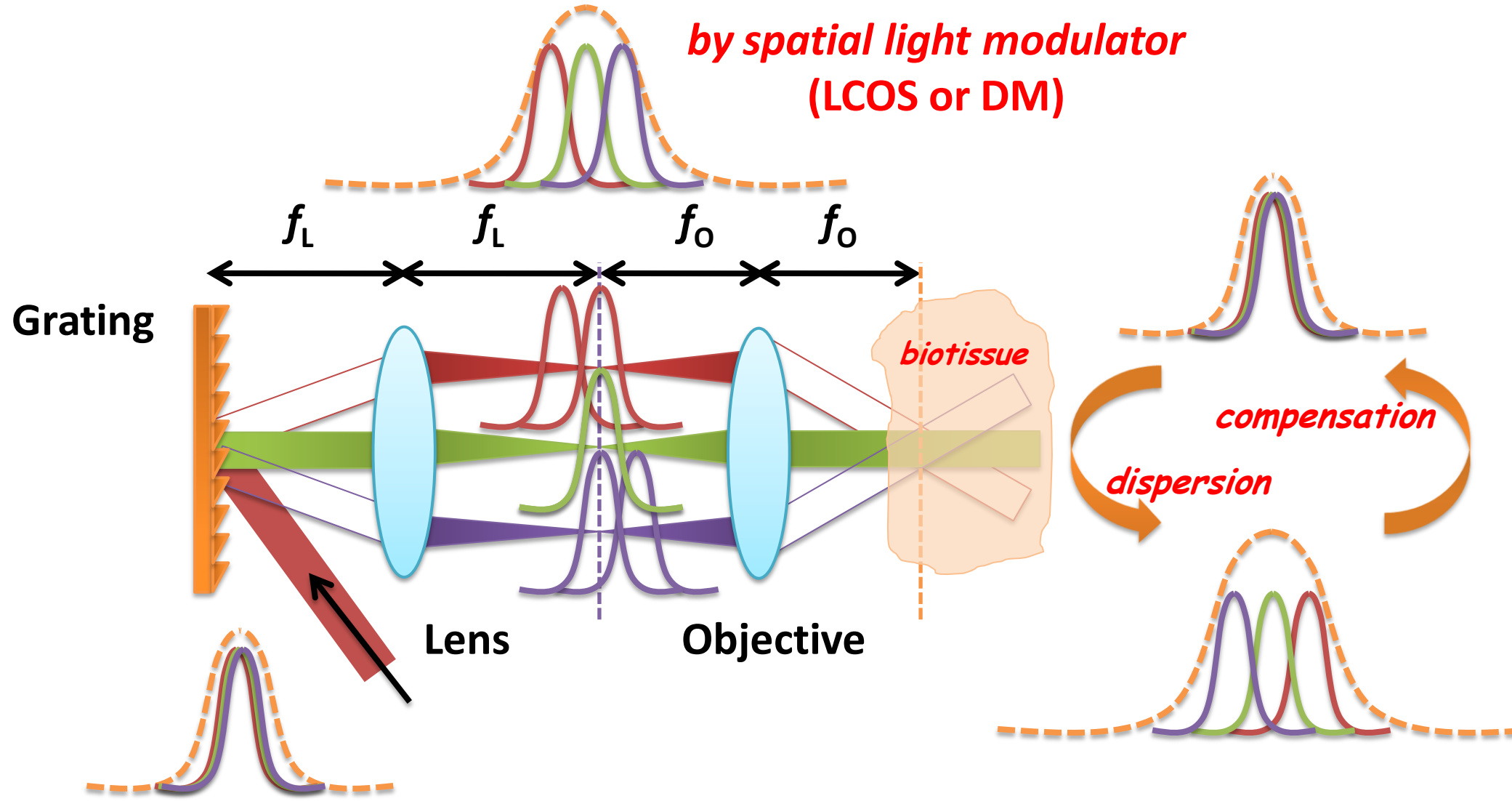
- Main parts of AOS:
  - Wavefront sensors
  - Wavefront correctors
  - Multichannel controllers



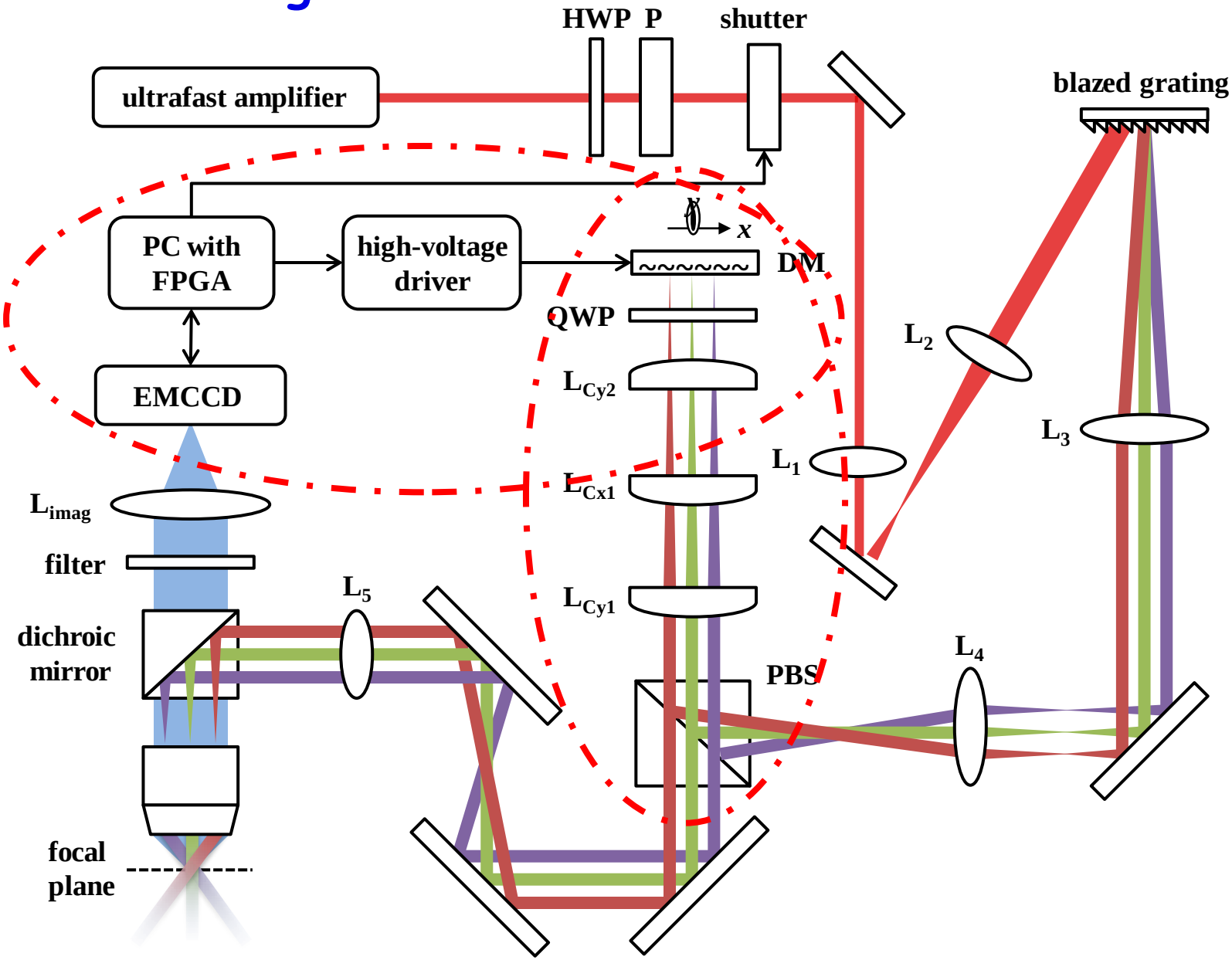
Easily implementable FPGA-based adaptive optics system with state-space multichannel control

C.-Y. Chang *et al., Rev. Sci. Instrum.* 84 (2013) 095112.

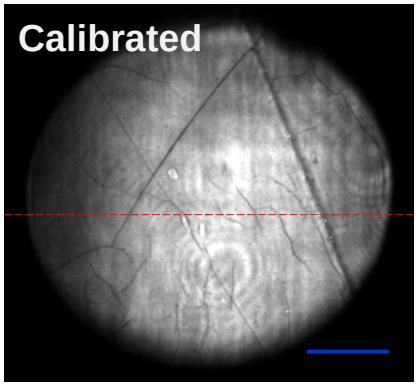
Basic Concept: Temporal Compensation

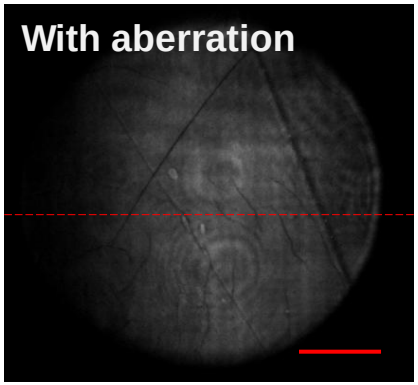


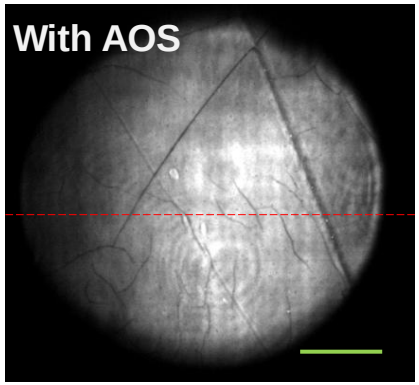
Schematic Diagram

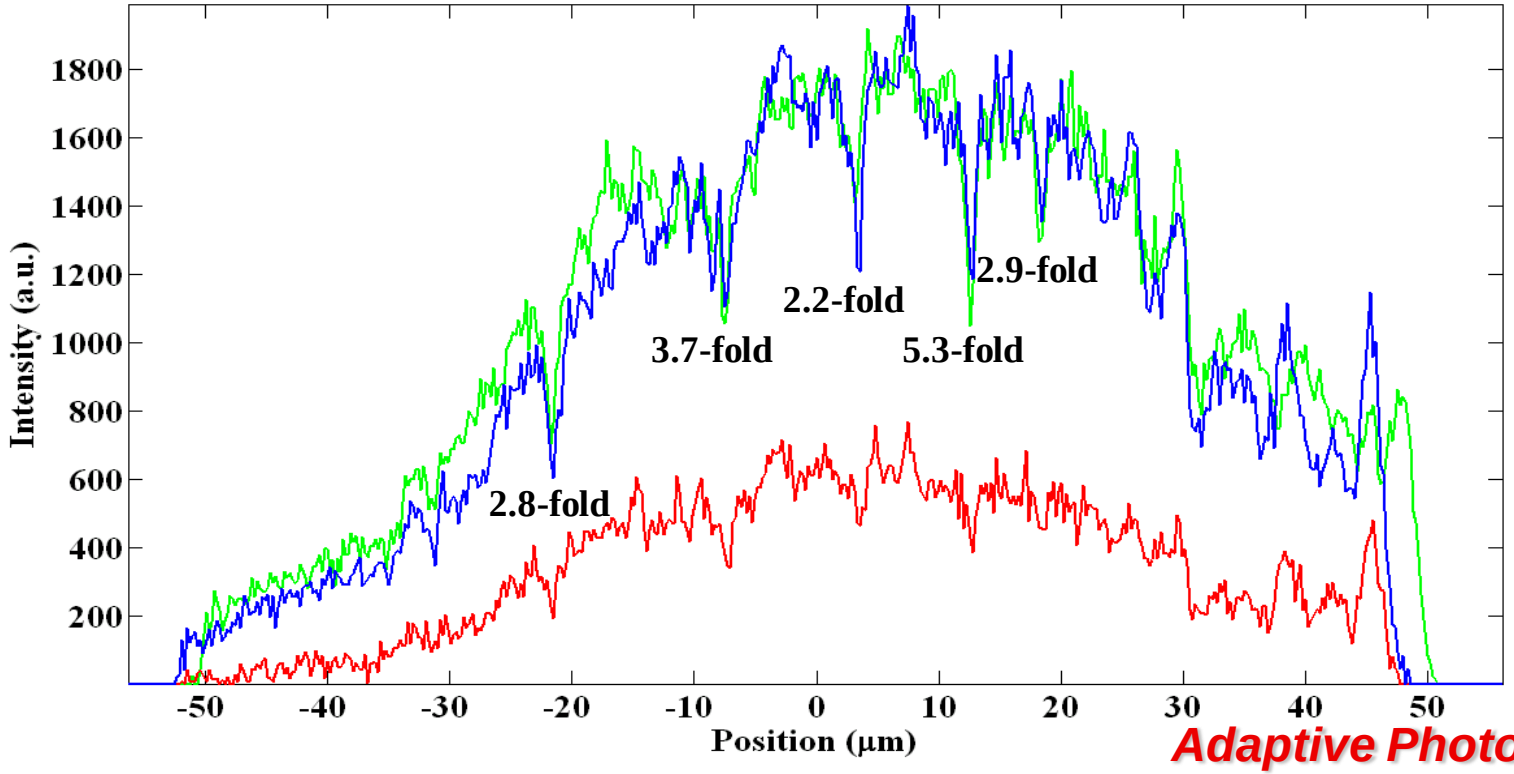


R6G Fluorescent Thin Film



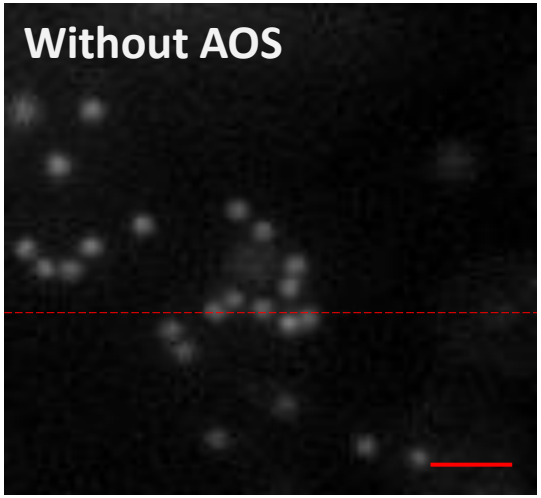


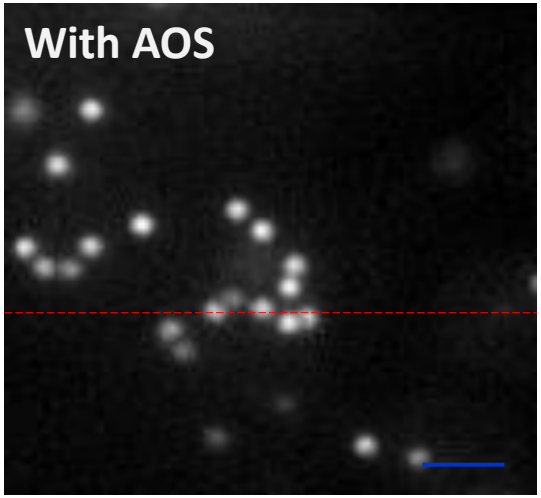


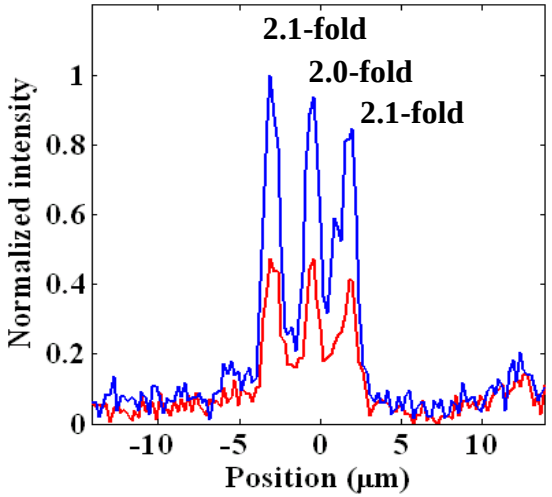


Int. time: 100 ms
Size: 100×100 μm²
(512×512)
Power: 12.3 mW

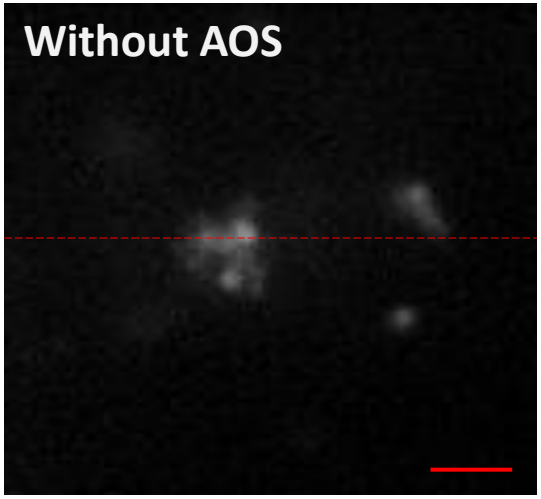
1 μm Fluor. Beads at Different Depths in Agarose Gel

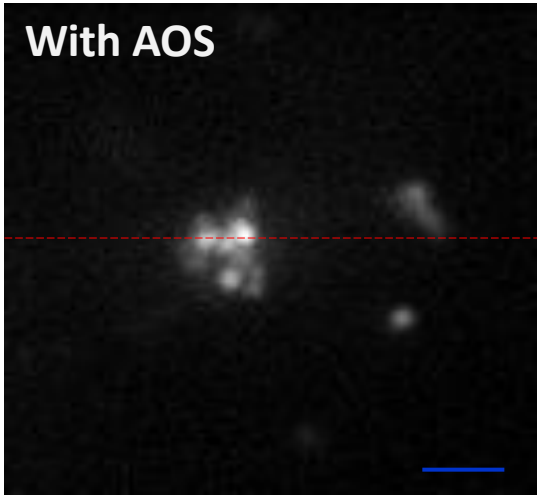


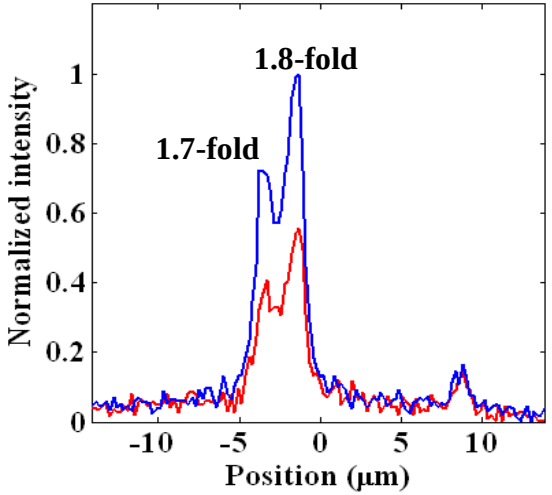




z= -38 μm







z= -120 μm

Int. time: 50 ms
Size: 25×25 μm^2 (128×128)
Power: 17.5 mW

C.-Y. Chang *et al., Biomed. Opt. Express 5* (2014) 1768.

Outlines

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- High-speed 3D Sectioning Images (over 100 Hz)
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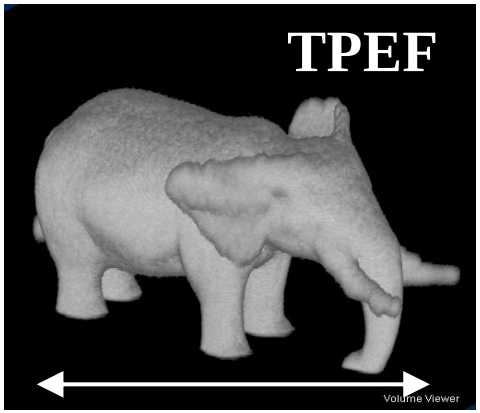
- To Improve Deep Imaging with Adaptive Optics
- **Fast Multiphoton Microfabrication (3D Lithography)**
- High-throughput Multiphoton-induced Laser Ablation
- Summary

3D Multiphoton Microfabrication (Lithography)

- ✓ Conventional fabrication technique, such as E-beam lithography, nanoimprinting lithography, etc. Limited to 2D applications
- ✓ Two-photon excited (TPE) microfabrication achieves 3D resolution by spatially focusing light to induce nonlinear excitation within focal volume. Low fabrication speed

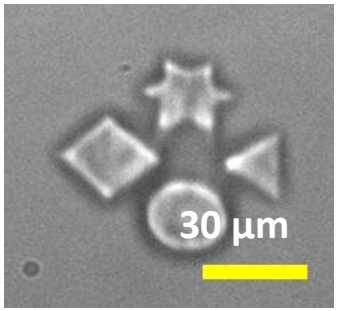
Goal: To develop a high-speed fabrication technique (mass production) which can make arbitrary 3D structure. Also, the resolution can achieve sub-micro level.



~50 μm

Multi-objects & Inspection

Solution: 2 mM RB + 75% TMPTA
Objective: 40X oil 1.3
Height: ~40 μm
Fabrication time: 1 s



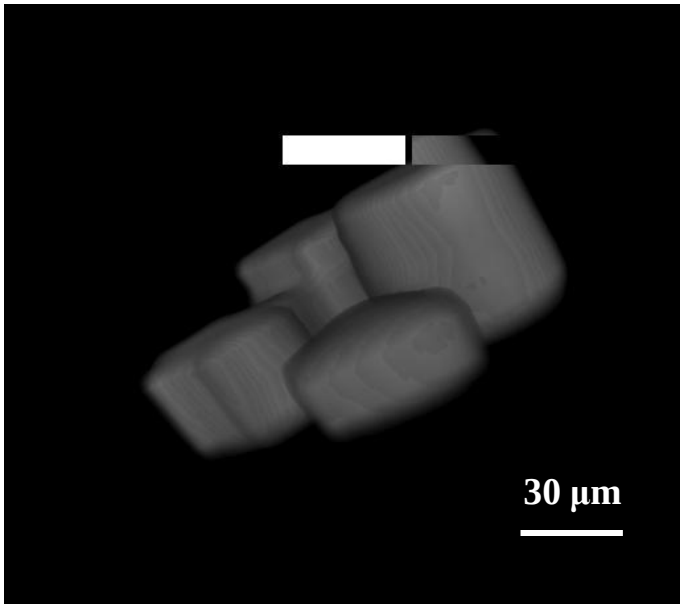


Image acquired during fabrication process

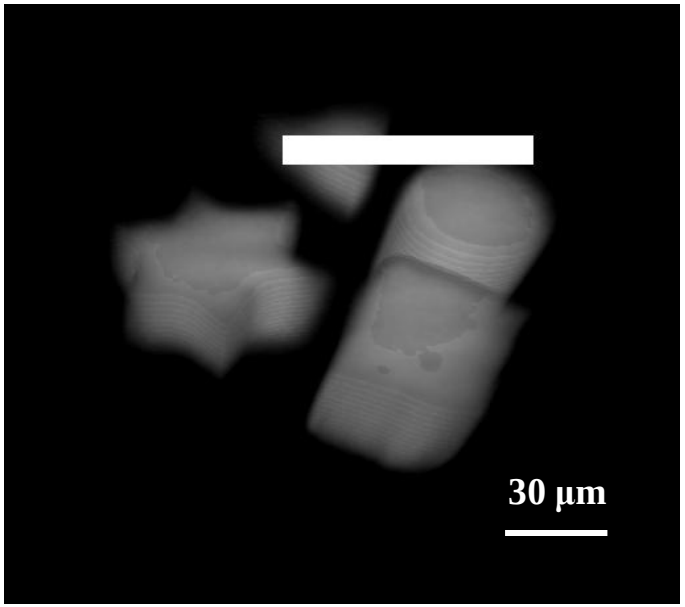


Image acquired using serial scanning microscope

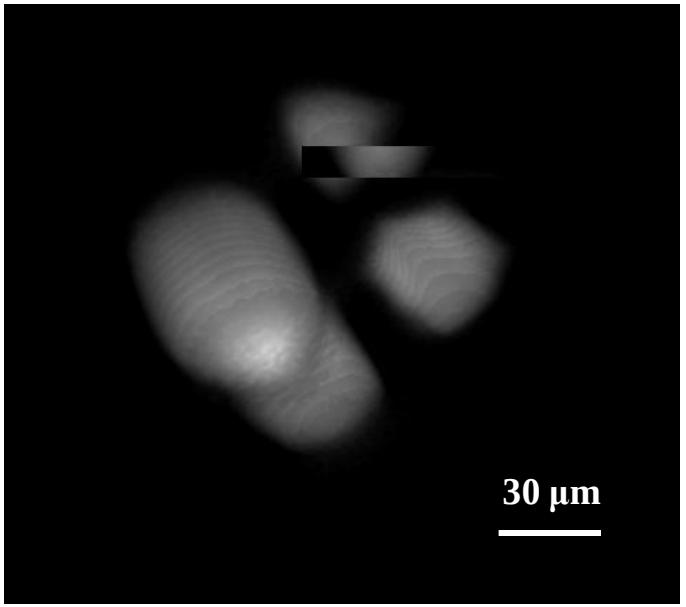
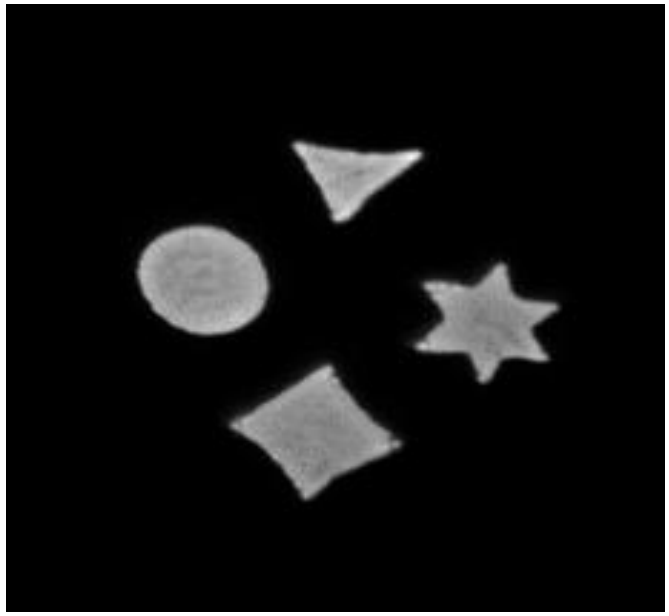


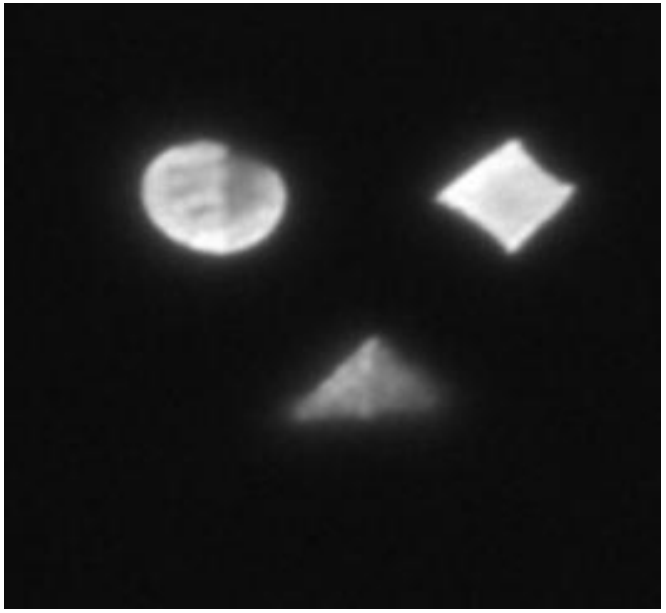
Image acquired using widefield microscope

Y.-C. Li *et al.*, *Opt. Express* 20 (2012) 19030.

Mass-Production via High-Throughput Multiphoton 3D Lithography

- Gray-Level BSA Microstructures





Multiple BSA structures of different concentrations can be simultaneously achieved by selecting different pulse numbers in the designated regions with an appropriate femtosecond laser power.

Time: 0.01 sec/layer, Speed enhanced: 300 times

C.-Y. Lin *et al.*, *Opt. Express* 20 (2012) 13669.
Y.-C. Li *et al.*, *J. Biomed. Opt.* 18 (2013) 075004.

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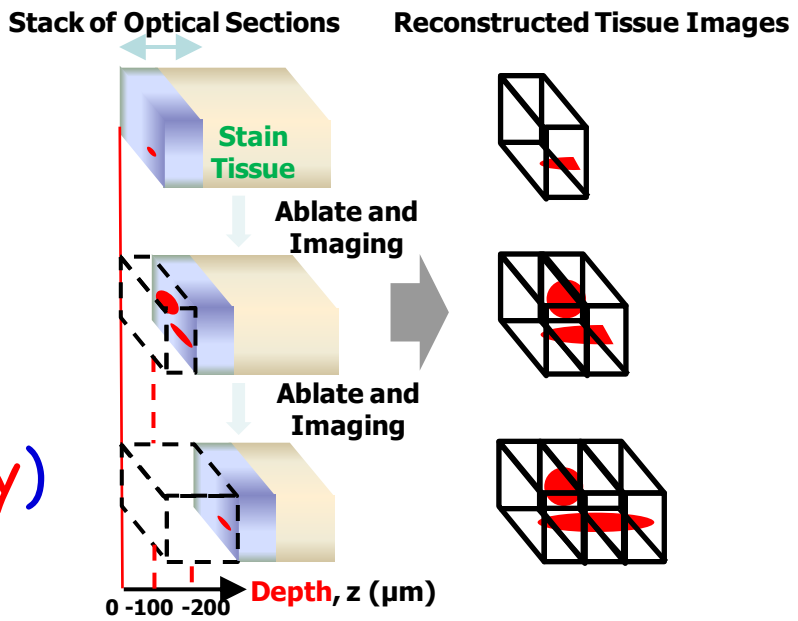
- **High-throughput Multiphoton-induced Laser Ablation**
- Summary

Multiphoton-induced Laser Ablation & Imaging

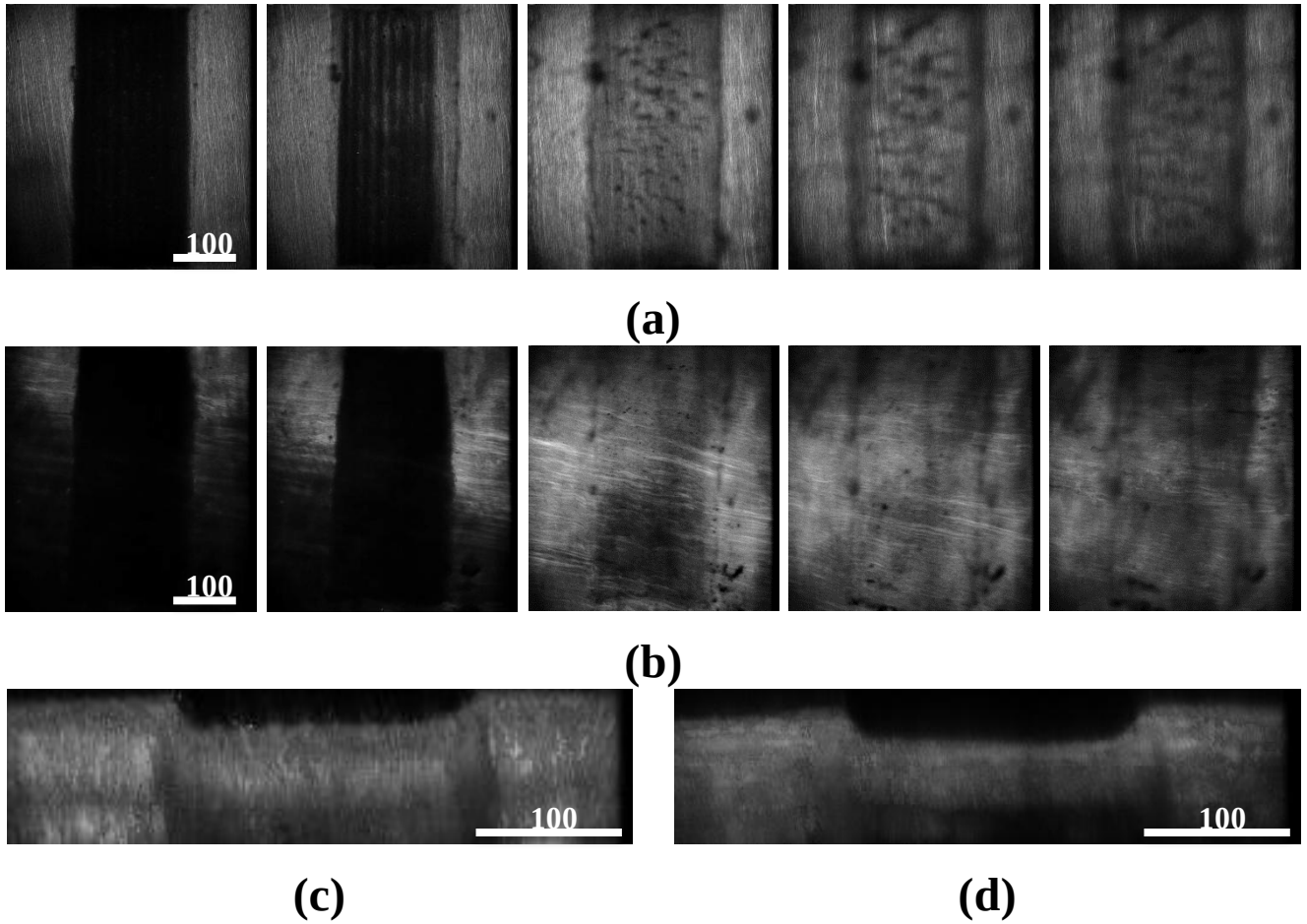
- ✓ Conventional method for micro-sectioning tomography, such as diamond knife to remove the tissue is very useful. Limited to sample preparation.
- ✓ Point-scanning femtosecond laser achieves automatically and iteratively ablation and imaging at sub-micron level for fresh tissue. Low throughput.

Goal: To develop high-throughput multiphoton-induced laser ablation and fast nonlinear optical imaging simultaneously. (for all optical histology)



Multiphoton-induced Laser Ablation of Tissues

SHG images in different depths for chicken tendon after multiphoton-induced ablation machining

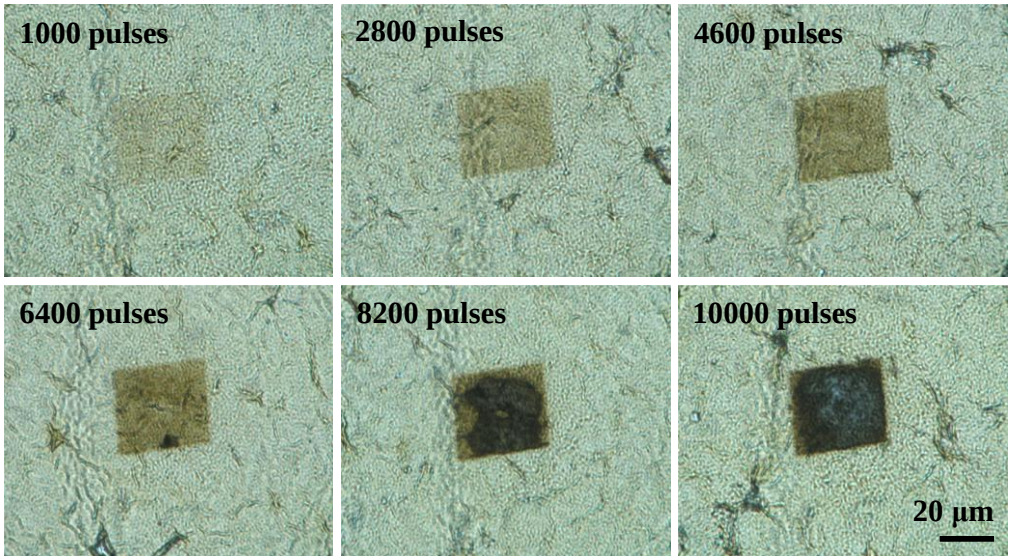


SHG images in different depths @ 10, 20, 40, 60 and 70 μm with the machining pitches of (a) 30.0 μm & (b) 20.0 μm.

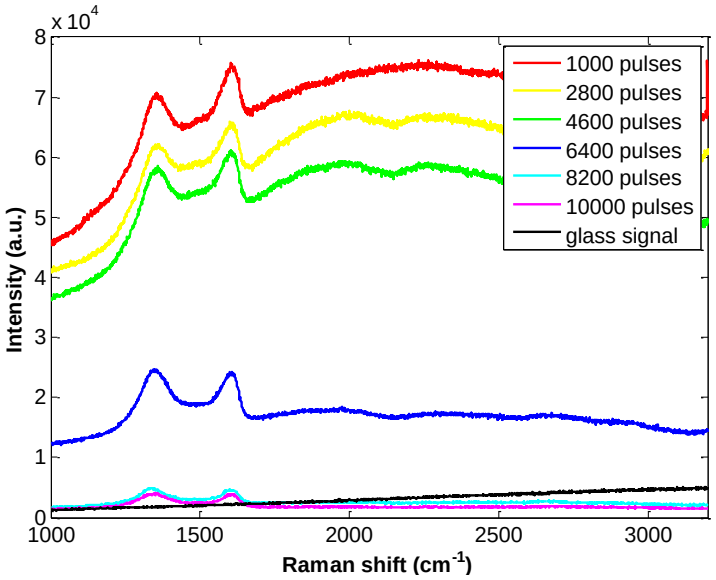
Projective images (c) & (d) from the 3D reconstructed images (a) & (b).

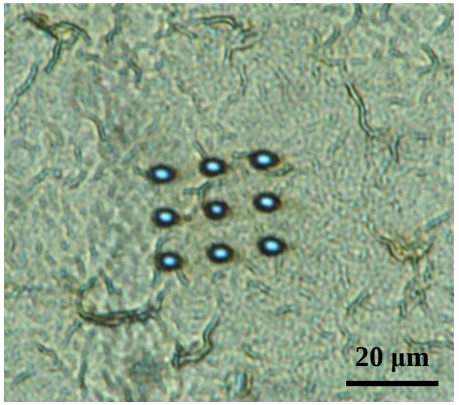
C.-Y. Lin *et al., Biomed. Opt. Express* 22 (2014) submitted.

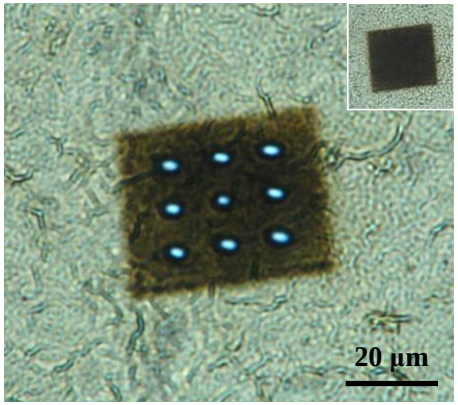
GO-based Micropatterns via Multiphoton-induced Reduction and Ablation



Bright-field images of reduced GO patterns with different pulse number. The yellow words indicate the pulse number used for each pattern.







Bright-field images of GO array patterns. (a) Direct ablation of GO film. (b) Combination of reduction and ablation of GO film.

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Summary

- ✓ High-speed sectioning images (up to 200 Hz) via temporal focusing-based widefield multiphoton microscopy.
- ✓ To approach super-resolution microscopy with nonlinear structured illuminlation microscopy (NSIM) & Astigmatisum imaging.
- ✓ To improve deep imaging with adaptive optics (AO).
- ✓ High-throughput multiphoton microfabrication: To develop a high-speed fabrication technique which can make arbitrary 3D bio-microstructures.
- ✓ Multiphoton-induced laser ablation: To develop high-throughput multiphoton-induced laser ablation and fast nonlinear optical imaging simultaneously.

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- **Contributors:**
  Yi-Cheng Li, Li-Chung Cheng,
  Chia-Yuan Chang, Chi-Hsiang Lien,
  & Chun-Yu Lin

- **Collaborators:**
  C. Y. Dong (Physics, NTU)
  P. Campagnola (Biomedical Eng., UW-Madison)
  C. Xu (Appl. Phys., Cornell U.)
  F.-C. Chien (Photonics, NCU)

Thanks for Your Attention!