光片荧光显微镜

Light-sheet Fluorescence Microscopy

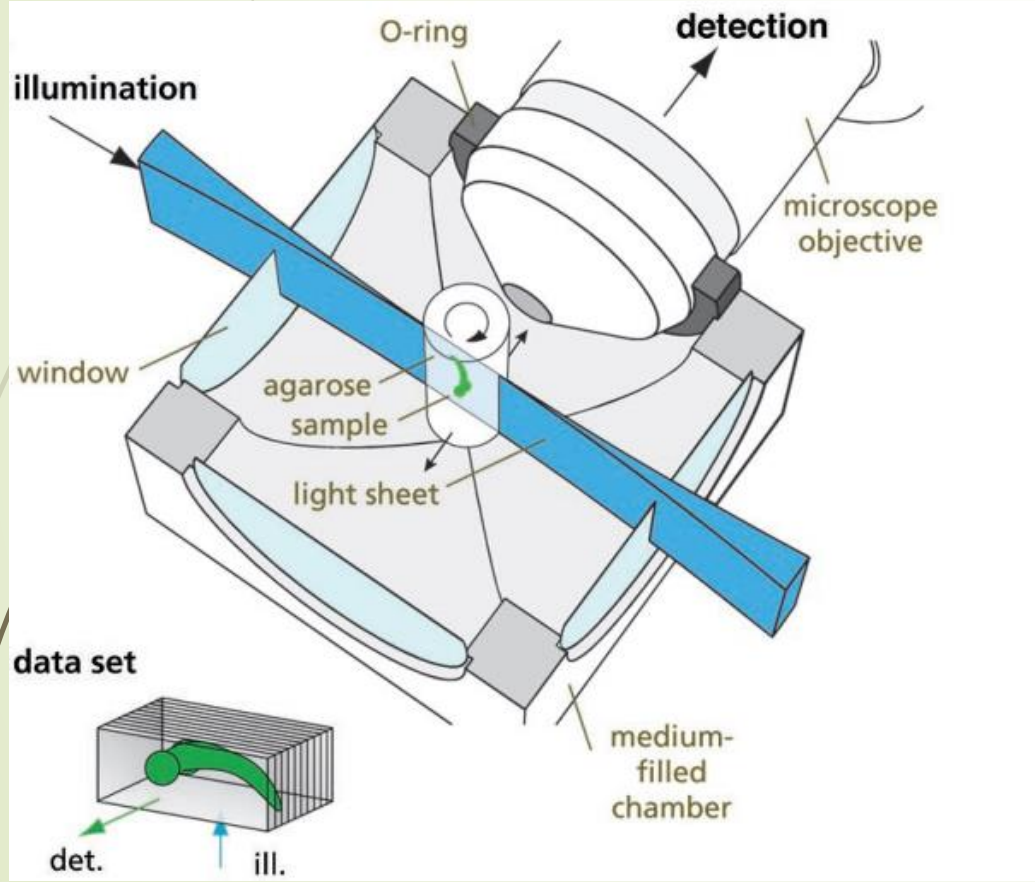
畅星兆

日期：2018.11.28

目录

- LSFM的变种（Varients）

光片荧光显微镜



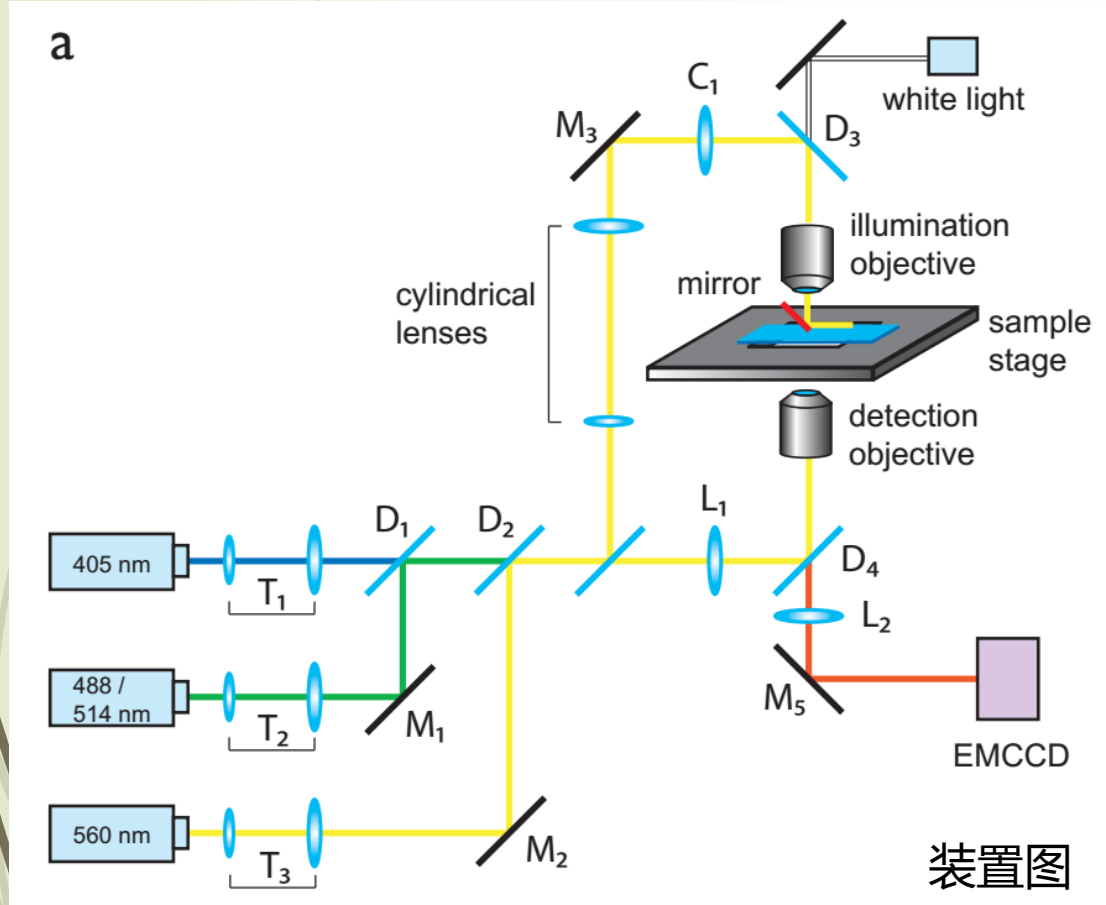
光片荧光显微镜（又称 light sheet fluorescence microscopy， LSFM；或者 selective plane illumination microscopy, SPIM）

LSFM共包含两束光路，分别是照明光路（illumination）和探测光路（detection）。照明光路用来产生光片状结构光，探测光路用来收集荧光分子释放出的荧光信号，再通过扫描，便可以实现对样品的三维扫描成像。

之后，根据观测样品尺寸、时间空间分辨率等要求的不同以及某些特殊需要，产生了不同的显微镜变种。以下是几个光片荧光显微镜的变种。

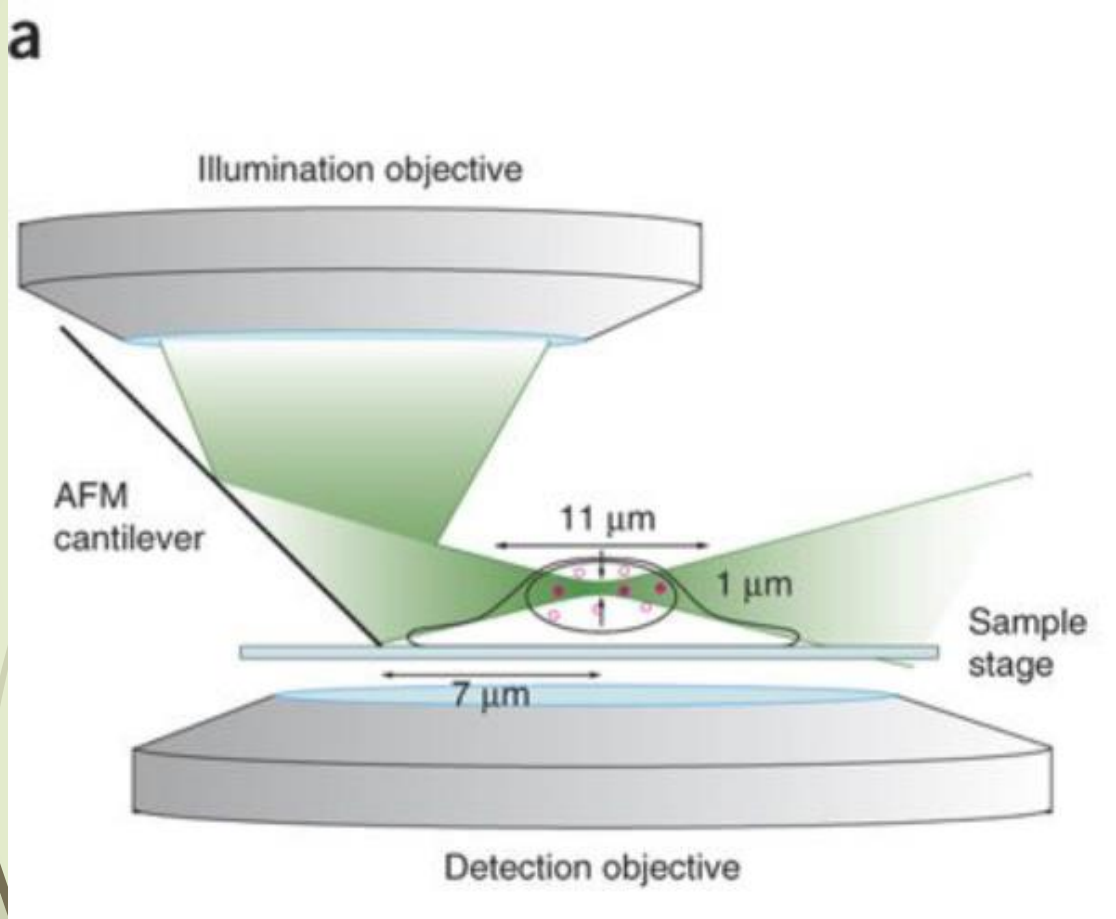
- RLSM (Reflected light sheet microscope)
- soSPIM (Single-objective selective plane illumination microscopy)
- APOM (axial plane optical microscopy)
- IML-SPIM (individual molecule localization-selective plane illumination microscopy)
- πSPIM
- FCS (Fluorescence correlation spectroscopy)
- DSLM (digital scanned laser light sheet fluorescence microscopy)
- Bessle beam plane illumination microscope
- iSPIM (Inverted selective plane illumination)

1.Reflected Light Sheet Microscope (RLSM)

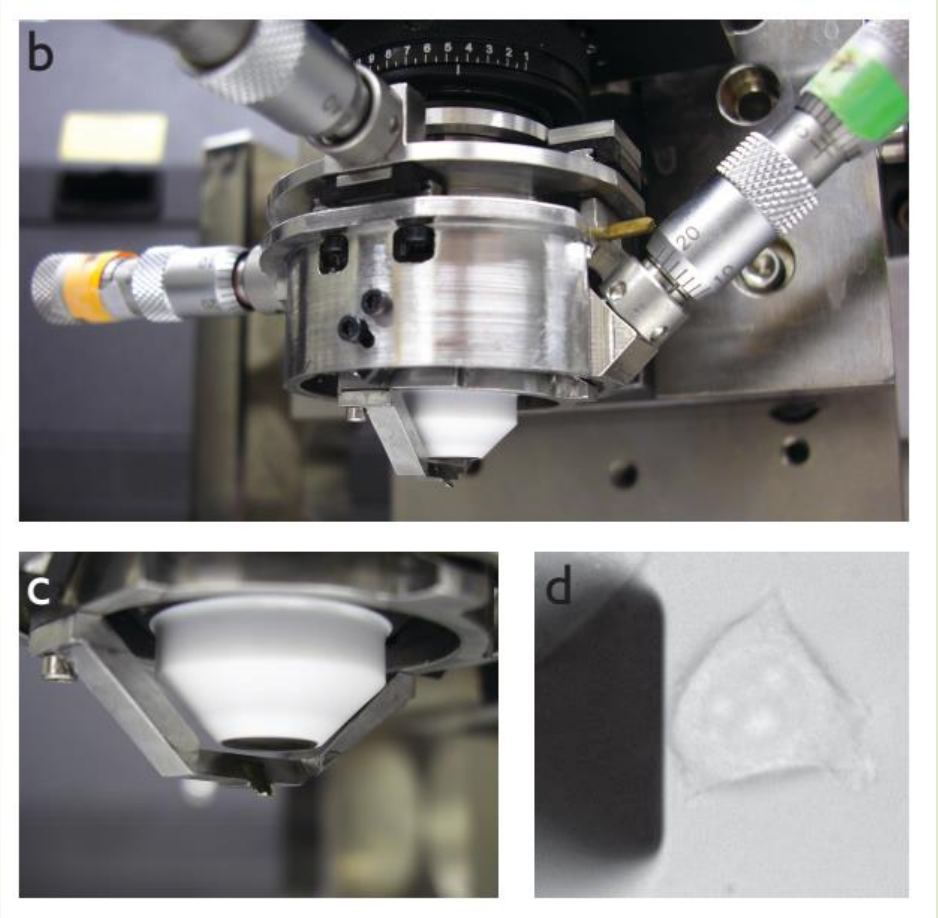


在普通的LSFM结构中，照明物镜与探测物镜相对于待测样品正交放置，这限制了高数值孔径物镜的使用。

RLSM通过用反射镜将从顶部入射的的光片反射，使得两个物镜可以相向放置，从而解决了上述问题。

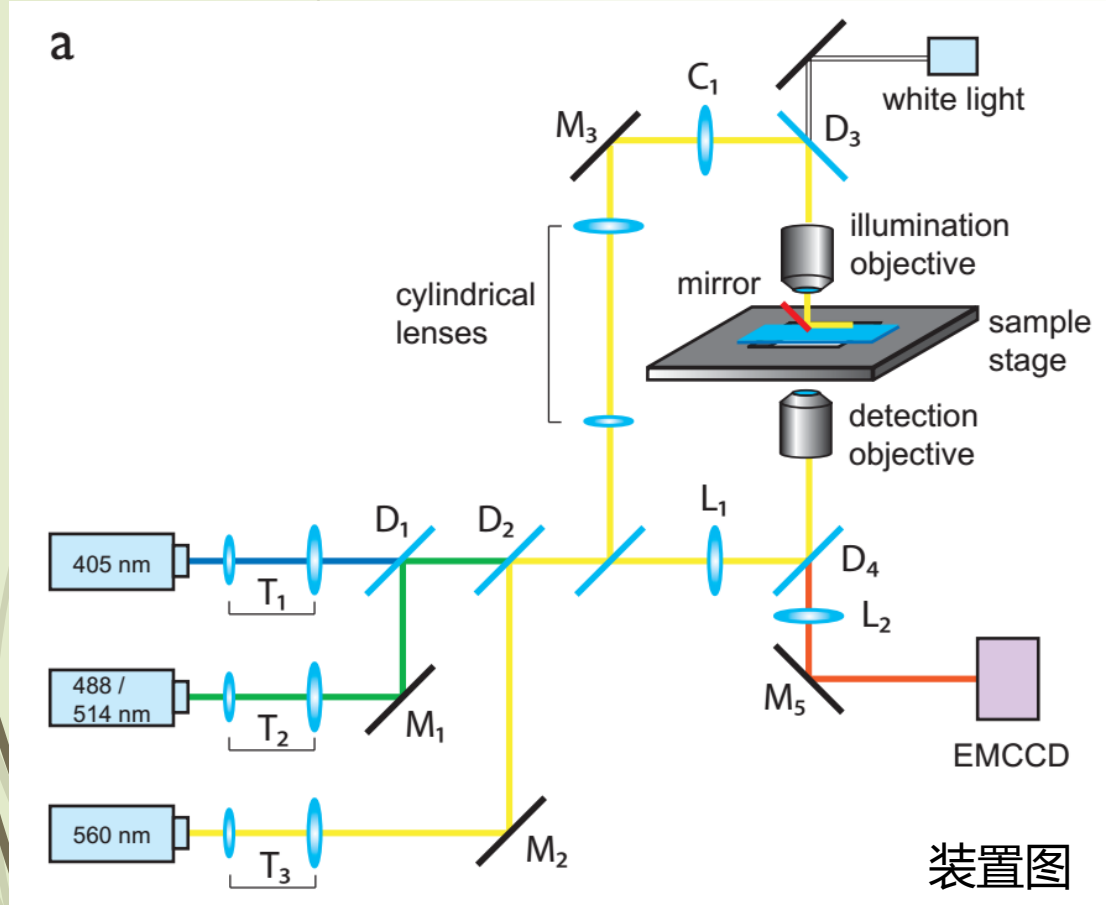


样品处光束图



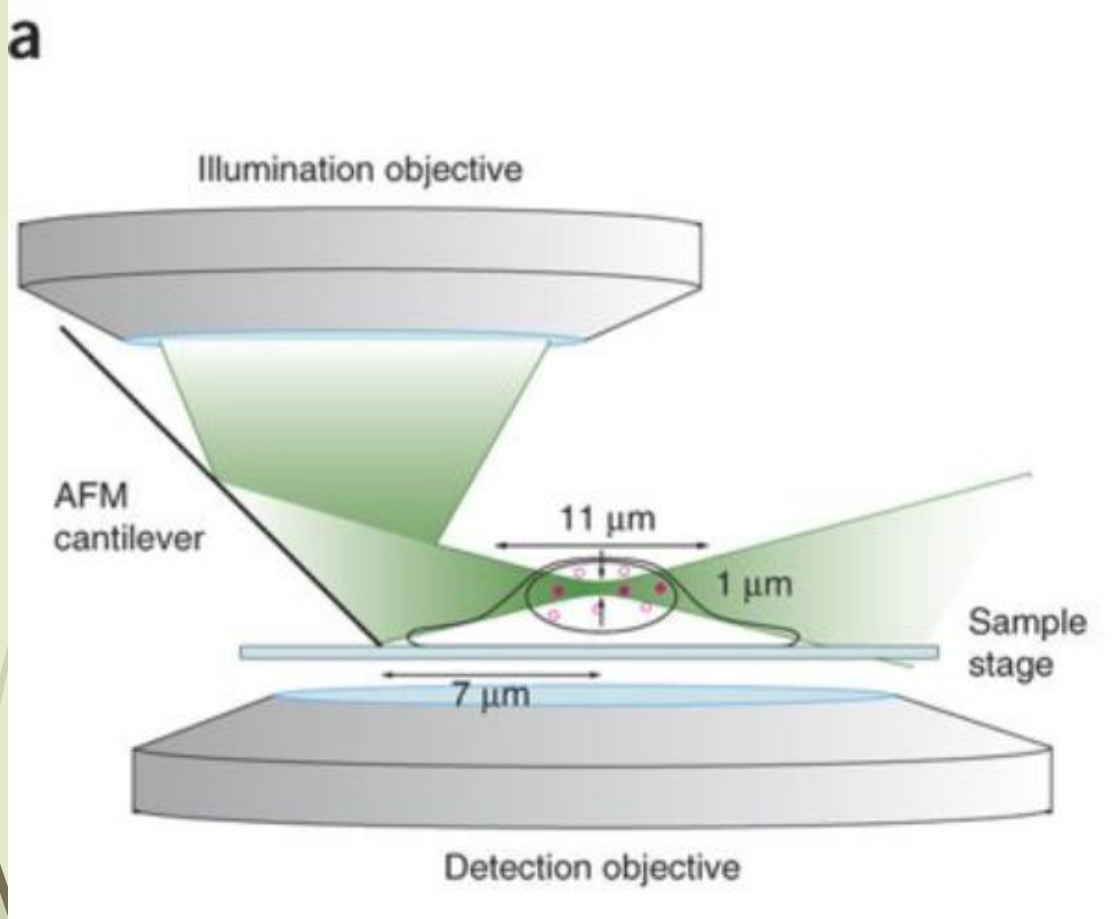
AFM Cantilever

1.Reflected Light Sheet Microscope (RLSM)

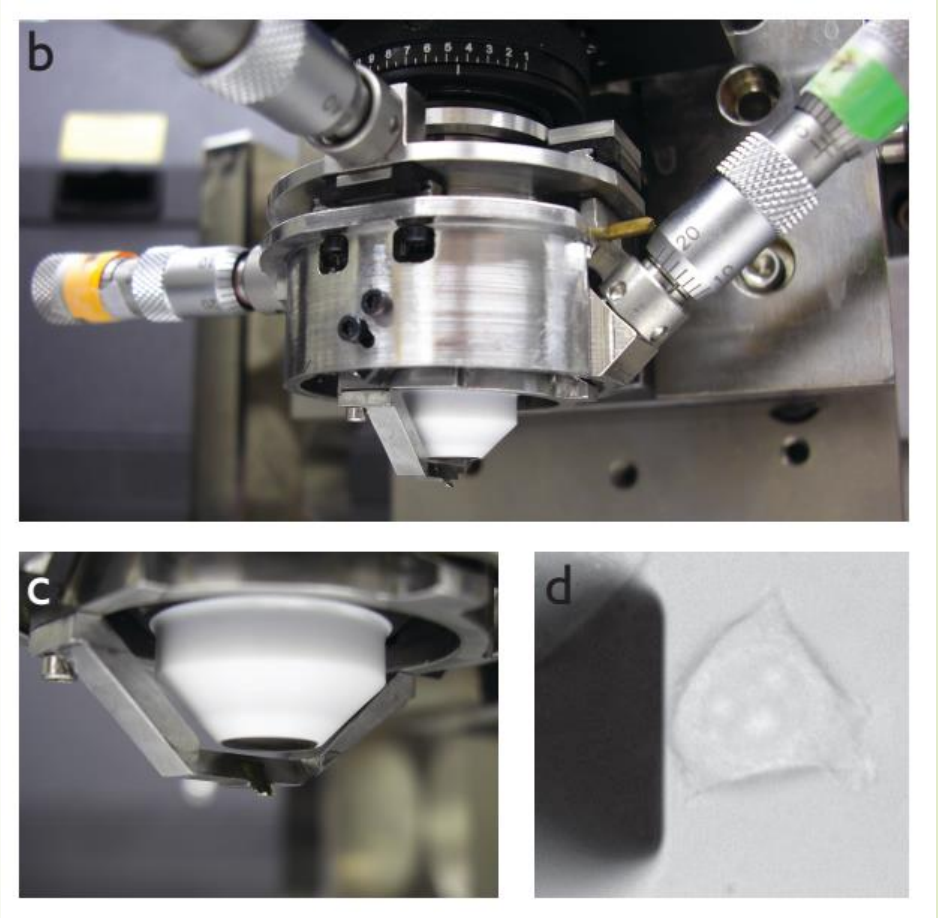


在普通的LSFM结构中，照明物镜与探测物镜相对于待测样品正交放置，这限制了高数值孔径物镜的使用。

RLSM通过用反射镜将从顶部入射的的光片反射，使得两个物镜可以相向放置，从而解决了上述问题。



样品处光束图



AFM Cantilever

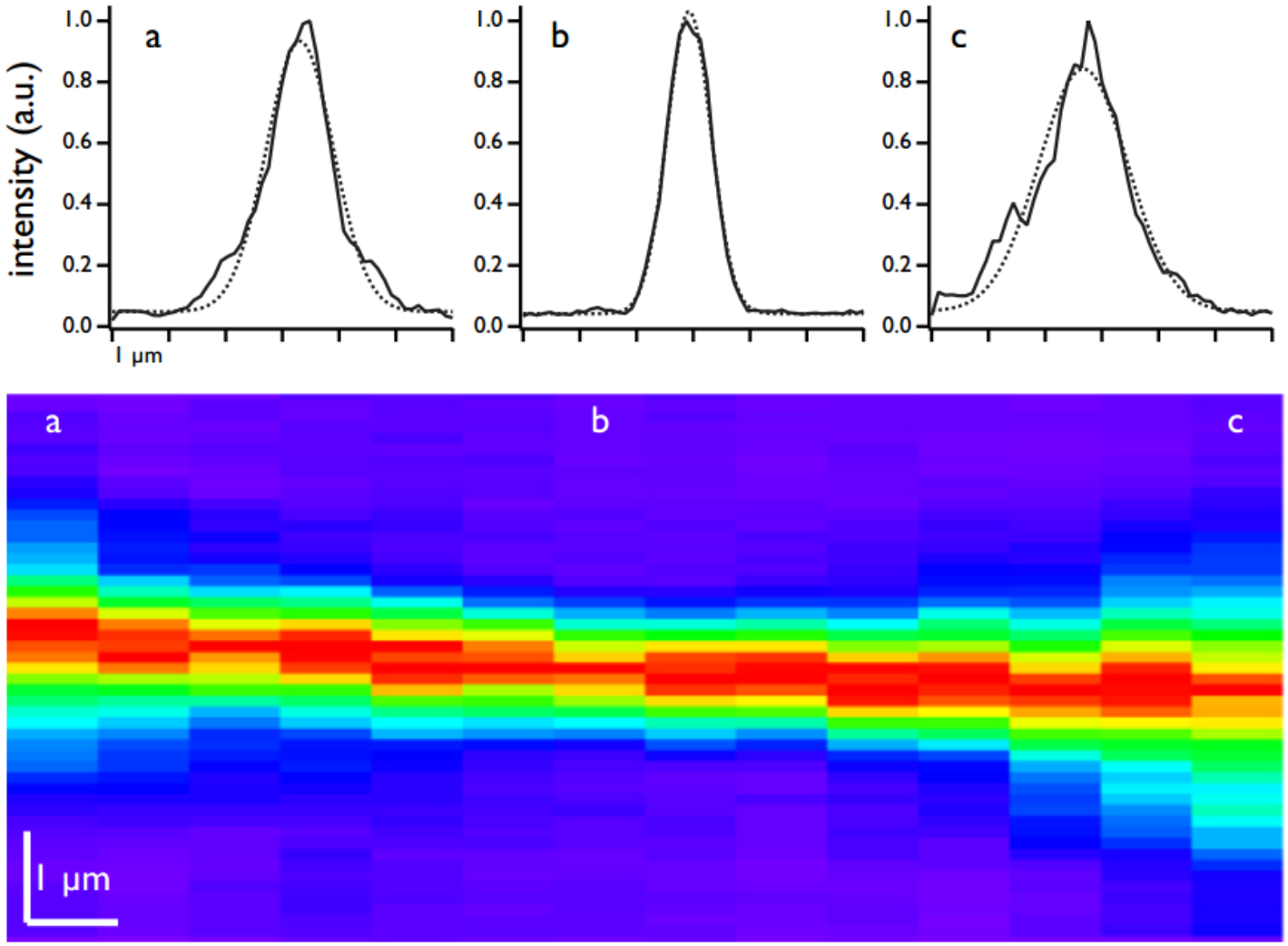
1装置

- 模板：IX71, Olympus
- 光源：405 nm, 50 mW, Electra-40, Laserglow; 488/514nm, 1000 mW, Innova300, Coherent; 560 nm, 1000 mW, VFL-P-1000-560, MPB communications
- 扩束：f = 40 mm, LJ1402L1-A and f = 400 mm, LJ1363L1-A, both Thorlabs
- 照明物镜：LUMPLFLN 40x Water, NA 0.8, Olympus
- 探测物镜：UPlanApo 100x 1.35 Oil or UPlanSApo 100x 1.4 Oil, both Olympus
- EMCCD: iXon+, DU-897E-CSO-BV, Andor
- 扫描：Nano-Bio3200, Mad City Labs

1软件控制

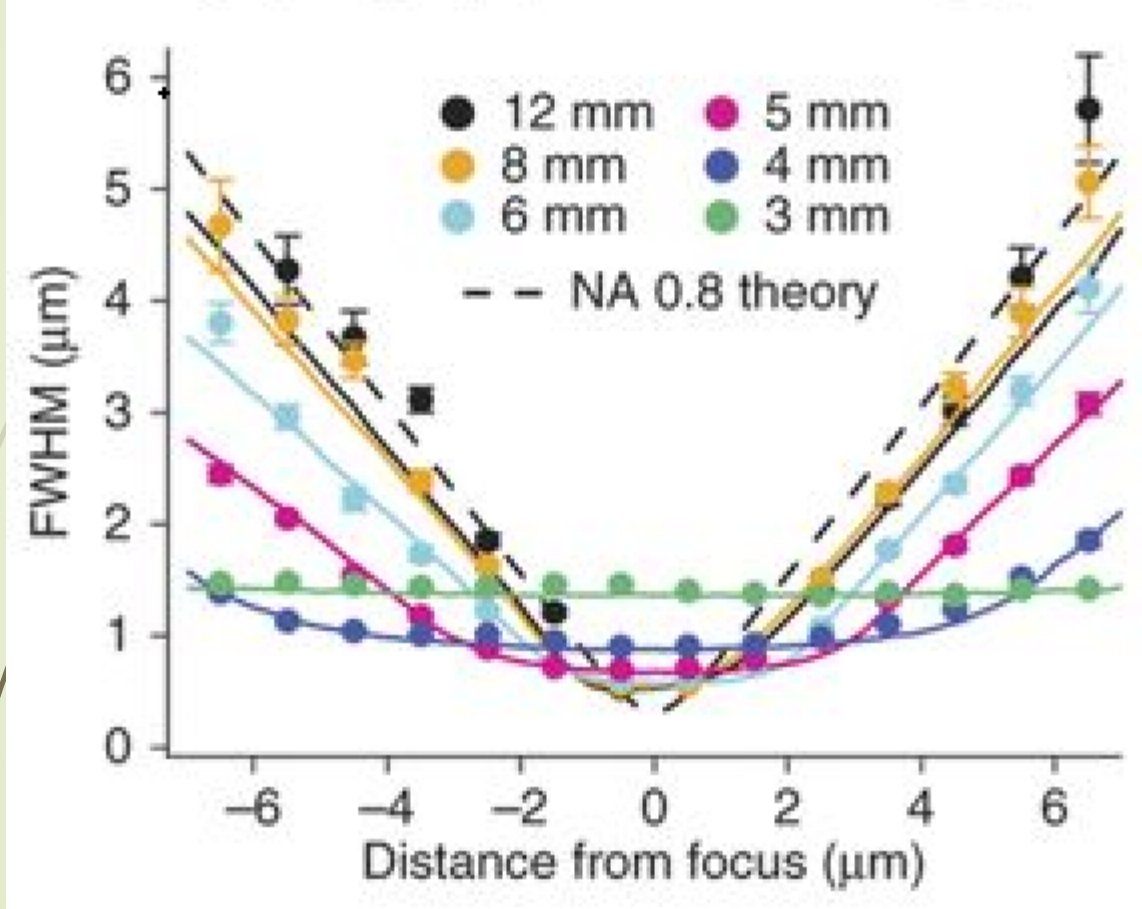
- LabView: 样品扫描
- MetaMorph Software: 控制显微镜，快门，EMCCD相机

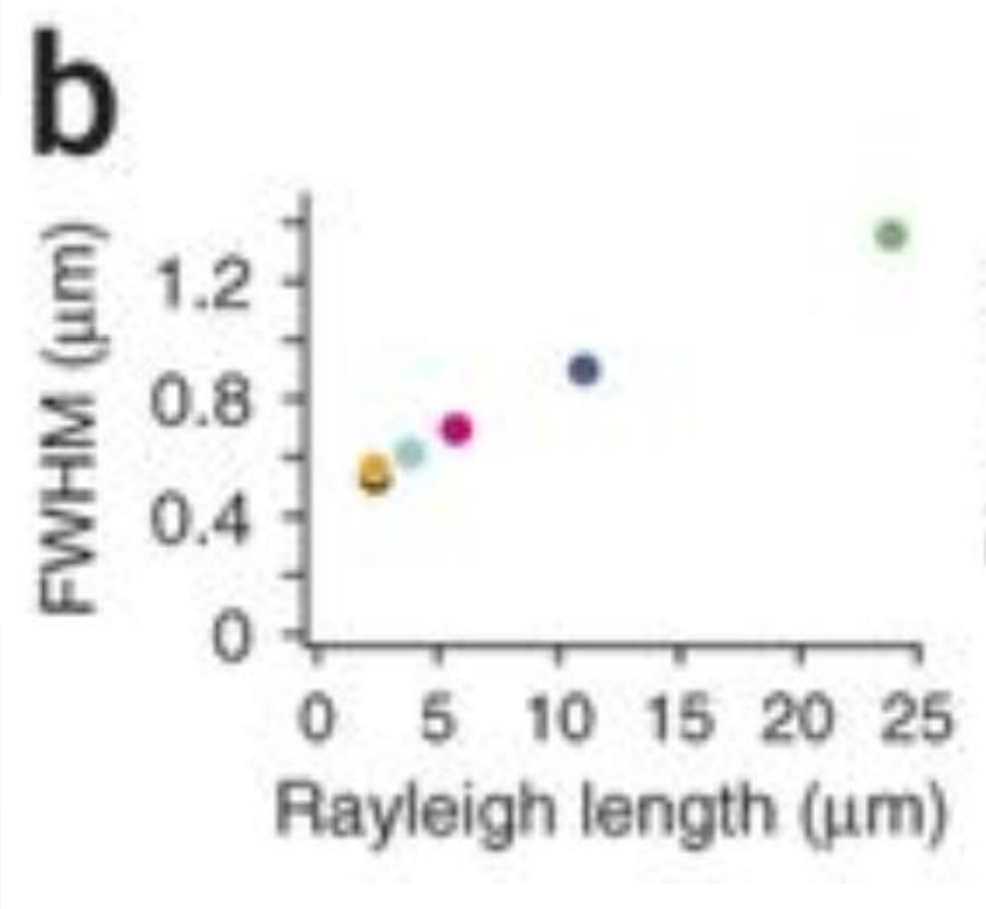
光片特性



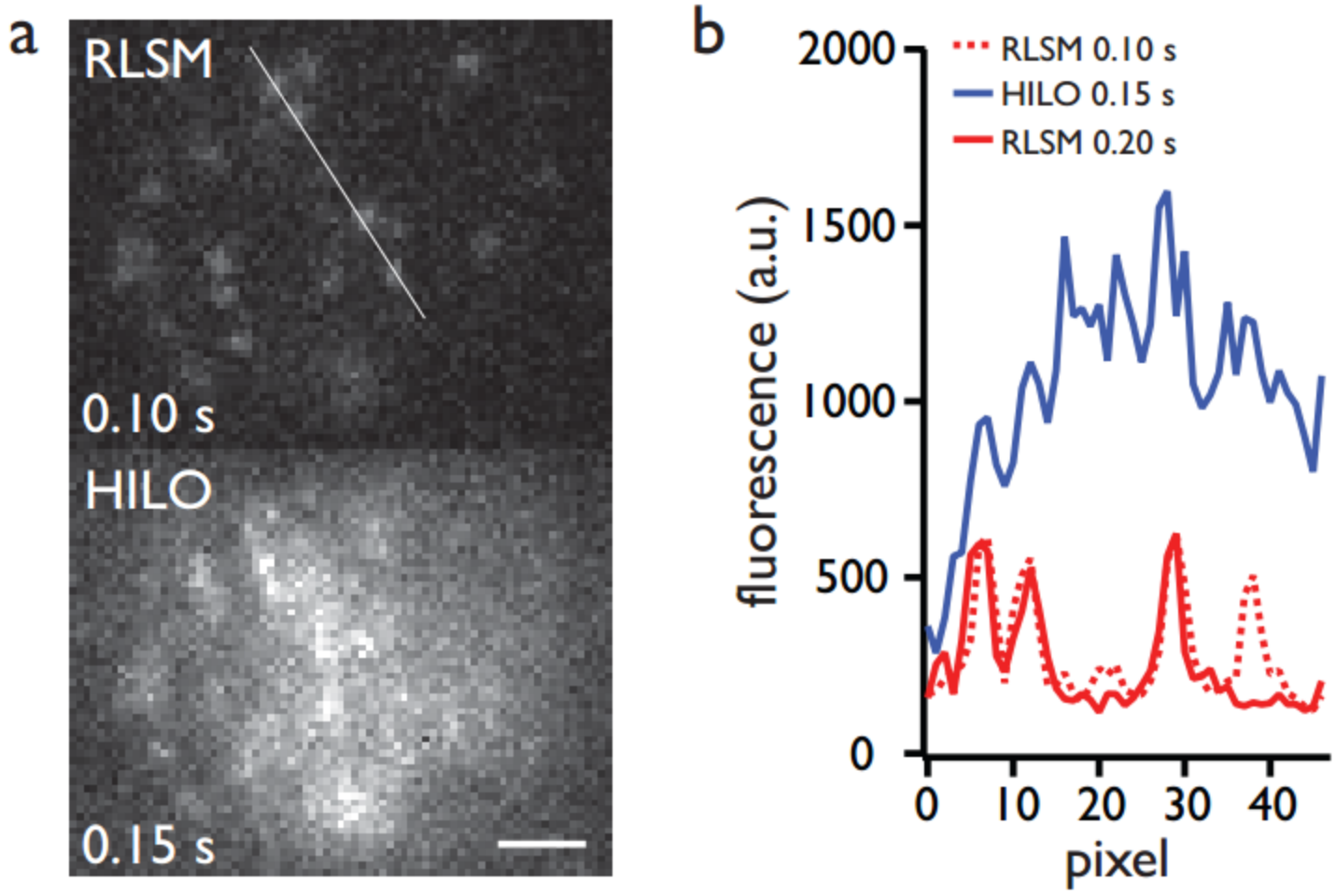
方法:
荧光珠（fluorescent bead）扫描

光片特性


12 mm
8 mm
6 mm
5 mm
4 mm
3 mm
NA 0.8 theory
FWHM (μm)
Distance from focus (μm)
0
1
2
3
4
5
6
−6
−4
−2
0
2
4
6


b
FWHM (μm)
0
0.4
0.8
1.2
0
5
10
15
20
25
Rayleigh length (μm)

结果图



采用RLSM HILO两种方法对同一样本成像

RLSM的背景光大大减小

RLSM可以清晰地分辨出三个荧光蛋白的存在

特点

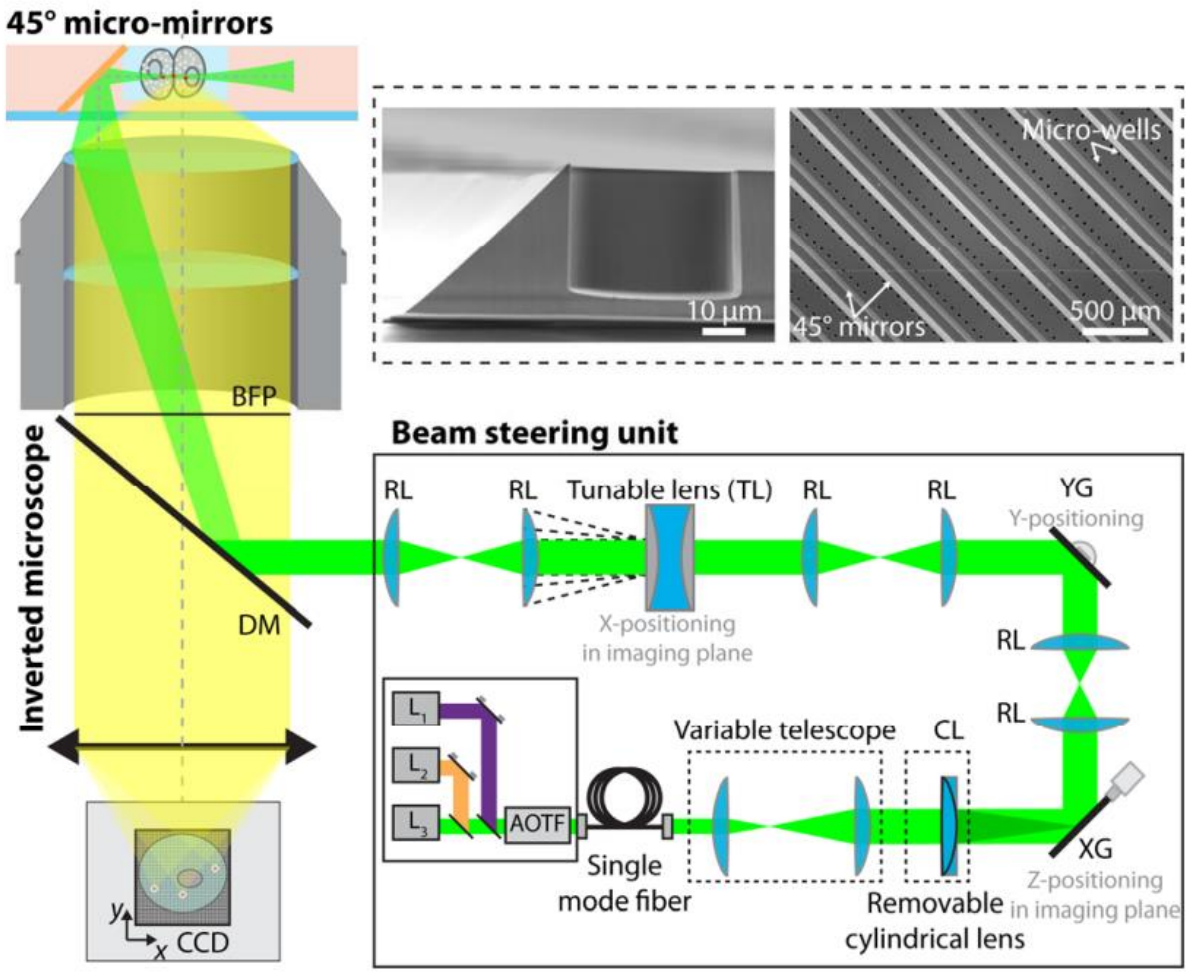
- 以倒置型显微镜为模板
- 可以使用标准的生物样本
- 扫描通过样本的移动完成（压电陶瓷平台）

优势

- 照明物镜与探测物镜均可以使用高数值孔径物镜

2. Single-objective selective-plane illumination microscopy (soSPIM)

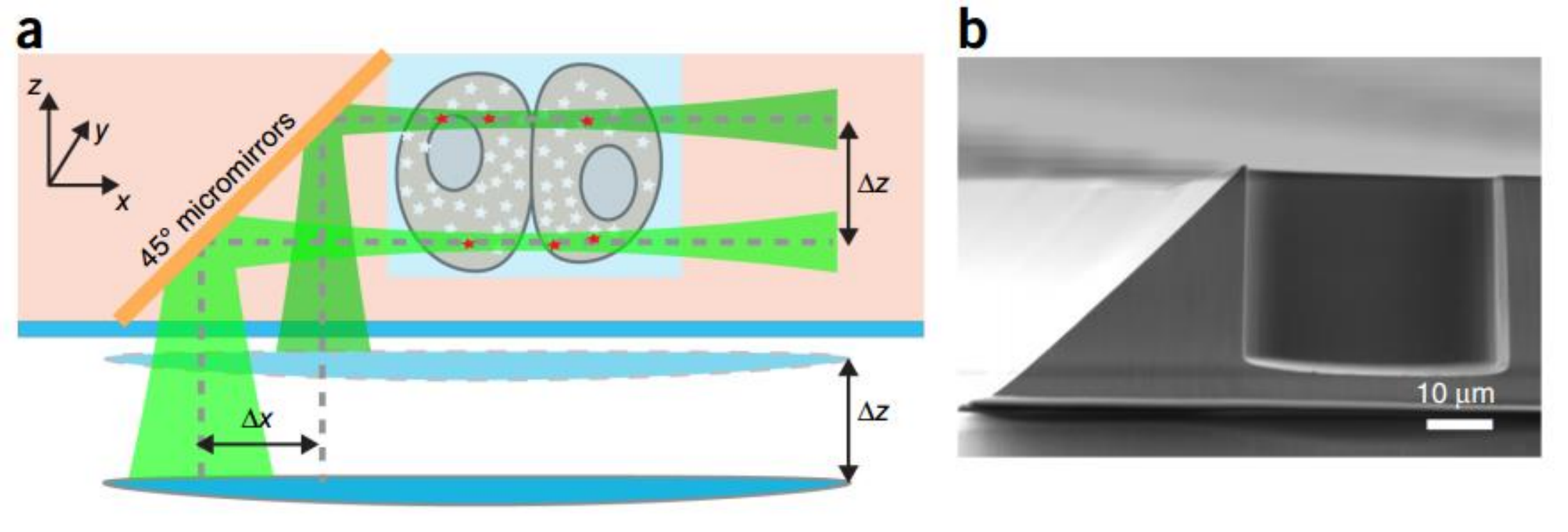
装置图



Supplementary Figure 1

soSPIM principle and representation of the soSPIM beam-steering unit.

装置图



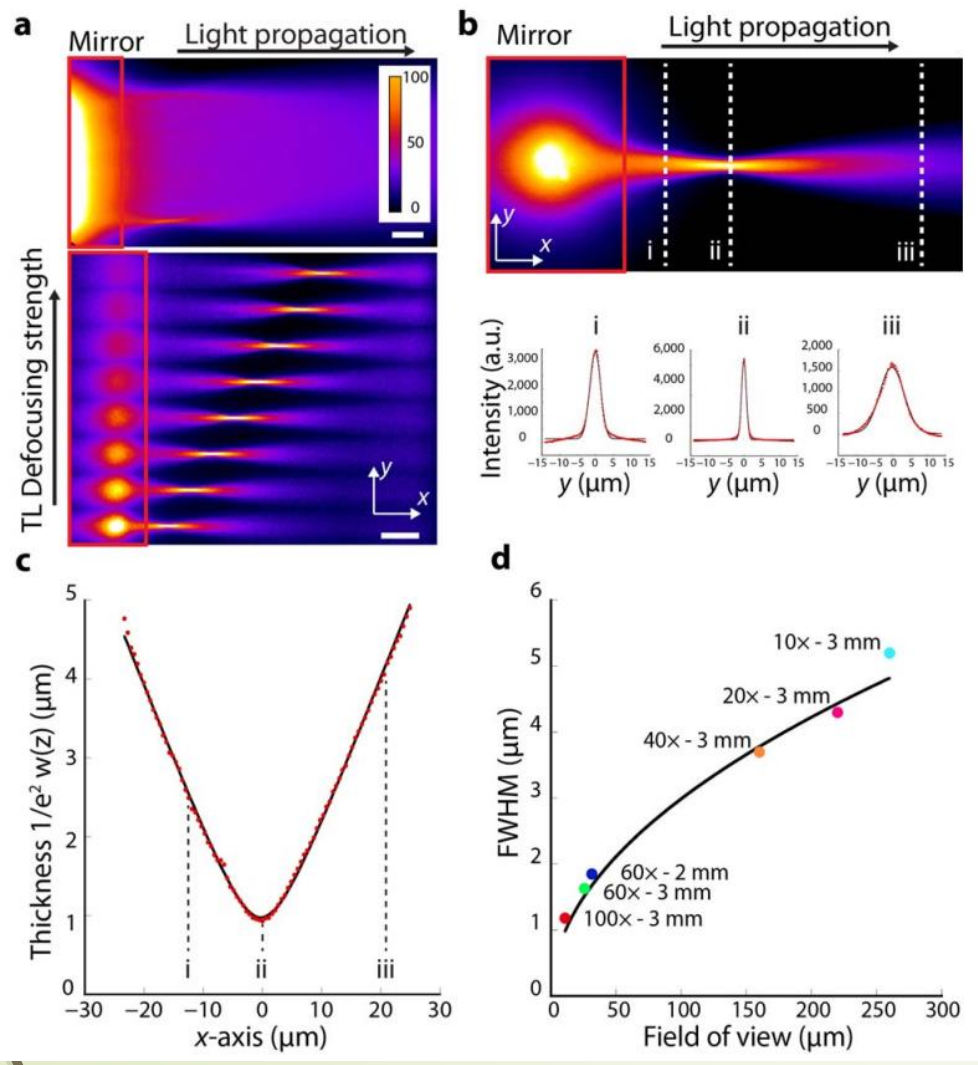
装置

- 模板： Nikon Ti-E
- 光源： 405 nm(200 mW; Errol), 488 nm (200 mW; Coherent), 561 nm (200 mW; Coherent) and 635 nm (150 mW; Errol))
- 扩束： AC254-050-A, Thorlabs (focal length of 50 mm), and AC254-75-A, Thorlabs (focal length of 75 mm)
- 光片形成： a cylindrical lens (ACY254-150-A, Thorlabs; focal length of 150 mm)
- 照明物镜（探测物镜）： CFI Plan Apochromat VC 60×/1.2- NA water-immersion objective or a CFI Plan Apochromat VC 100×/1.4-NA oil-immersion objective, Nikon. CFI Plan Fluor 10×/0.3-NA, CFI Plan Fluor 20×/0.5-NA or CFI Plan Fluor 40×/0.75-NA objective, Nikon
- EMCCD/cMOS: Evolve 512,Photometrics/ Neo 5.5, Andor (单细胞、高分辨率建议使用EMCCD)

软件控制

- LasershowDesigner (Pangolin Laser Systems) : 控制扫描
- MetaMorph Software: 图像重建、实现3D扫描

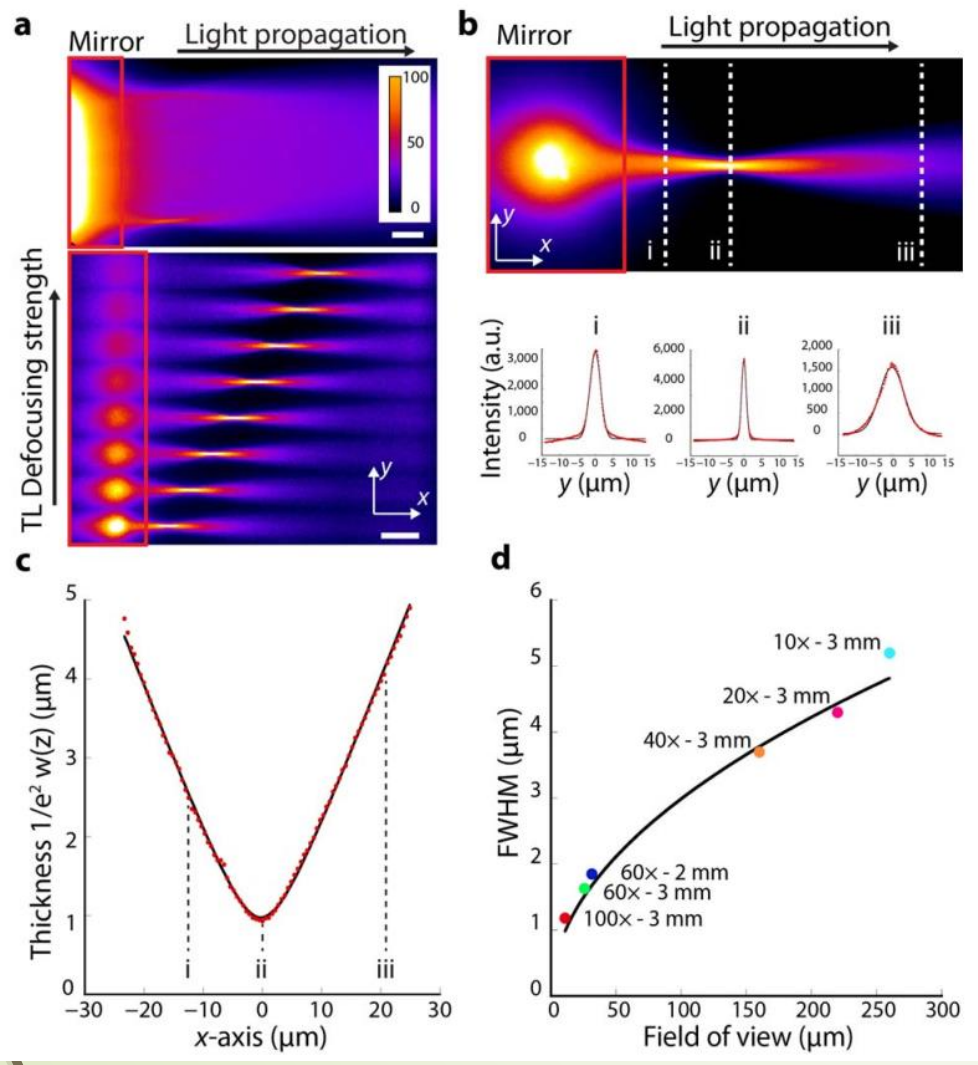
光片特性



方法:

荧光溶液（fluorescent solution）扫描

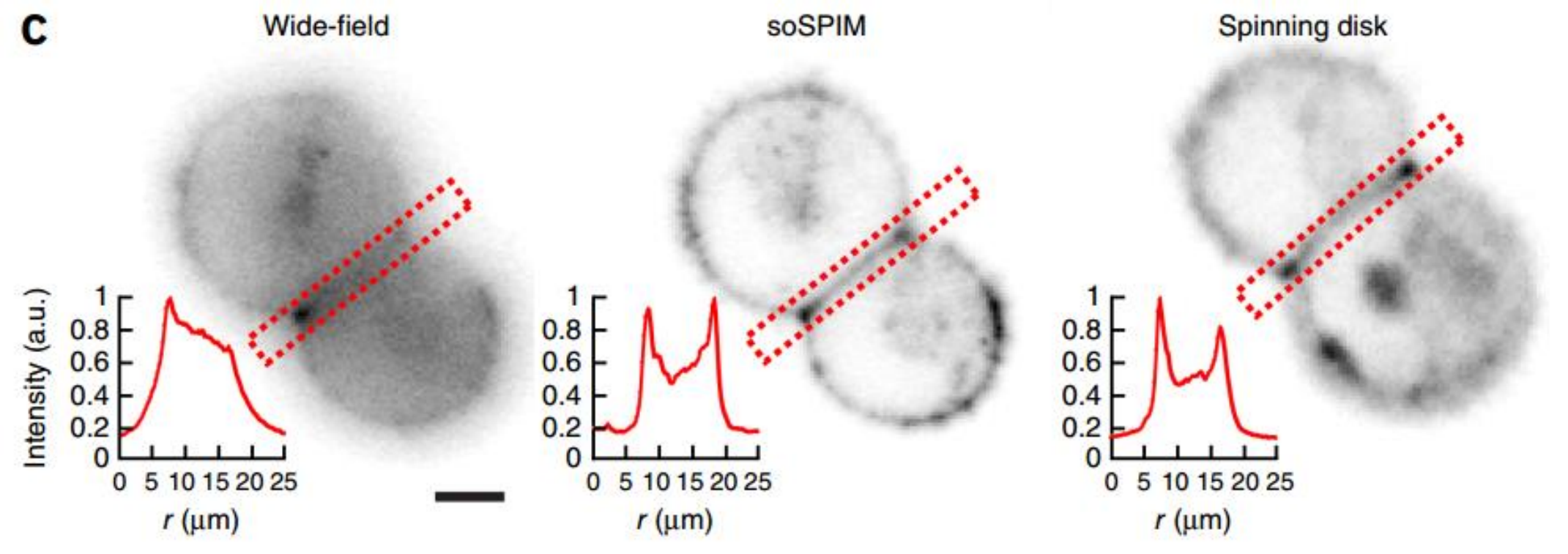
光片特性



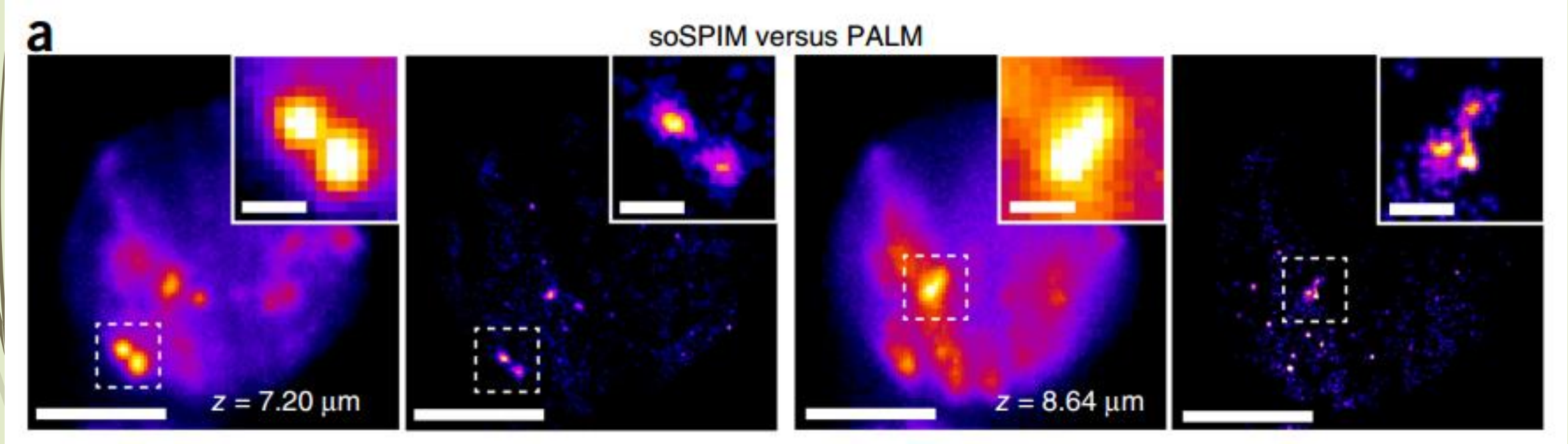
方法:

荧光溶液（fluorescent solution）扫描

切片能力


C
Wide-field
soSPIM
Spinning disk
Intensity (a.u.)
1
0.8
0.6
0.4
0.2
0
0 5 10 15 20 25
r (μm)

结合超分辨显微成像



从左侧起，第一幅第三幅图像为soSPIM采集所得图像，第二幅第四幅图像为soSPIM结合PALM超分辨显微技术所得图像
理论分辨率：40nm

其他信息

下表给出了6种显微镜形成光片的厚度以及瑞利长度（保持一定厚度的光片长度），其中第一行e为光片束腰处的光片厚度

Method		IML-SPIM	Bessel-Beam	iSPIM	RSLM	LSBM	Lattice light-sheet
Light-sheet	e (μm)	1.8 to 4	0.5 to 1	1.2	1	1.8	1
	ω (μm)	41.7 to 206	40	18.5	11	14	50

特点

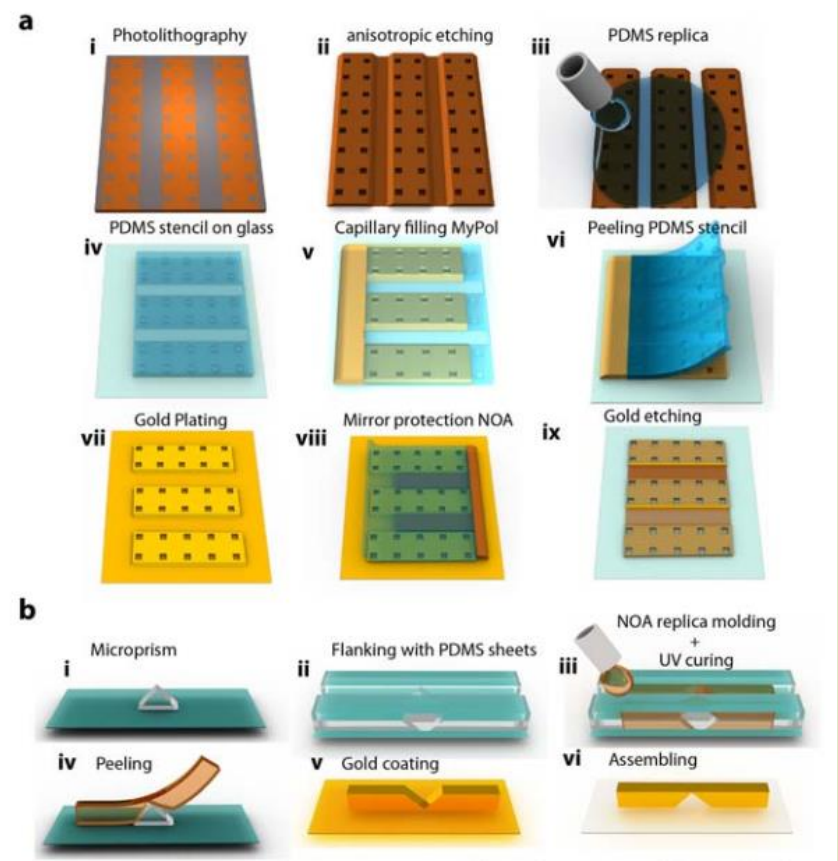
- 以倒置型显微镜为模板
- 可以使用标准的生物样本
- (optional)结合了超分辨显微技术（PALM，STORM, etc.)

优势

- 照明物镜与探测物镜均可以使用高数值孔径物镜

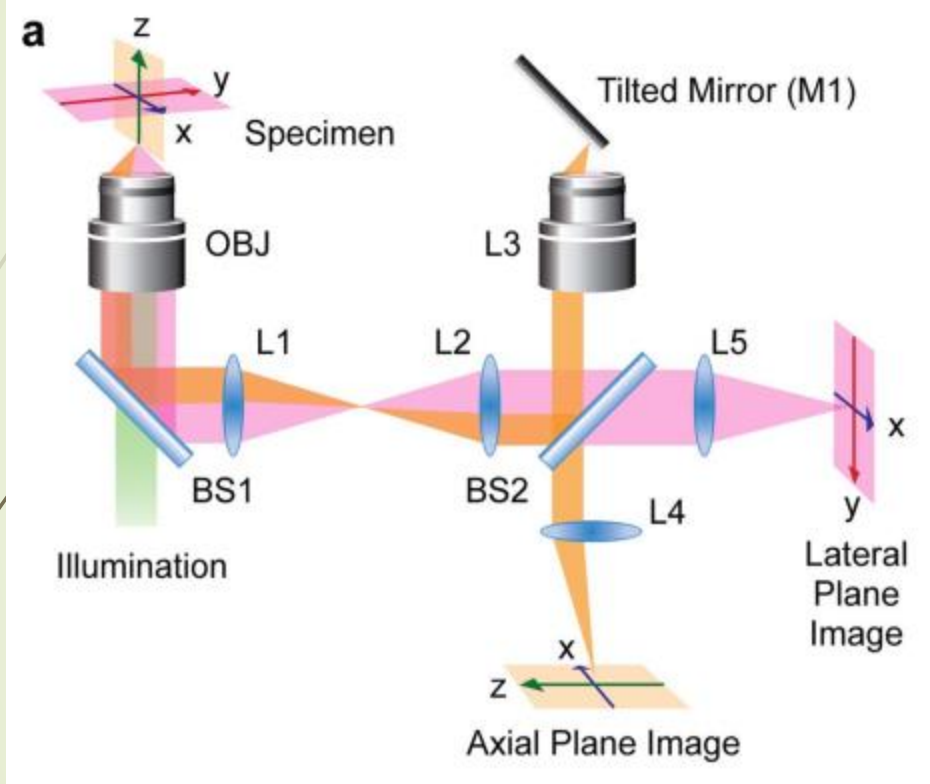
不足

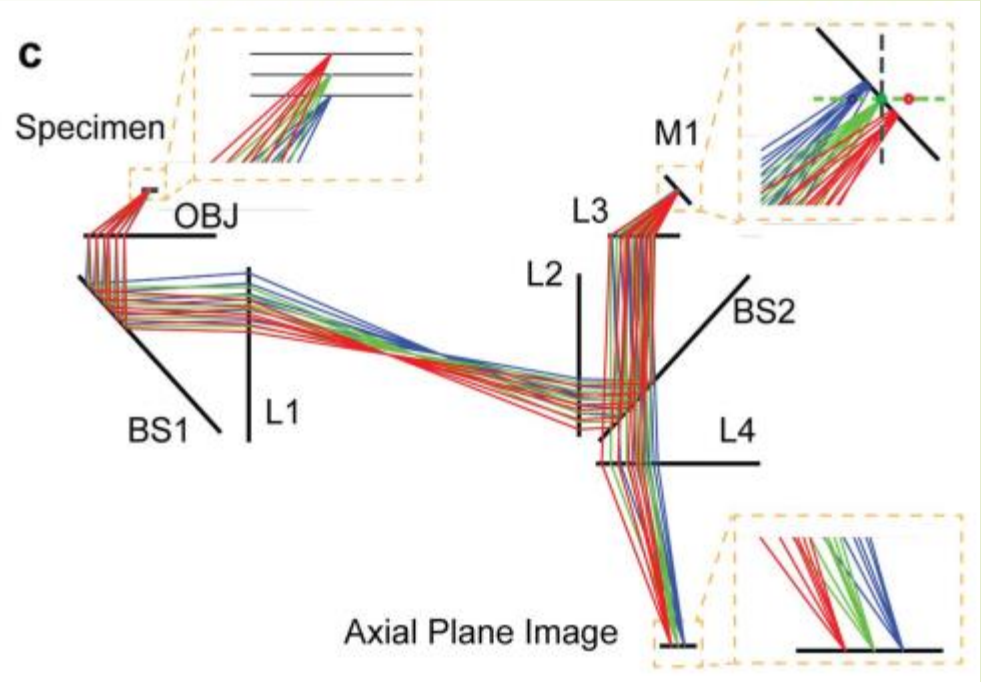
- 成像质量受反射镜表面粗糙度影响
- 反射镜制造过程复杂（如图）



3. Axial Plane Optical Microscopy (APOM)

装置图


a
z
y
x
Specimen
Tilted Mirror (M1)
OBJ
L3
L1
L2
L5
x
BS1
BS2
L4
y
Illumination
Lateral
Plane
Image
x
z
Axial Plane Image


c
Specimen
M1
OBJ
L3
L2
BS2
BS1
L1
L4
Axial Plane Image

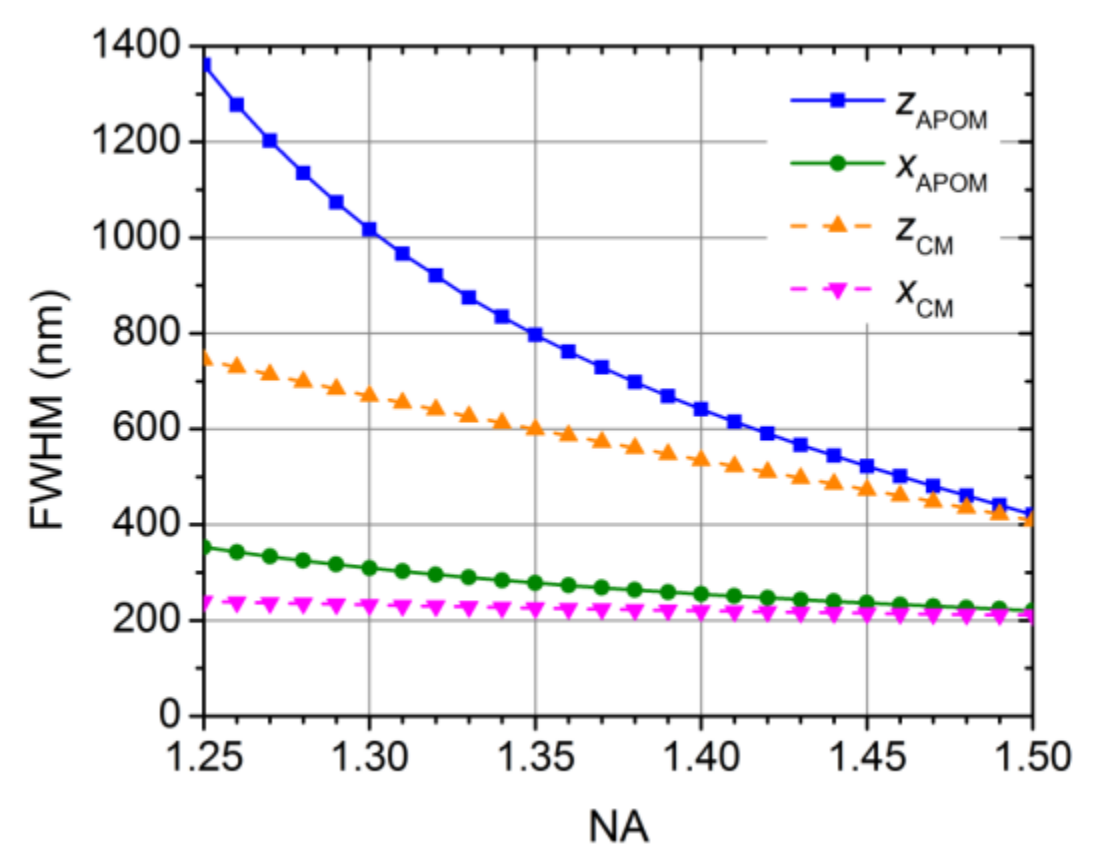
装置图

仿真图

装置

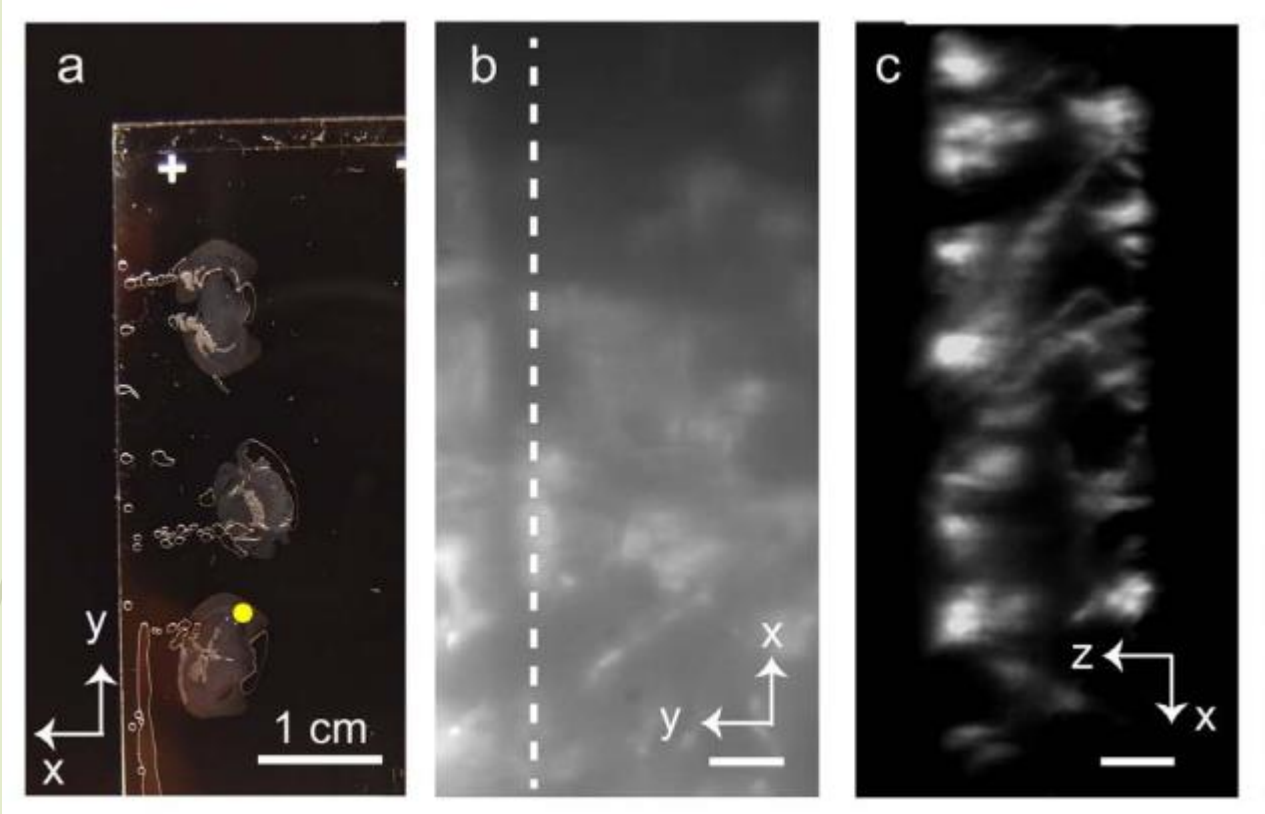
- 光源：532 nm laser
- 扩束： f=15 mm and f=200 mm
- 照明物镜（探测物镜）： Zeiss PlanApochromat 1003/NA 1.4 oil immersion objective lenses
- EMCCD：Luca R EMCCD , Andor

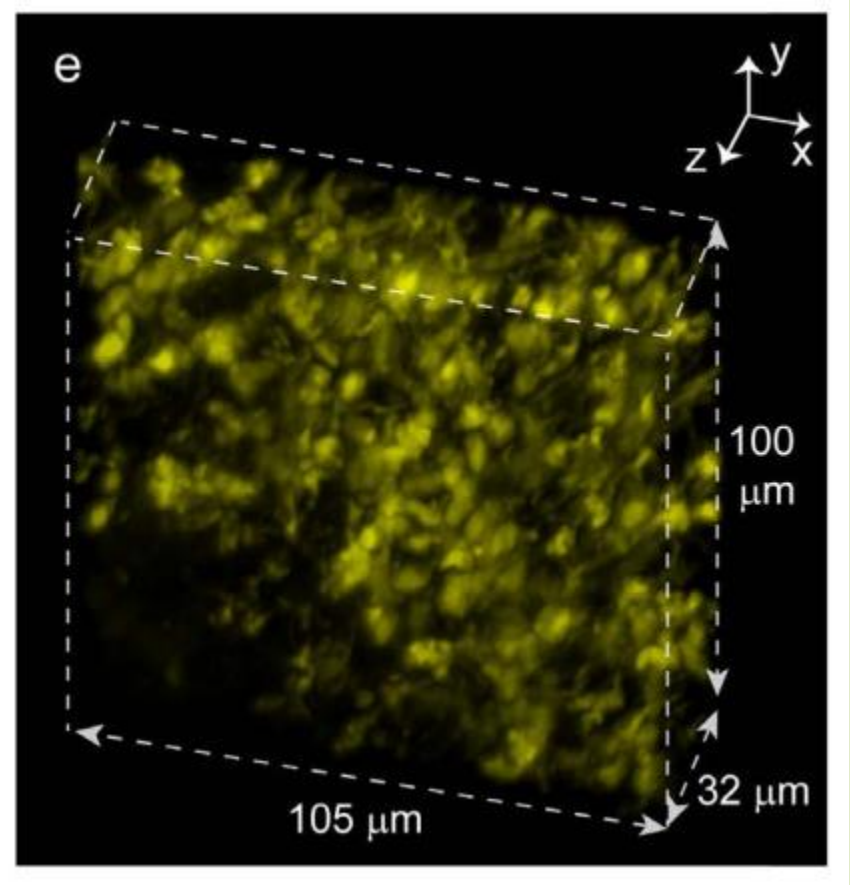
性能参数



APOM与普通的宽场显微镜的光片在束腰处的半高全宽（FWHM）与物镜的数值孔径的函数关系。

性能参数

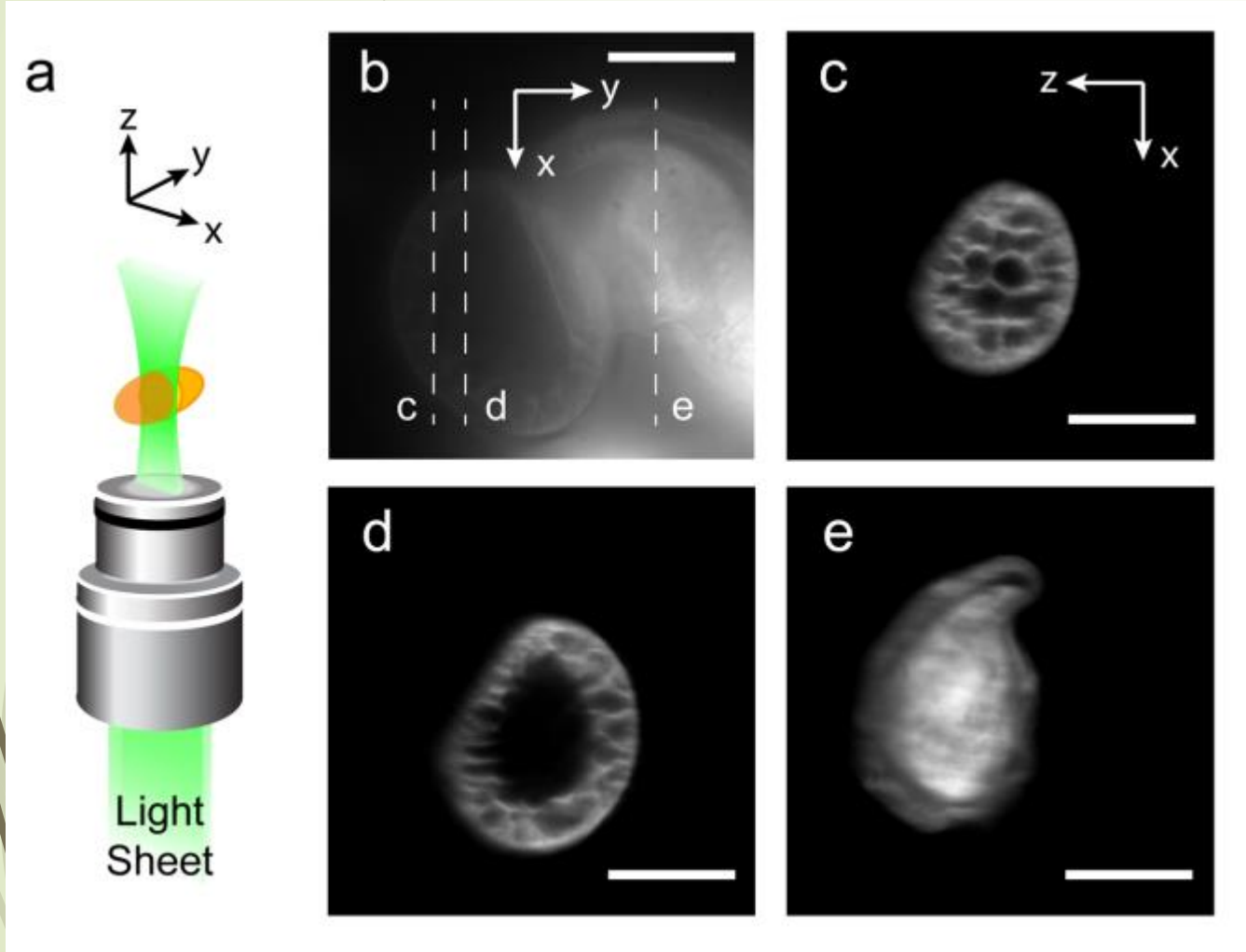




a,b: lateral; c: axial

3D

性能参数



b: lateral image by wide-field microscopy,
c, d, e: axial plane image in b

其他信息

下表给出了6种显微镜形成光片的厚度以及瑞利长度（保持一定厚度的光片长度），其中第一行e为光片束腰处的光片厚度

Method		IML-SPIM	Bessel-Beam	iSPIM	RSLM	LSBM	Lattice light-sheet
Light-sheet	e (μm)	1.8 to 4	0.5 to 1	1.2	1	1.8	1
	ω (μm)	41.7 to 206	40	18.5	11	14	50

特点

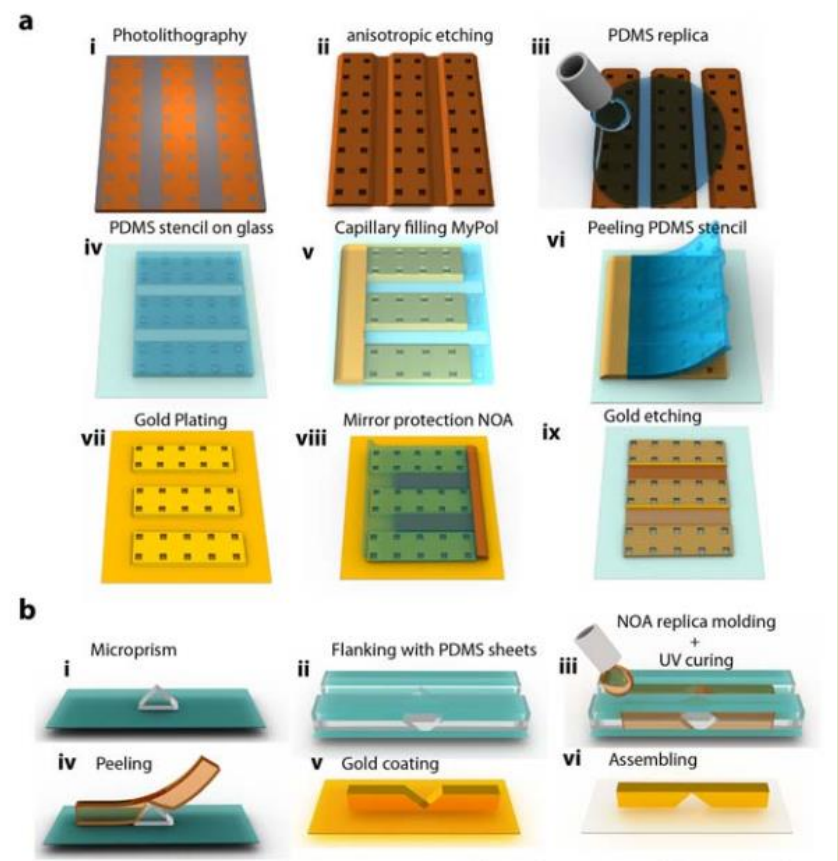
- 可以直接得到轴向图像

优势

- 照明物镜与探测物镜均可以使用高数值孔径物镜

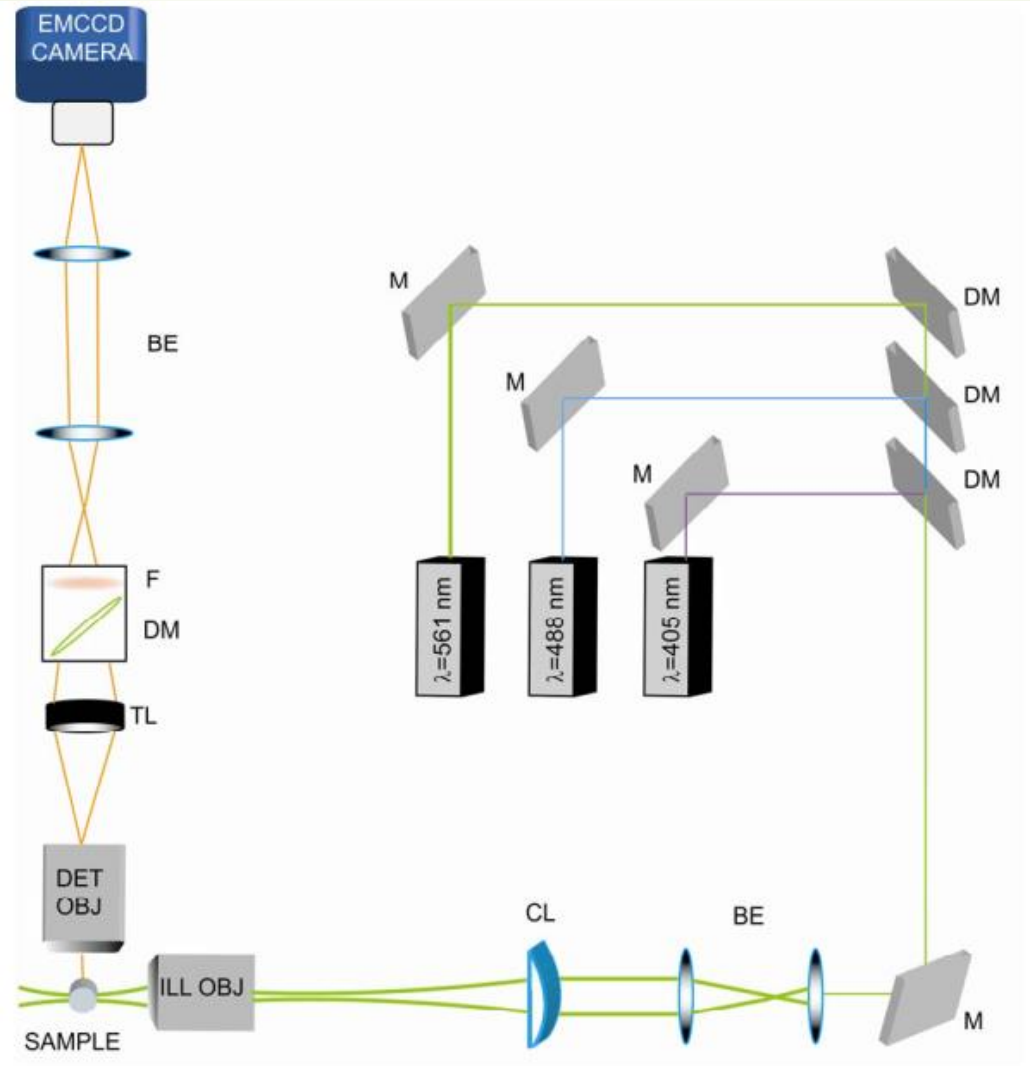
不足

- 成像质量受反射镜表面粗糙度影响
- 反射镜制造过程复杂（如图）

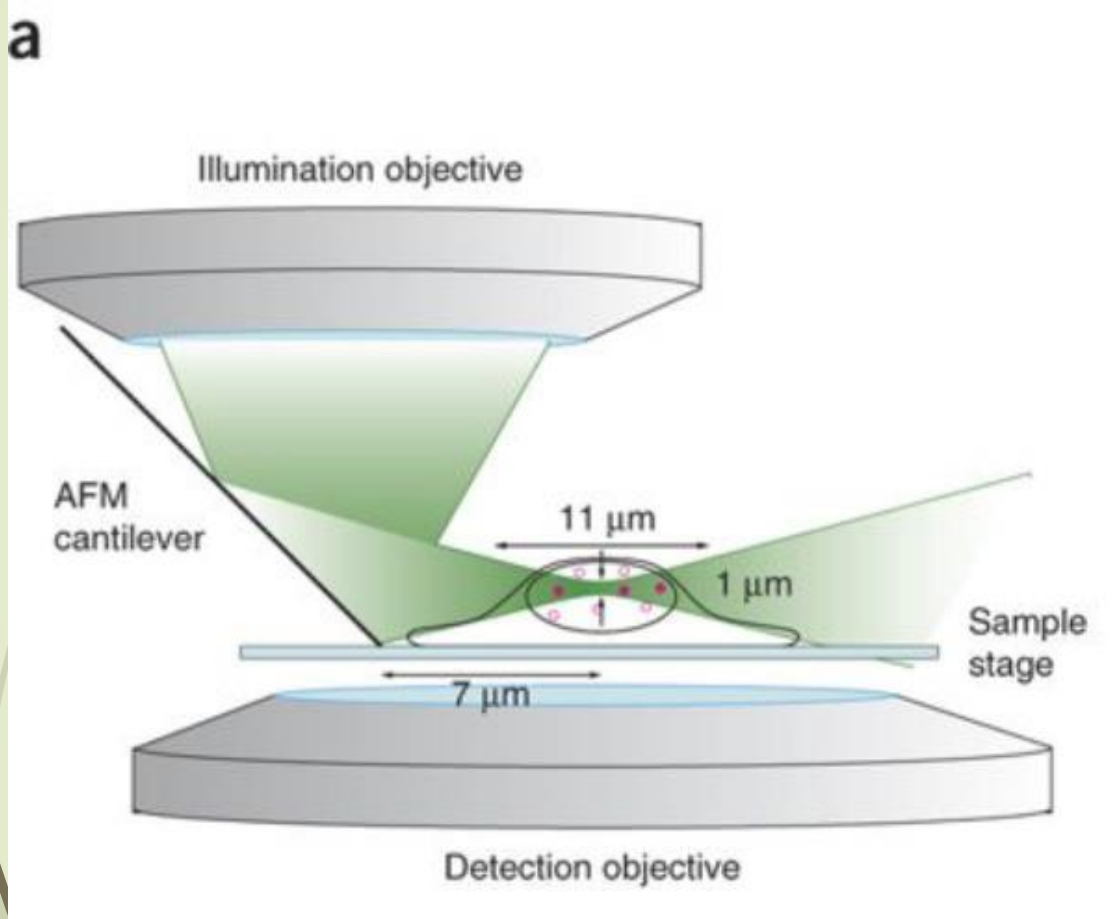


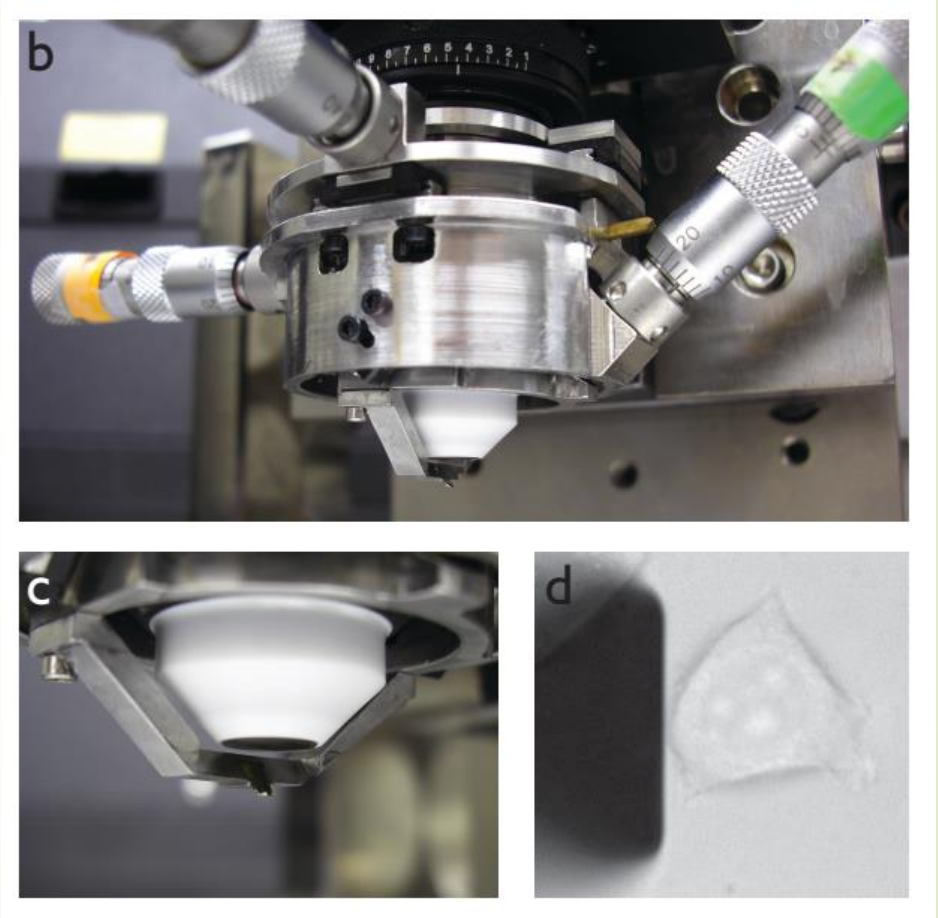
4. Individual Molecule Localization-Selective Plane Illmination Microscope (IML-SPIM)

装置图


EMCCD CAMERA
BE
F
DM
TL
DET OBJ
ILL OBJ
SAMPLE
CL
BE
M
M
M
M
DM
DM
DM
λ=561 nm
λ=488 nm
λ=405 nm

装置图





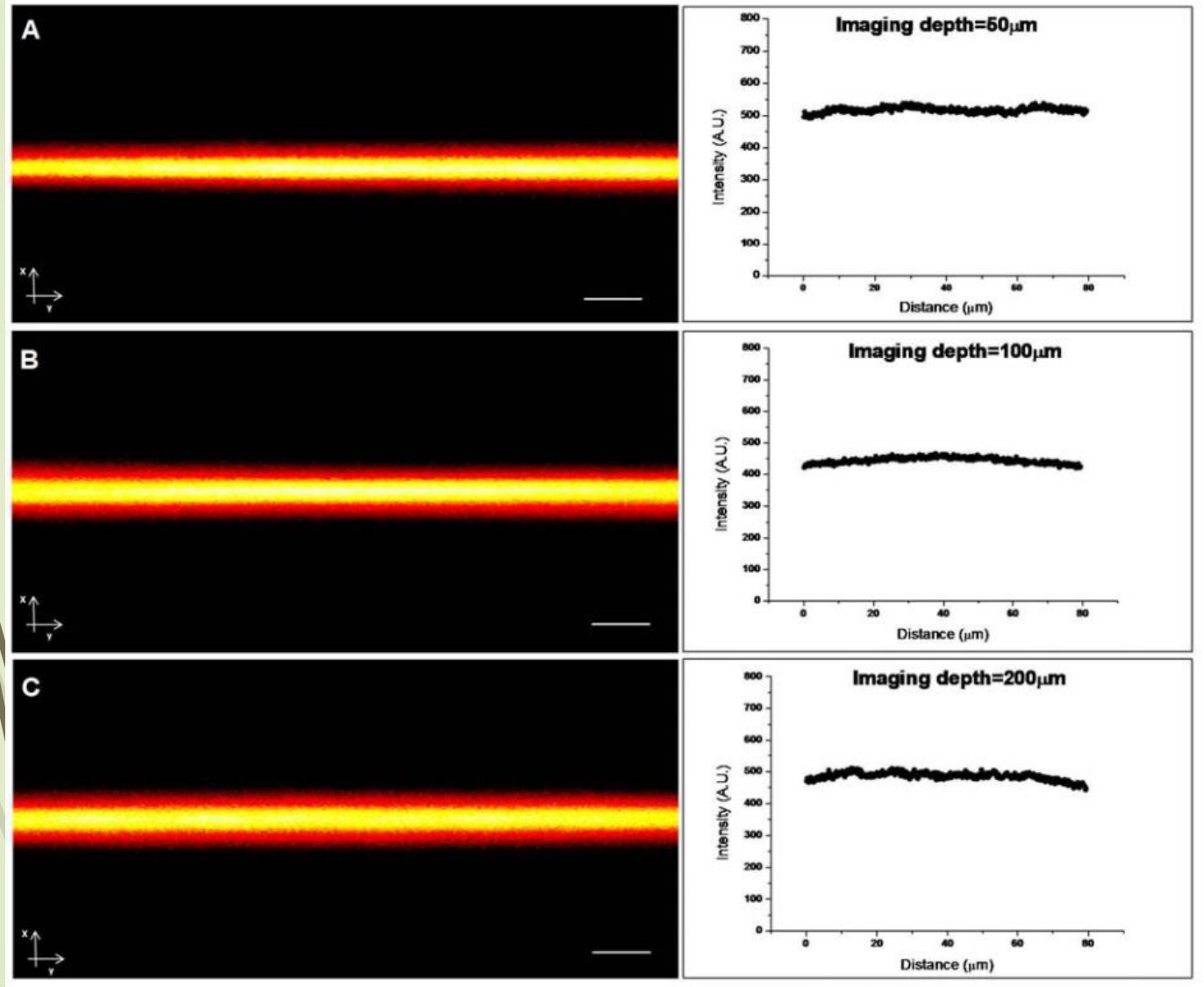
样品处光束图

AFM Cantilever

装置

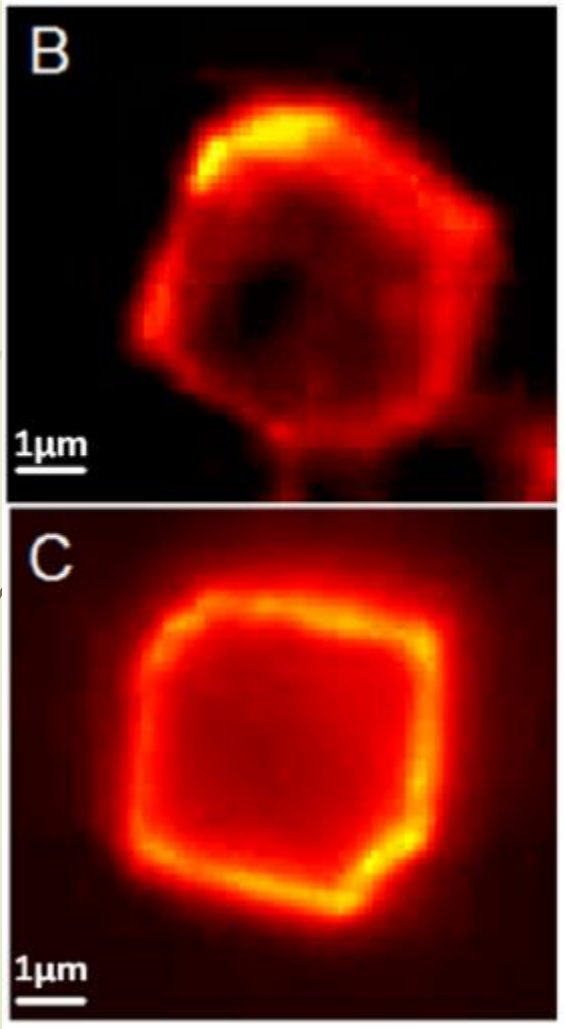
- 模板：普通SPIM
- 光源：Coherent Cube 405 nm–100 mW, Coherent Sapphire OPSL 488 nm, 200 mW, Coherent Sapphire OPSL 561 nm, 200 mW
- 扩束：Thorlabs AC254–030–A–ML, focal length *f*1 = 30 mm and Thorlabs AC254–060ML, *f*2 = 60 mm
- 光片形成：Thorlabs LJ1653L1–A *f* = 200 mm
- 照明物镜：Nikon Plan, 10 × , NA 0.3
- 探测物镜：HCX APO L U–V–I 40 × , NA 0.8 or CFI Plan 100 × W, NA 1.1
- 探测光路：Thorlabs AC254200–A–ML
- EMCCD: Andor Ixon DU–897E–CS0BV
- 扫描：（移动样品）Physik Instrumente PI M-105.10 （x, y）, Physik Instrumente PI M–110.1DG (z)

光片特性



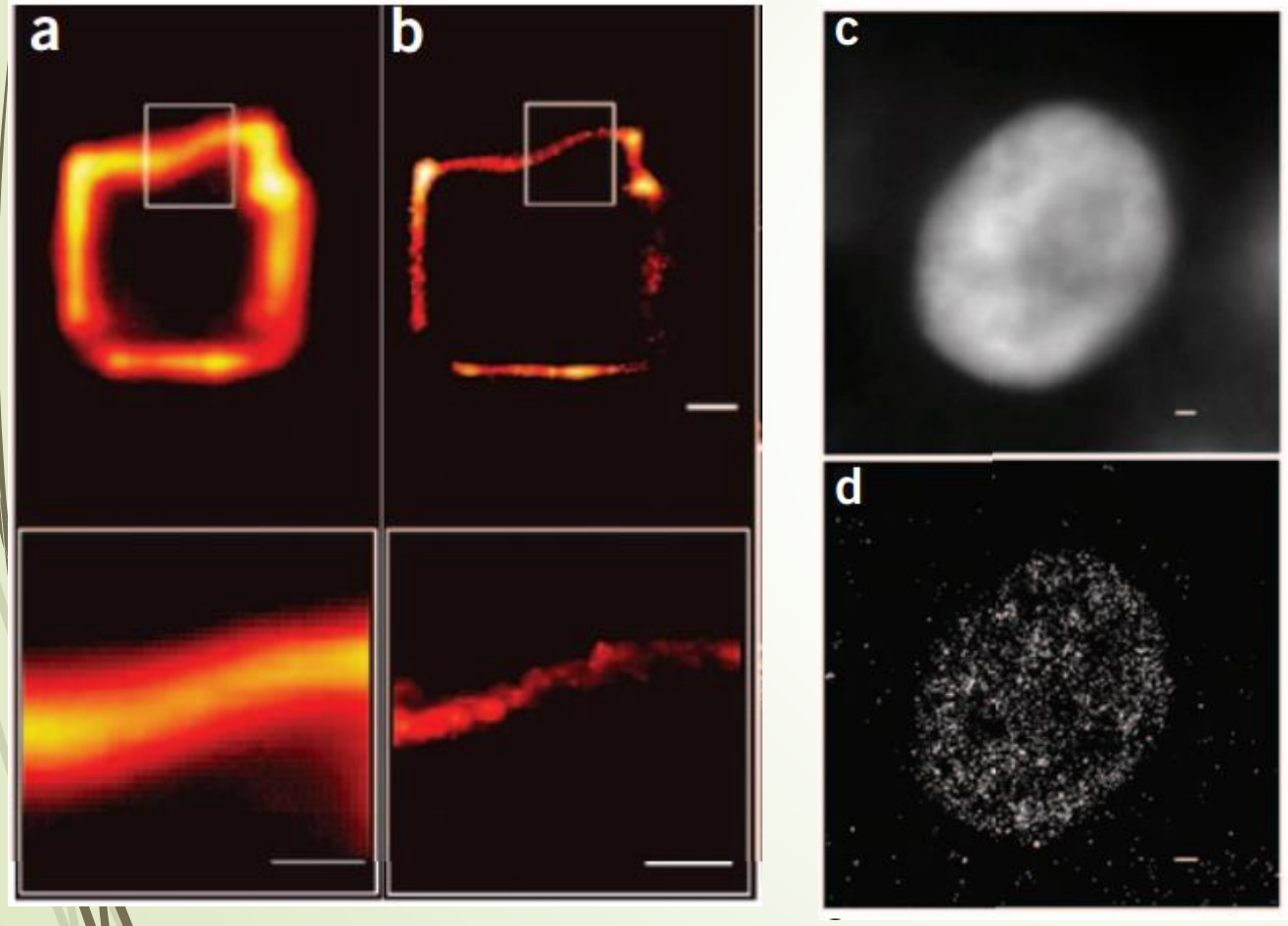
因为结合了超分辨显微技术，顾对于光片厚度没有很高的要求，这里仅对光强度进行了分析。
证明了光片在整个视场中没有明显的衰减

信噪比提高



b: SPIM c: wide-field
488 nm (0.9 kW/cm2) and same exposure time (100ms)

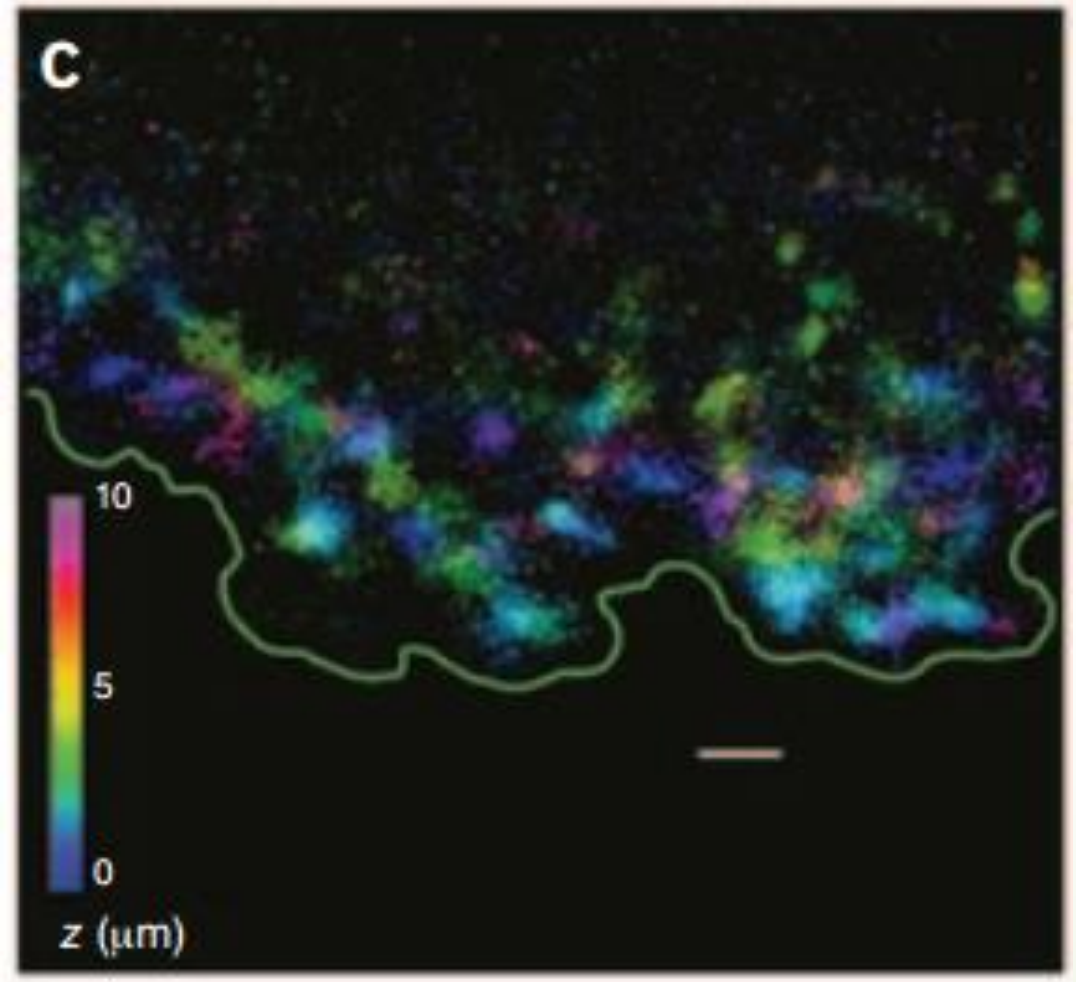
结果图



a, c: 普通的SPIM显微镜，
b, d: 结合单分子定位技术之后，IML-SPIM是通过分析10000帧图像后得出的（33帧/秒）
比例尺：1微米

实现了横向分辨率小于35nm，轴向分辨率65nm-1

结果图



IML-SPIM得到的3D重建图像，其中颜色代表深度，比例尺：1微米

特点

- 将SPIM技术与远场单分子定位技术相结合

5. πSPIM

装置图

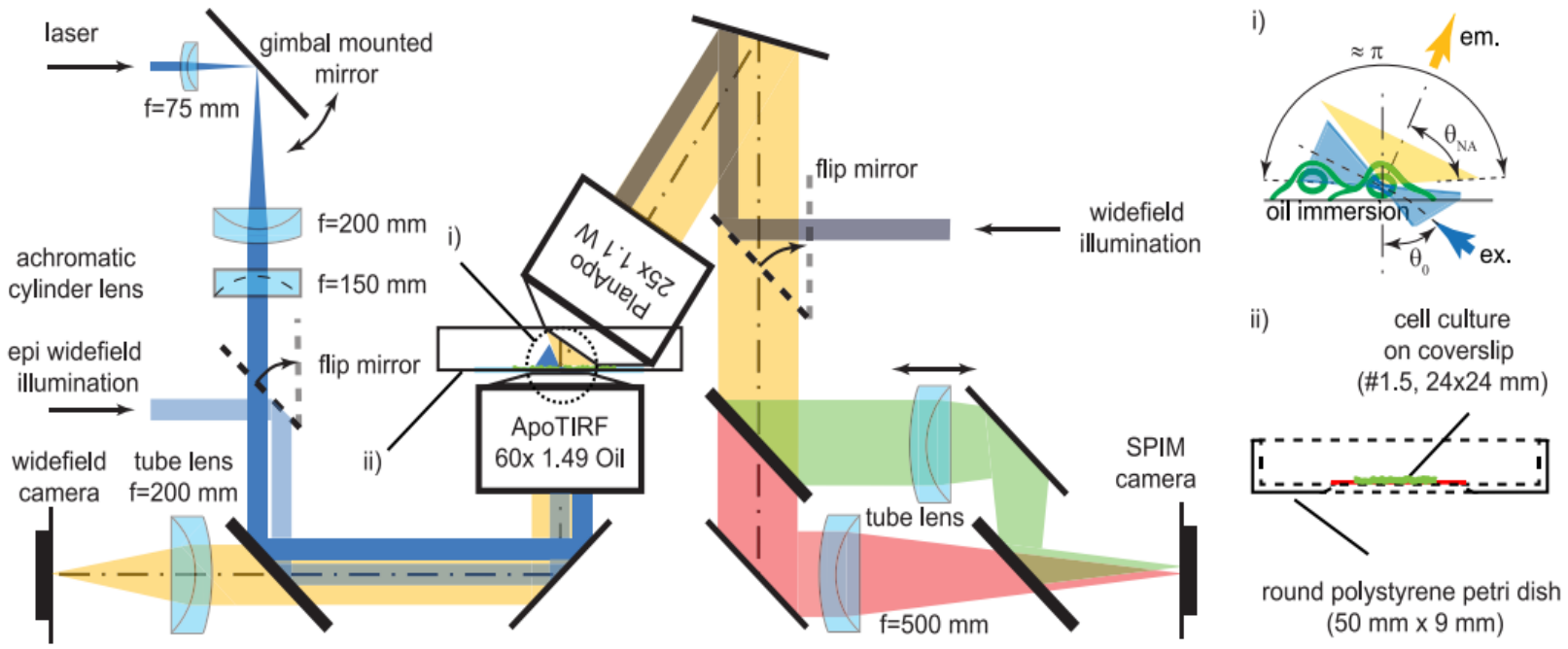
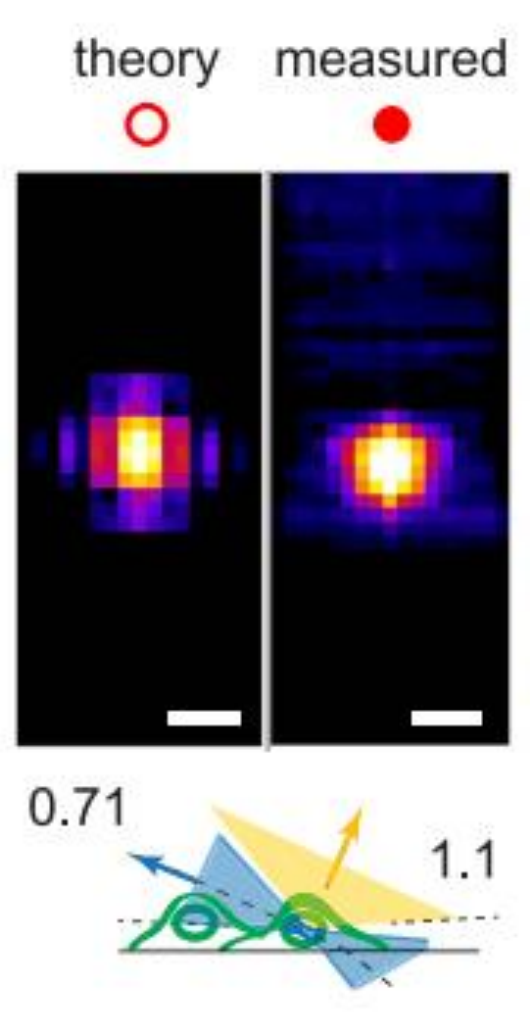


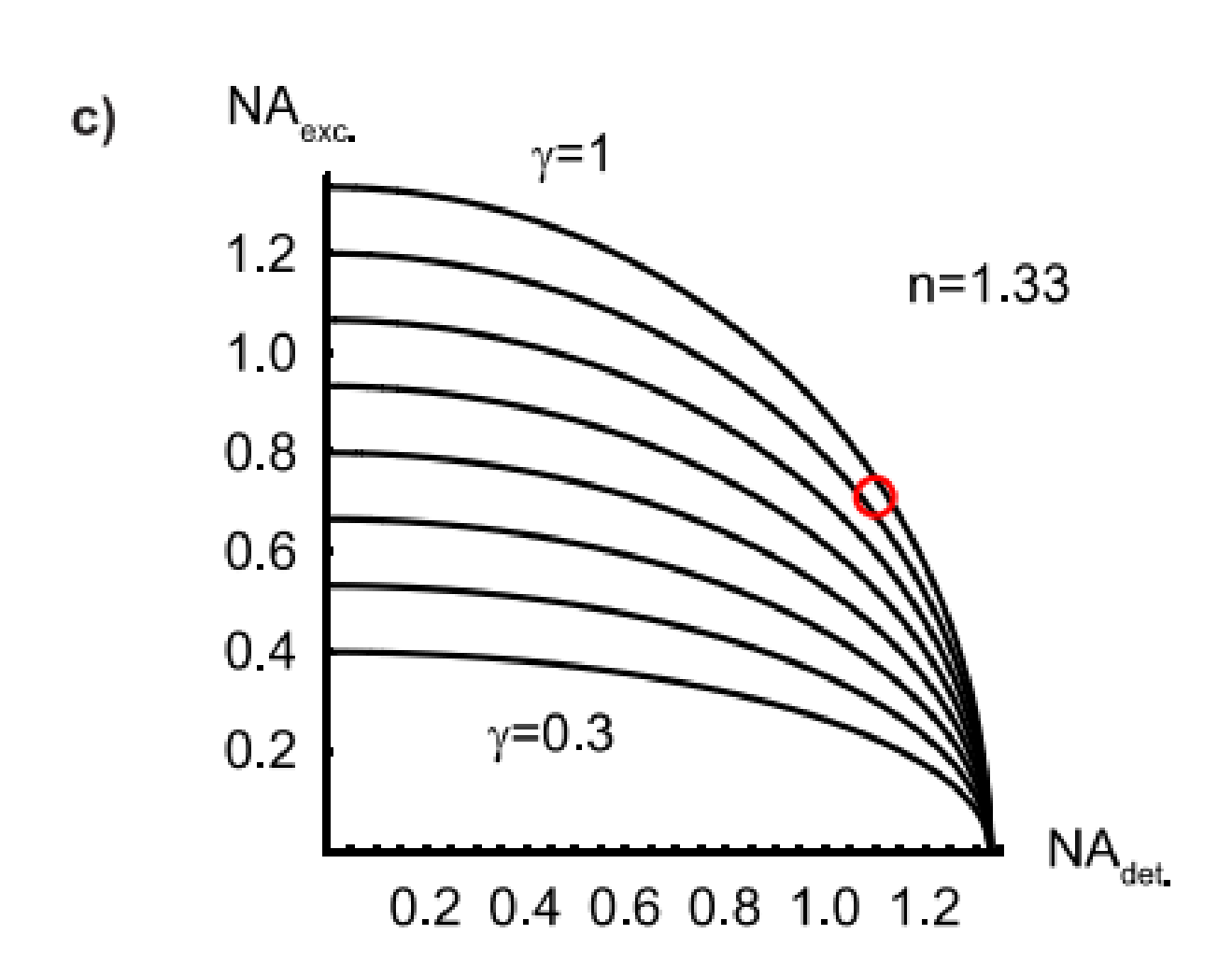
Figure 1. πSPIM set-up. Schematic dual-color πSPIM set-up with an oblique light-sheet produced by off-center passage of the beam through the illumination lens (1.49 ApoTIRF 60×), and the detection lens (1.1 W, 25×) arranged orthogonally to the oblique light-sheet. (i) Close-up of the focal region showing the angular range of the complementing illumination and emission cones. (ii) Mid-plane cross-section of the glass-bottom dish used for sample mounting.

装置

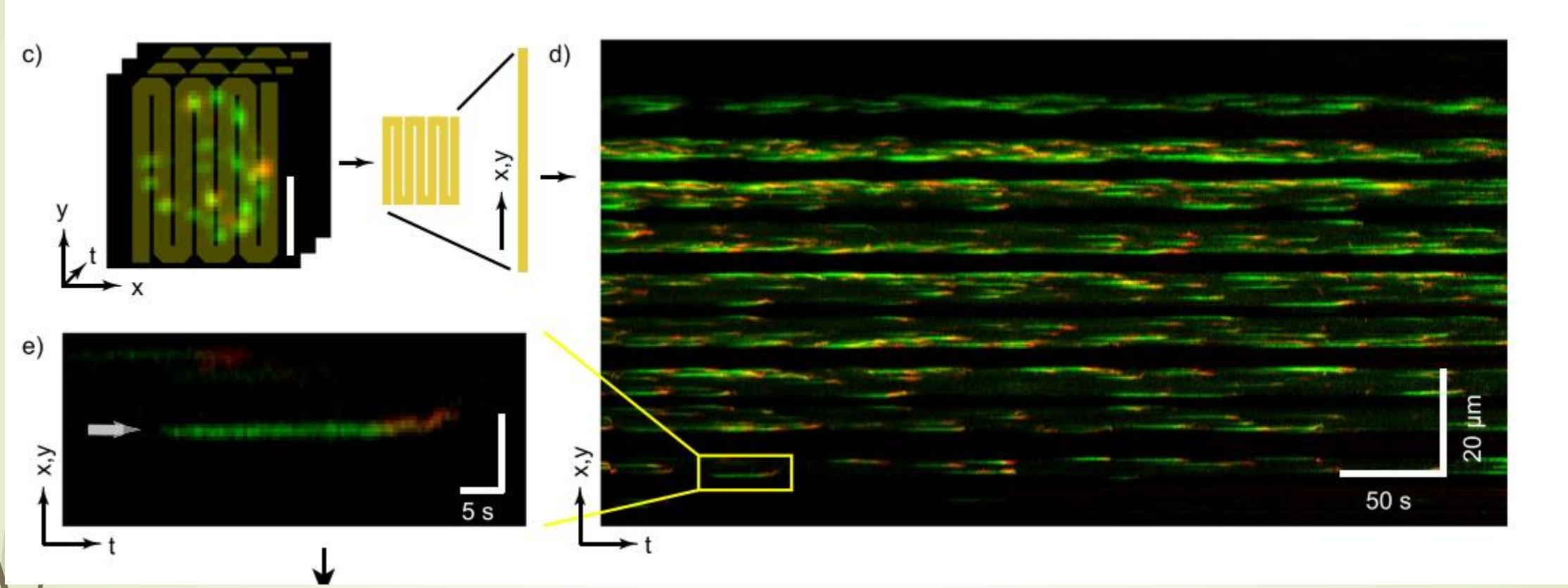
- 光源：Mambo 594 nm 25 mW and MLD 488 nm 60 mW, both Cobolt
- combiner dichroic (LM01-503-25, Semrock),用来耦合激光
- 扩束：AC254-075-A-ML f = 75 mm, Torlabs and MXA20696 f = 200 mm, Nikon)
- 光片形成：ACY254-150-A, f= 150 mm, Torlabs
- 照明物镜：ApoTIRF 60 × 1.49 NA, Nikon or PlanApo 100 × 1.27 NA
- 探测物镜：（water dipping） HCX APO L U–V–I 40 × , NA 0.8 or CFI Plan 100 × W, NA 1.1, with correction ring for spherical aberration
- 探测光路：MXA20696, f= 200 mm, Nikon
- CMOS: GS3-U3-23S6M-C, Point Grey Research Inc
- 扫描：样品架由沿x-y方向的两个微米平移台驱动 (Physik Instrumente PI M-105.10) (Physik Instrumente PI M–110.1DG) (Physik Instrumente PI M–116.DG)

性能参数


theory
measured
0.71
1.1


c)
NA exc.
γ=1
n=1.33
γ=0.3
1.2
1.0
0.8
0.6
0.4
0.2
0.2 0.4 0.6 0.8 1.0 1.2
NA det.

结果图



特点

- 以倒置型显微镜为模板

其他信息

Mode	Objective(s)	NA	Resolution (FMWH)/nm		Anisotropy	Focal volume/fl
type			*x,y (theory)*	*z (theory)*	*(theory)*	*(theory)*
widefield	PlanApoW25×	1.1	274 (240)	785 (801)	0.35 (0.30)	0.072 (0.057)
πSPIM	PlanApoW25× & ApoTIRF60×	1.1/1.49*	284 (212)	339 (285)	0.84 (0.74)	0.057 (0.023)
widefield	PlanApoW60×	1.27	250 (203)	648 (510)	0.39 (0.40)	0.052 (0.027)

其他信息

Technique	Resolution (FMWH)/nm		Reference
	x, y	*z*	
4π microscopy	280	190	Bahlman *et al.*[23]
LSM	435[1]	482[1]	Capoulade *et al.*[11]
LSM (Bessel 2PE)	—	490	Planchon *et al.*[24]
Bessel plane SR-SIM	185/238	348	Gao *et al.*[12]
Lattice light-sheet	230[2]	370[2]	Chen *et al.*[13]
πSPIM	284	339	this work
widefield 1.27 NA	250	648	this work
widefield 1.49 NA (TIRF)	222	504	Theer *et al.*[14]

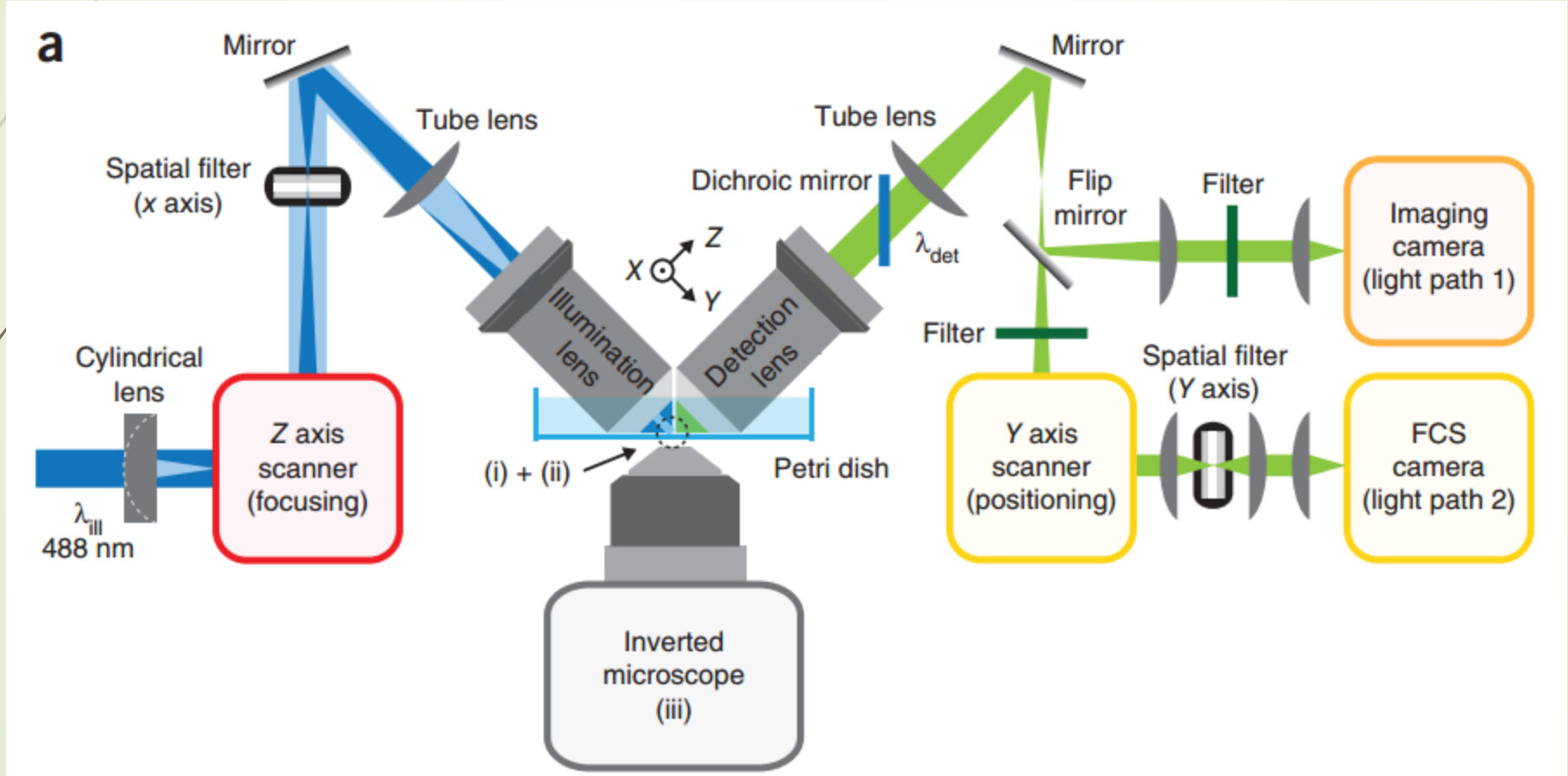
Table 2. Overview of previously published resolution performances. Abbreviations: LSM, light-sheet microscopy; 2PE, two-photon excitation; SIM, structured illumination microscopy; [1]calculated from given $1/e^2$ radii; [2]theoretical value.

6. SPIM combined with fluorescence correlation spectroscopy (FCS)

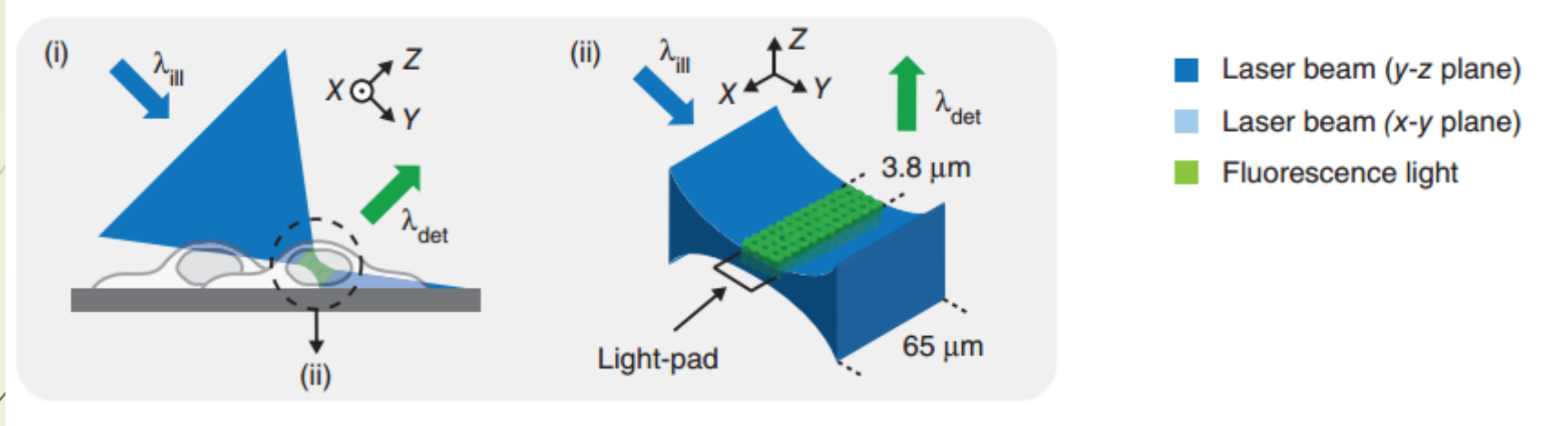
简单介绍

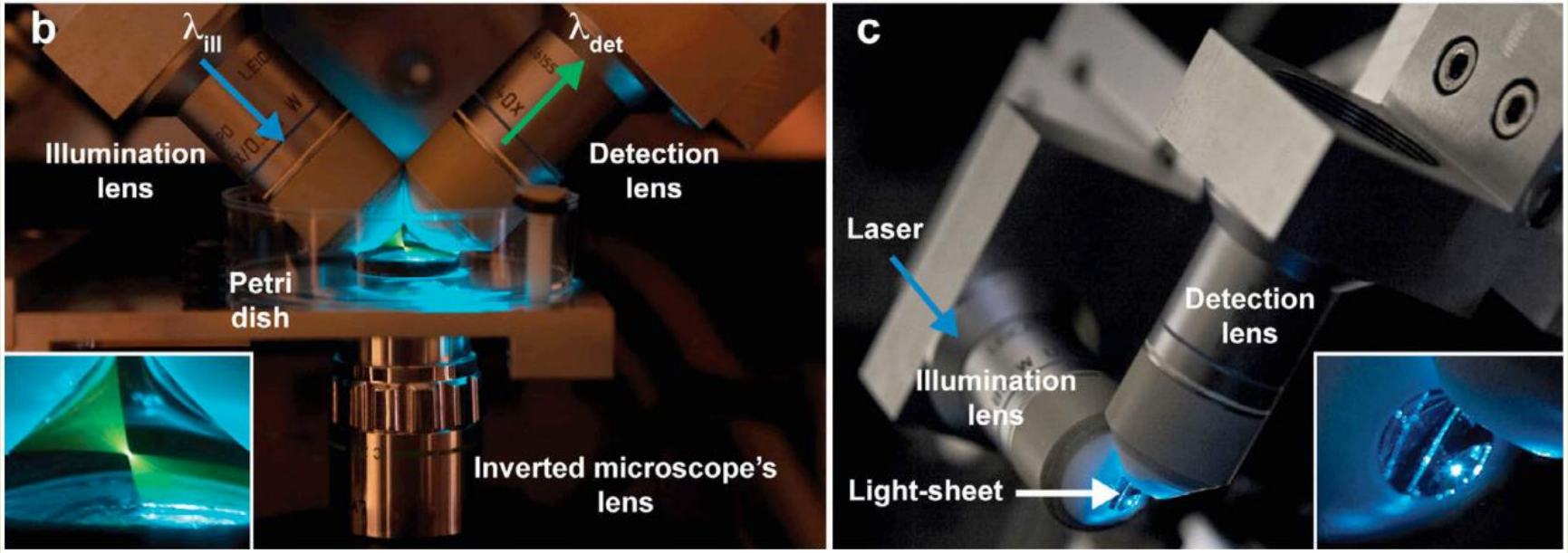
- FCS技术介绍：Fluorescence correlation spectroscopy (FCS) is an experimental technique using statistical analysis of the fluctuations of fluorescence in a system in order to decipher dynamic molecular events, such as diffusion or conformational fluctuations of biomolecules.
- FCS技术仅仅是出于特定目的的数字信号处理技术，在硬件光路部分和普通的SPIM基本一致。

装置图


a
Mirror
Tube lens
Spatial filter
(x axis)
Cylindrical
lens
λill
488 nm
Z axis
scanner
(focusing)
Illumination
lens
Detection
lens
X
Z
Y
(i) + (ii)
Petri dish
Inverted
microscope
(iii)
Dichroic mirror
λdet
Tube lens
Mirror
Flip
mirror
Filter
Imaging
camera
(light path 1)
Filter
Spatial filter
(Y axis)
Y axis
scanner
(positioning)
FCS
camera
(light path 2)

光片位置图


(i)
λill
X
Z
Y
λdet
(ii)
(ii)
λill
Z
X
Y
λdet
3.8 µm
Light-pad
65 µm
Laser beam (y-z plane)
Laser beam (x-y plane)
Fluorescence light


b
λill
λdet
Illumination lens
Detection lens
Petri dish
Inverted microscope's lens
c
Laser
Detection lens
Illumination lens
Light-sheet

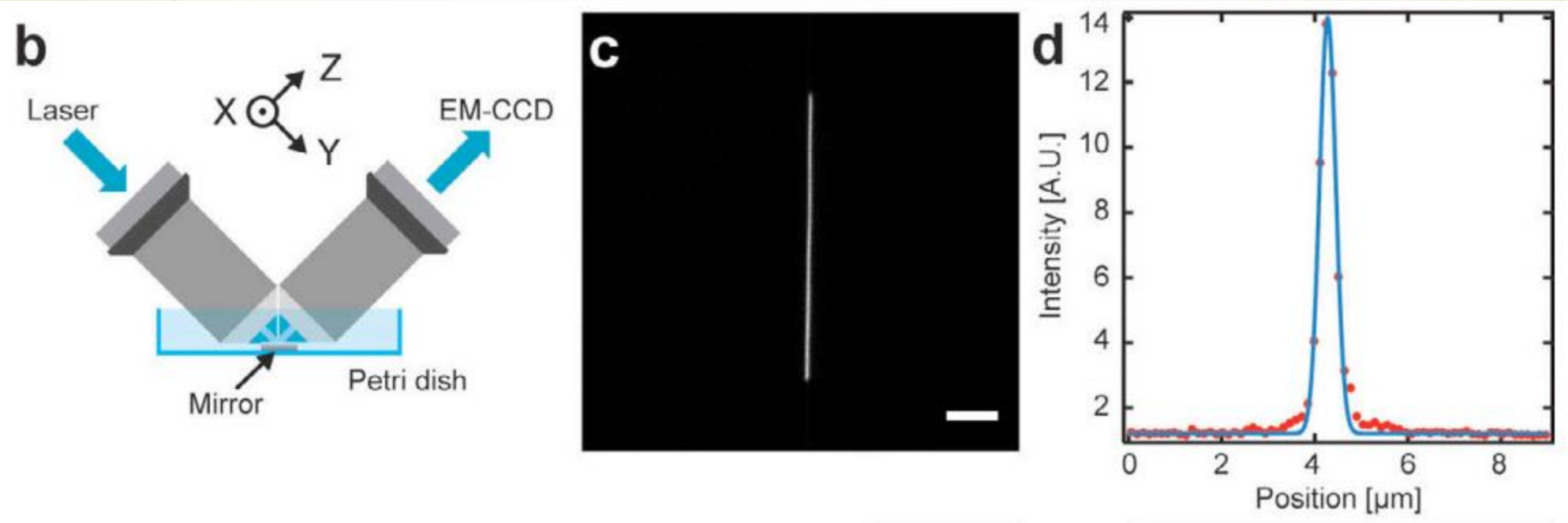
装置

- 光源：488 nm line of an argon laser (Innova Sabre, Coherent) , power: 2w
- 扩束： ?
- 光片形成： a cylindrical lens (*f* = 75.6 mm, Thorlabs)
- 照明物镜（探测物镜）：(water-dipped)Plan-Apochromat 40×/0.8 NA, Leica
- EMCCD/cMOS: QuantEM:512SC, Photometrics
- 扫描：three-axis motorized stage (stepper motors: LN-Mini23 manipulator block XY and LN-Mini Z vario, Cell Biology Trading/Luigs & Neumann)， 位移精度 50nm

软件控制

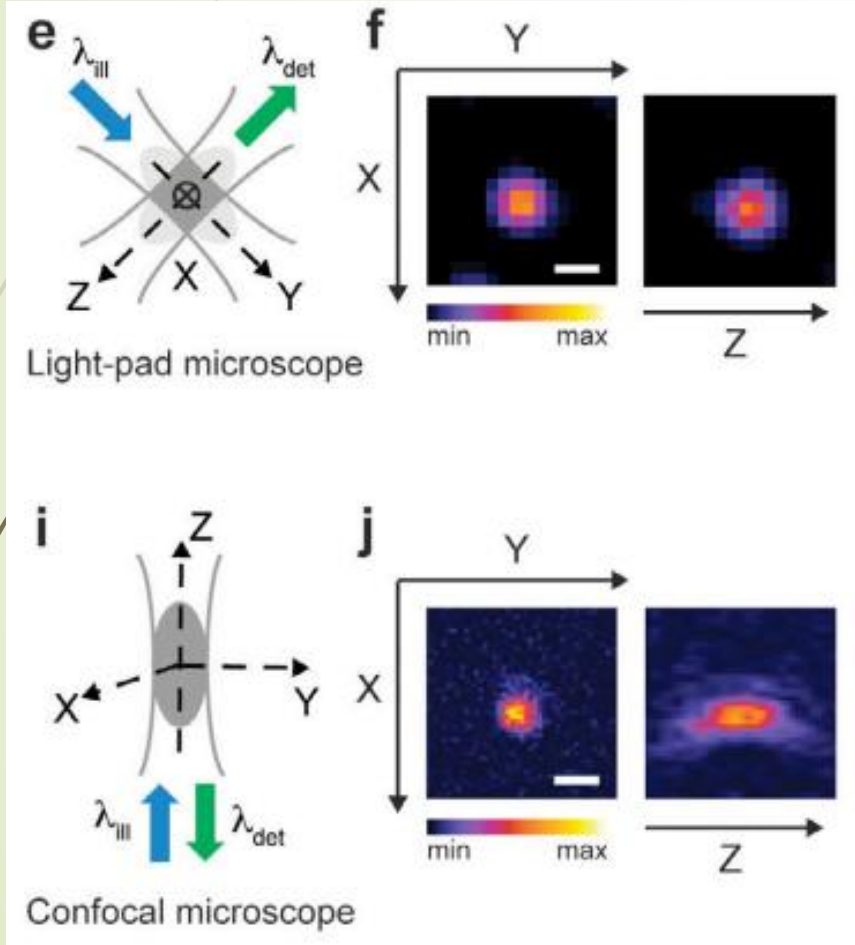
- LabView: 控制扫描
- Self-written C++: 存储图像

光片特性



测得的光片厚度：700nm(误差10nm)

成像特性

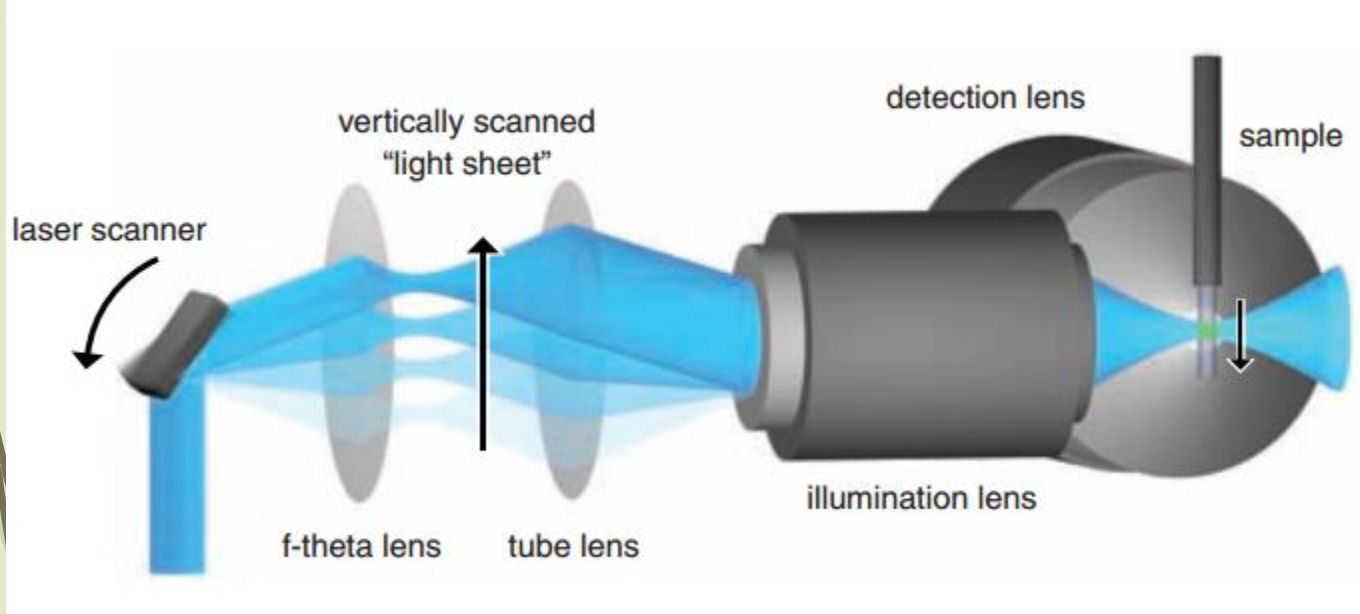


方法：
通过对一个直径20nm的荧光珠进行成像

点扩散函数性质：
Lateral: 370nm(误差20nm)
Axial: 410nm(误差40nm)

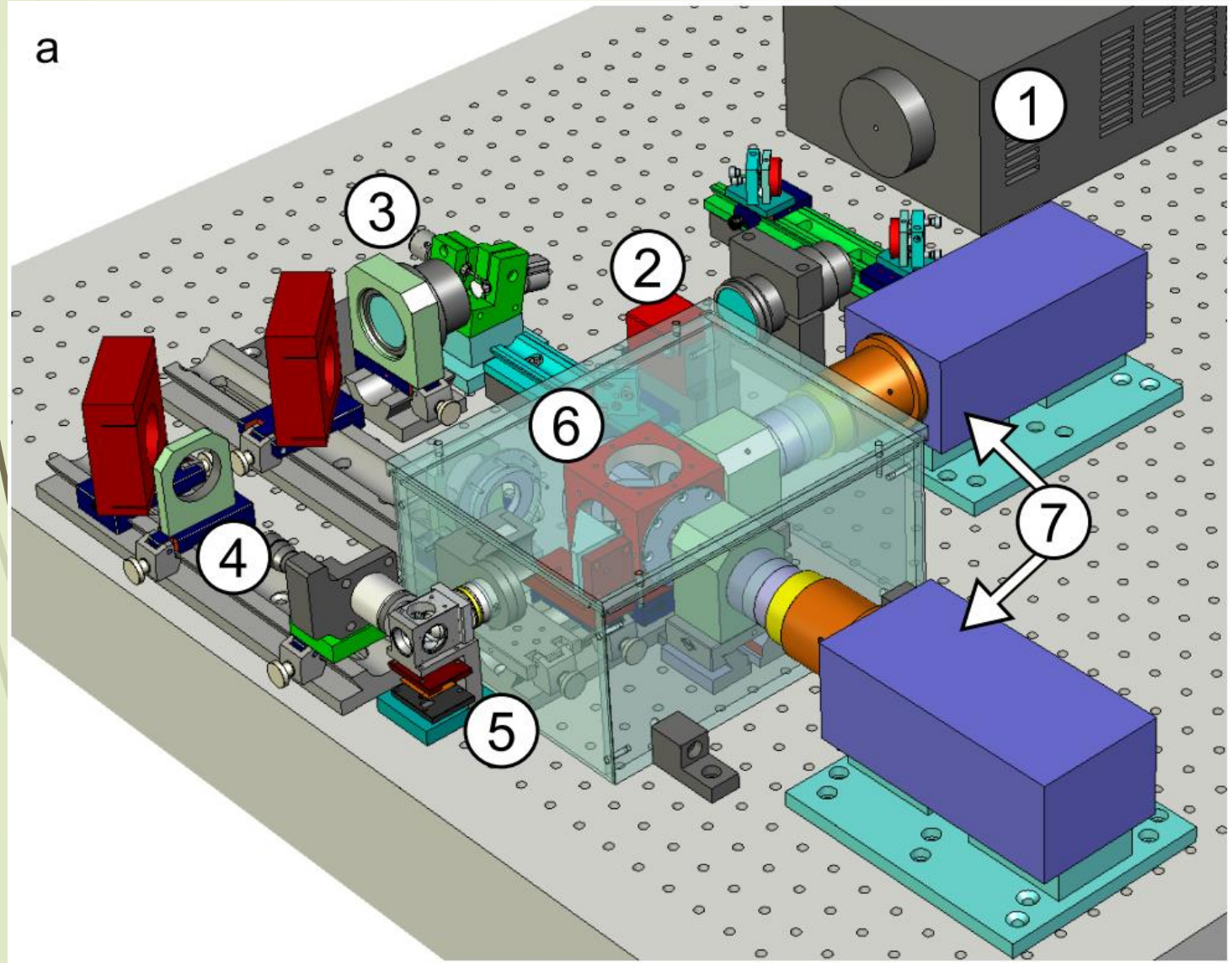
7. Digital scanned laser light sheet fluorescence microscopy(DSLM)

扫描原理



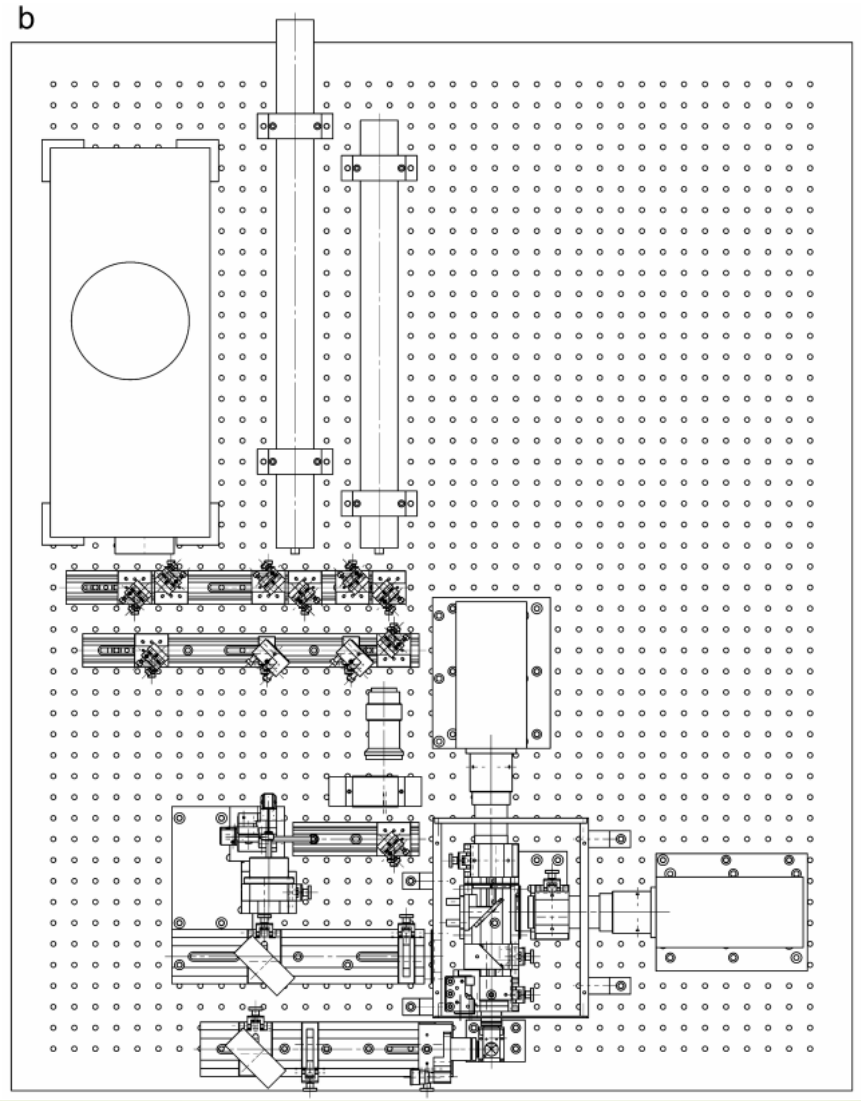
DSLM的原理是使用一束快速摆动的激发光形成一个虚拟的“光片”，通过恒定扫描速度的控制来实现均匀光片的产生。
f-theta透镜的作用是讲光束的倾斜摆动转化为光束在垂直方向的位移，从而实现扫描。

示意图



1. 光源 laser light source
2. 声光可调谐滤波器 acousto-optical tunable filter
3. 扫描装置（f-theta 透镜） the laser scanner
4. 照明光路 beam illumination arm
5. 样品温控装置 the temperature-controlled specimen chamber
6. 探测光路
7. 两个独立的相机

示意图



装置

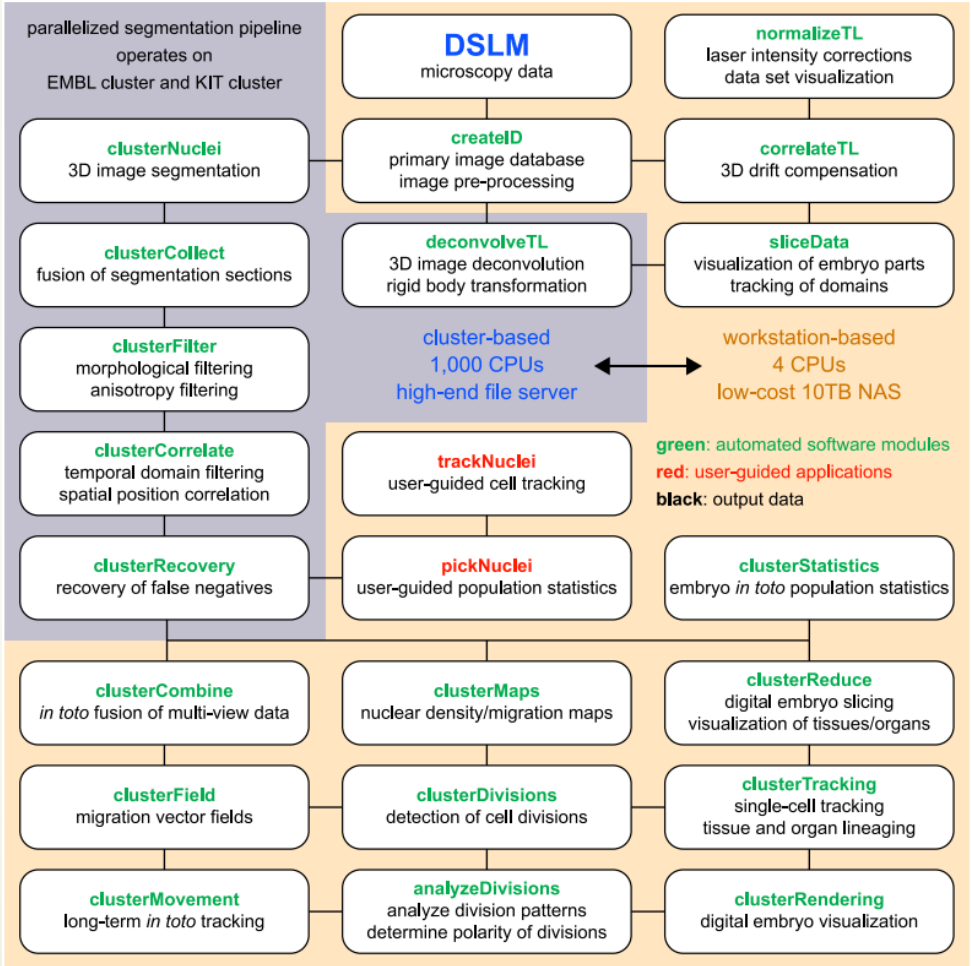
- 光源：多线氩氪激光器 Melles Griot, 35 LTL 835-230
- ATOF: AA Opto-Electronic, AA.AOTF.nC-400-650nm-PV-TN
- 扫描：一个双轴高速扫描头 GSI Lumonics, VM500+，f-theta透镜：Sill Optics, S4LFT0061/065*
- 照明物镜：Carl Zeiss, Plan-Apochromat 5x/0.16
- 探测物镜：（多种选择） Carl Zeiss: Plan-Neofluar 2.5x/0.075, Fluar 5x/0.25, C-Apochromat 10x/0.45 W, PlanApochromat 20x/1.0 W or Plan-Apochromat 63x/1.0 W
- 镜头对焦：a piezo nanofocus (Physik Instrumente, P-725.CLQ)
- CCD: the pco.2000 CCD camera from PCO AG
- a specimen positioningsystem: three linear translation stages (Physik Instrumente, M-111K028) and one micro-rotation stage (Physik Instrumente, M-116.DG)

软件控制

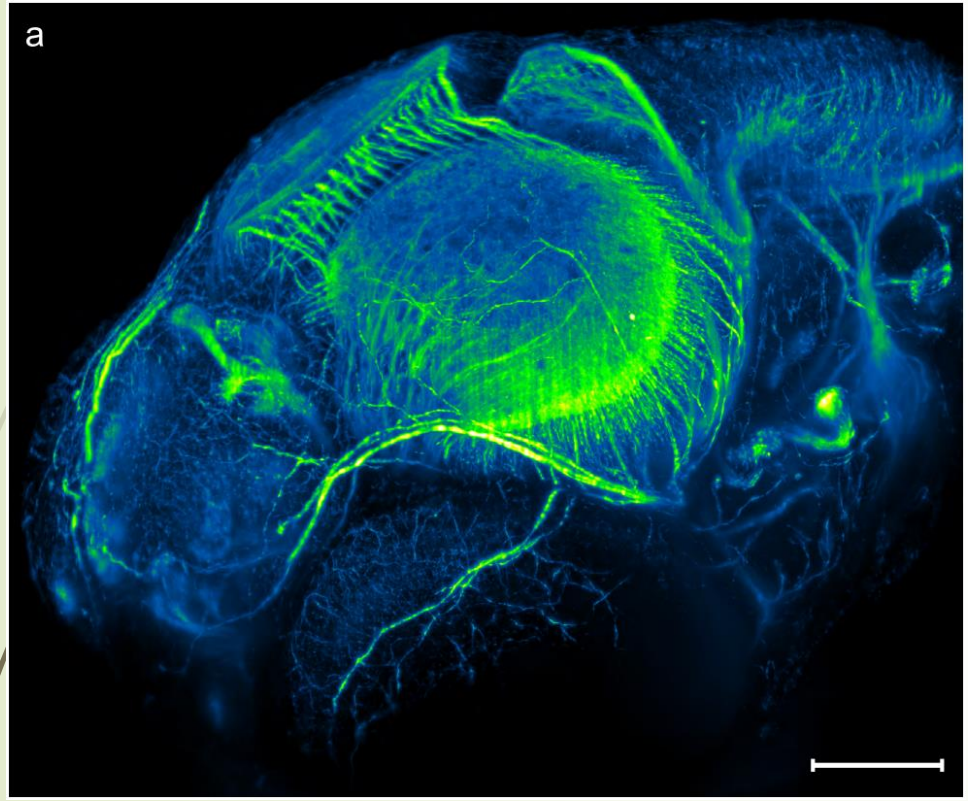
- 系统环境：.NET framework 3.0
- C#: 用户界面，高级控制层
- C++: 低层硬件通信
- Matlab: 图像处理（分为图像分割、数据分析）

图像处理过程

Keller *et al.* (2008); supplementary figure 4



结果图

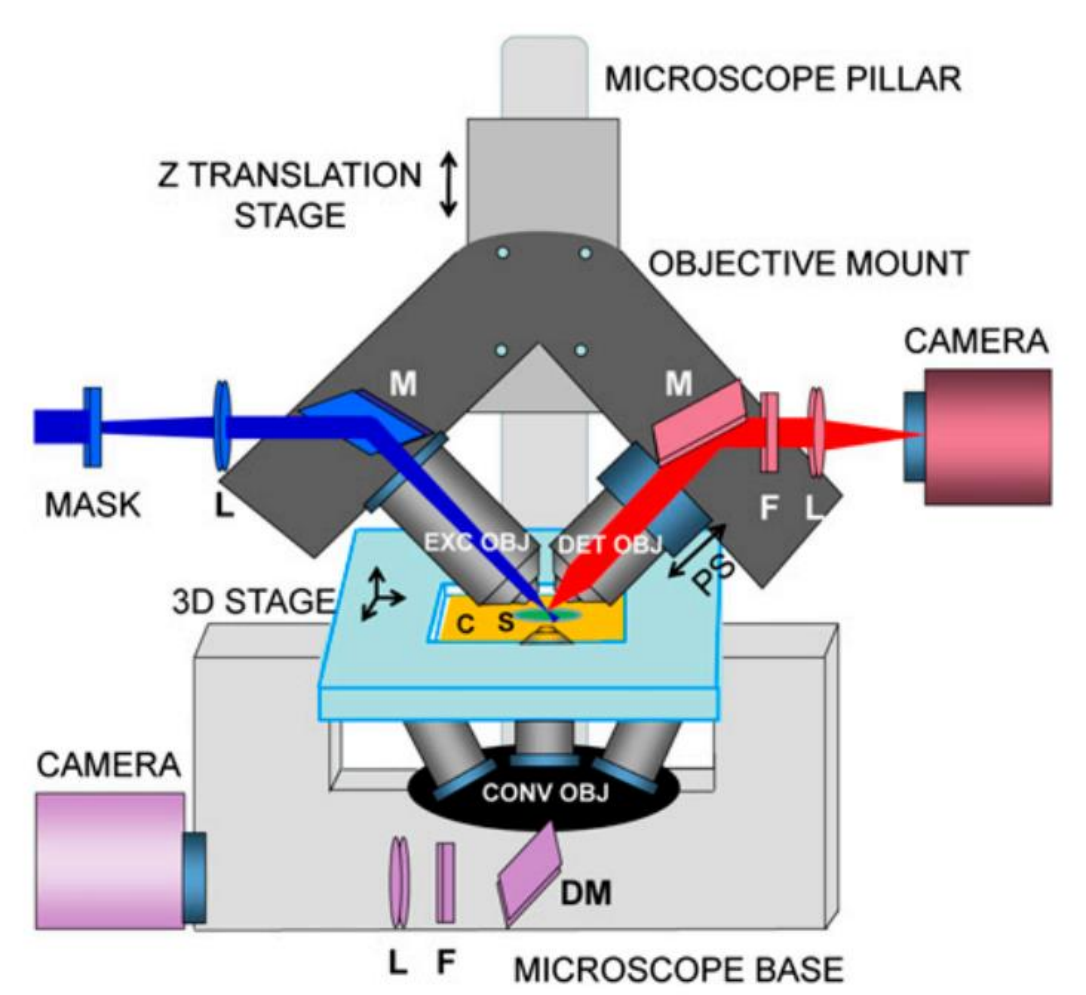


DSLM优势

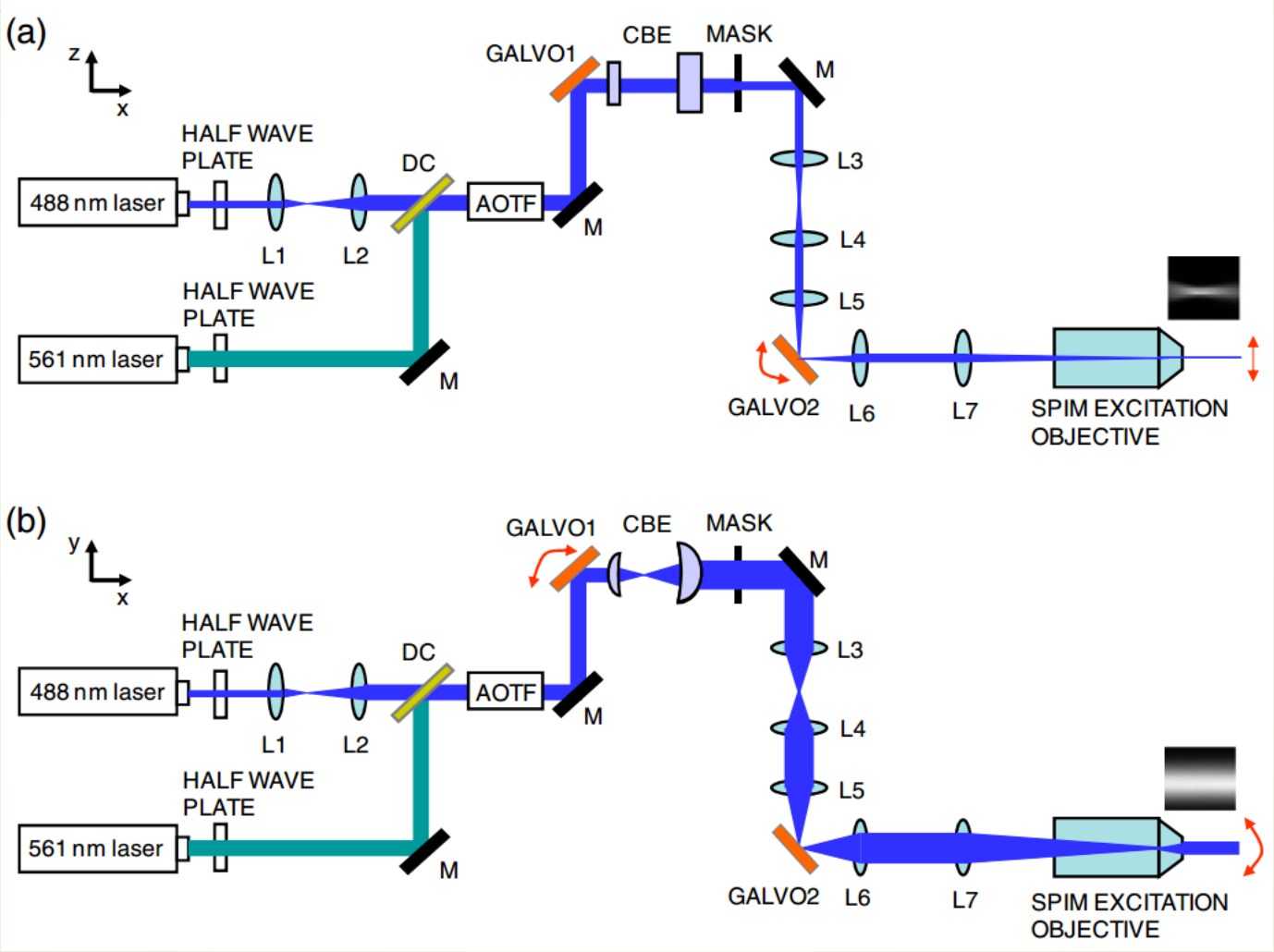
- 光路得到简化
- 照明效率高：由于无需光圈来对光束进行整形，照明效率可达95%，传统的SPIM平均只有3%
- 成像速度比普通SPIM提高了至少了两倍
- 装置紧凑：除去光源外，占地0.3平方米
- 对于扫描的控制更加灵活
- 费用相对较低：除去电子系统（电脑）以及软件，共耗费8万欧

9. Inverted selective plane illumination microscopy(iSPIM)

装置图


MICROSCOPE PILLAR
Z TRANSLATION STAGE
OBJECTIVE MOUNT
CAMERA
M
M
MASK
L
F
L
EXC OBJ
DET OBJ
PS
3D STAGE
C
S
CAMERA
CONV OBJ
DM
L
F
MICROSCOPE BASE

装置图


(a)
z
x
GALVO1
CBE
MASK
M
HALF WAVE PLATE
DC
488 nm laser
AOTF
M
L1
L2
L3
L4
L5
HALF WAVE PLATE
561 nm laser
M
GALVO2
L6
L7
SPIM EXCITATION OBJECTIVE
(b)
y
x
GALVO1
CBE
MASK
M
HALF WAVE PLATE
DC
488 nm laser
AOTF
M
L1
L2
L3
L4
L5
HALF WAVE PLATE
561 nm laser
M
GALVO2
L6
L7
SPIM EXCITATION OBJECTIVE

装置

- 模板：普通SPIM
- 光源：Coherent Cube 405 nm–100 mW, Coherent Sapphire OPSL 488 nm, 200 mW, Coherent Sapphire OPSL 561 nm, 200 mW
- 扩束：Thorlabs AC254–030–A–ML, focal length *f*1 = 30 mm and Thorlabs AC254–060ML, *f*2 = 60 mm
- 光片形成：Thorlabs LJ1653L1–A *f* = 200 mm
- 照明物镜：Nikon Plan, 10 × , NA 0.3
- 探测物镜：HCX APO L U–V–I 40 × , NA 0.8 or CFI Plan 100 × W, NA 1.1
- 探测光路：Thorlabs AC254200–A–ML
- EMCCD: Andor Ixon DU–897E–CS0BV
- 扫描：（移动样品）Physik Instrumente PI M-105.10 （x, y）, Physik Instrumente PI M–110.1DG (z)

结果图

特点

- 将SPIM技术与远场单分子定位技术相结合

LSFM（SPIM）的优势

- High sectioning capability
- High contrast
- Reduced photo bleaching and toxicity
- Imaging speed
- Prolonged duration

挑选LSFM时的关注点

- 示意图（setup)
- 分辨率（横向、纵向，极限200nm)
- 兼容性（工作距等)
- 数据采集、图像处理方法（图像重建)

谢谢！