

## Rapid three-dimensional isotropic imaging of living cells using Bessel beam plane illumination

Thomas A Planchon<sup>1,6</sup>, Liang Gao<sup>1,6</sup>, Daniel E Milkie<sup>2</sup>, Michael W Davidson<sup>3</sup>, James A Galbraith<sup>4</sup>, Catherine G Galbraith<sup>5</sup> & Eric Betzig<sup>1</sup>

A key challenge when imaging living cells is how to noninvasively extract the most spatiotemporal information possible. Unlike popular wide-field and confocal methods, plane-illumination microscopy limits excitation to the information-rich vicinity of the focal plane, providing effective optical sectioning and high speed while minimizing out-of-focus background and premature photobleaching. Here we used scanned Bessel beams in conjunction with structured illumination and/or two-photon excitation to create thinner light sheets ( $\sim 0.5 \mu\text{m}$ ) better suited to three-dimensional (3D) subcellular imaging. As demonstrated by imaging the dynamics of mitochondria, filopodia, membrane ruffles, intracellular vesicles and mitotic chromosomes in live cells, the microscope currently offers 3D isotropic resolution down to  $\sim 0.3 \mu\text{m}$ , speeds up to nearly 200 image planes per second and the ability to noninvasively acquire hundreds of 3D data volumes from single living cells encompassing tens of thousands of image frames.

A common theme in modern biological fluorescence microscopy is the quest to extract ever more information from a single cell, whether by higher spatial<sup>1</sup>, temporal<sup>2</sup> or spectral<sup>3</sup> resolution. As spatial resolution improves, each three-dimensional (3D) voxel encompasses fewer fluorescent molecules (Fig. 1a). In live-cell imaging, as temporal resolution improves, fewer molecules in each voxel can be sacrificed to fluorescence photobleaching in each 3D image stack (Fig. 1b). In short, the photon budget for each voxel at each point in time becomes increasingly constrained as spatiotemporal resolution improves. In contrast, photon statistics also demand that a minimum number of photons  $N$  be collected from each voxel at each time point to yield images of acceptable signal-to-noise ratio (SNR),  $N^{1/2}$  (Fig. 1c). Thus, as the quest for higher spatiotemporal resolution continues, it becomes imperative to devise methods to spend the photon budget wisely.

Unfortunately, many common methods of fluorescence microscopy (wide-field, structured illumination, 3D photoactivated localization, confocal and stimulated emission depletion microscopy) use an epi-illumination configuration that exposes

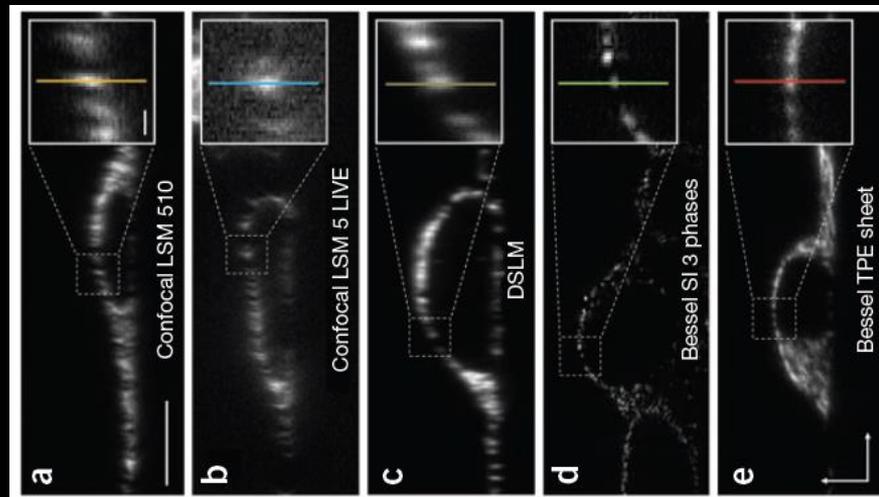
the entire sample thickness to the illuminating radiation. As high-resolution information is obtained only from the vicinity of the focal plane of the detection objective, with these methods the photon budget is increasingly wasted for increasingly thick samples. For live cells, the phototoxic effects of whole-cell illumination can prove even more limiting to physiological studies than photobleaching itself<sup>4</sup>.

A notable exception is plane-illumination microscopy<sup>5</sup>, which uses a separate excitation lens orthogonal to the detection objective to confine the excitation close to the focal plane of the latter. Although this has been successful for imaging large multicellular organisms at single-cell resolution<sup>6,7</sup>, a tradeoff exists between the minimum thickness of the light sheet and the field of view over which it remains reasonably uniform such that, when imaging a 50- $\mu\text{m}$ -diameter cultured cell, an optimized Gaussian light sheet diverges to a full-width at half-maximum (FWHM) thickness of  $2.8 \mu\text{m}$  at either end. As this is threefold greater than the depth of focus of a high-numerical-aperture (NA) detection objective, substantial out-of-focus excitation remains, and hence the benefits of background reduction and photobleaching mitigation that are the hallmarks of plane illumination are not fully realized. Furthermore, such light sheets are too thick to aid in improving axial resolution, which is  $\sim 3$ - $4\times$  poorer than transverse resolution, even when high-NA optics are used.

We describe the use of scanned Bessel beams<sup>8</sup> to generate much thinner light sheets. Bessel beams are created by projecting an annular illumination pattern at the rear pupil of an excitation lens<sup>9</sup>, their central peak width (Supplementary Fig. 1), unlike Gaussian beams, can be decoupled from their longitudinal extent (Supplementary Fig. 2) simply by changing the thickness of the annulus. The self-reconstructing property<sup>10</sup> of such beams has recently been used to reduce shadowing and scattering artifacts in plane illumination microscopy of multicellular organisms<sup>11</sup>. Here we use scanned Bessel beams of higher NA to create light sheets sufficiently thin to achieve isotropic 3D resolution and improve the expenditure of the photon budget to the point at which hundreds of 3D image stacks comprising tens of thousands of frames can be acquired from single living cells at rates of nearly 200 frames  $\text{s}^{-1}$ .

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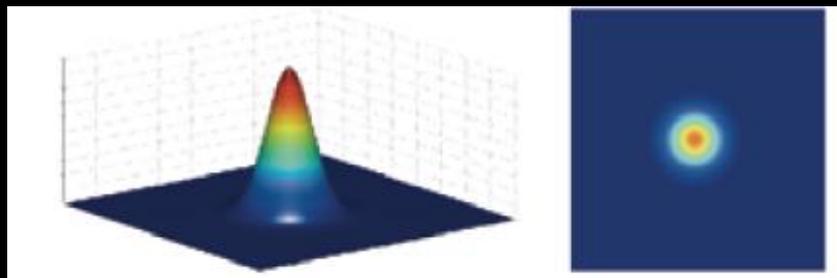
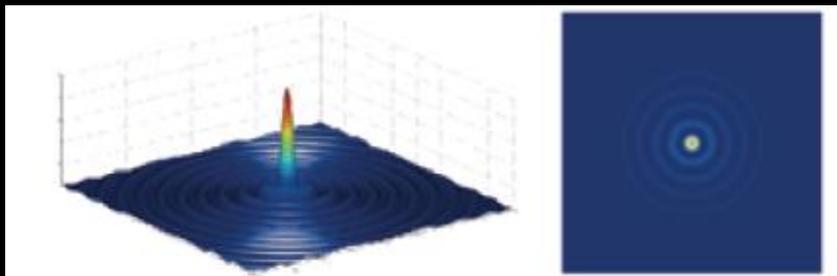
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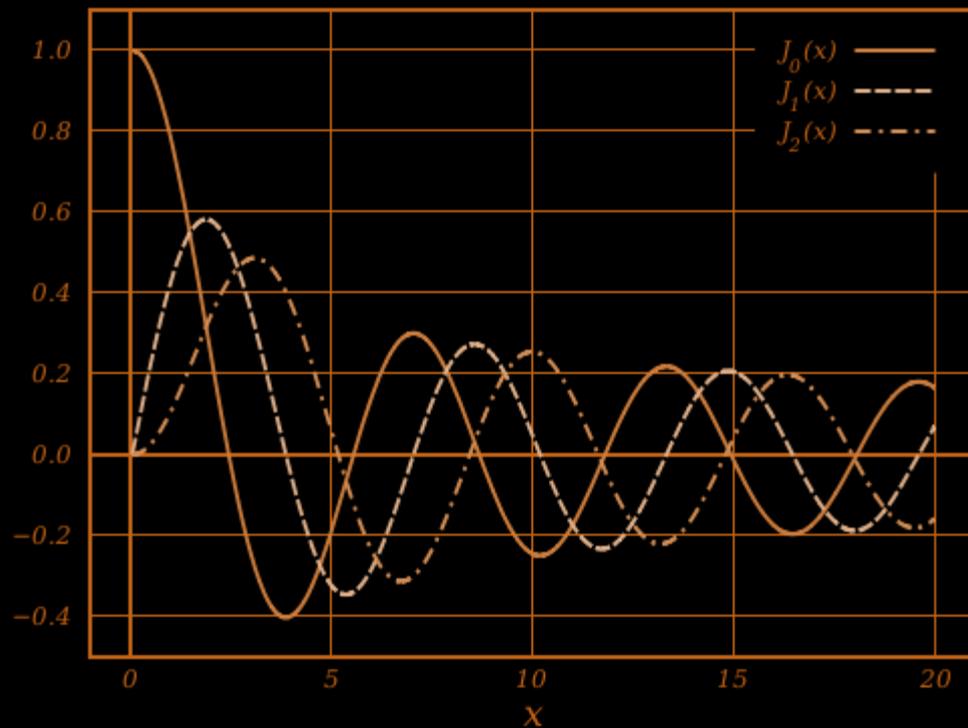
RECEIVED 14 SEPTEMBER 2010; ACCEPTED 27 FEBRUARY 2011; PUBLISHED ONLINE 4 MARCH 2011; DOI:10.1038/nature10788

# Bessel beam

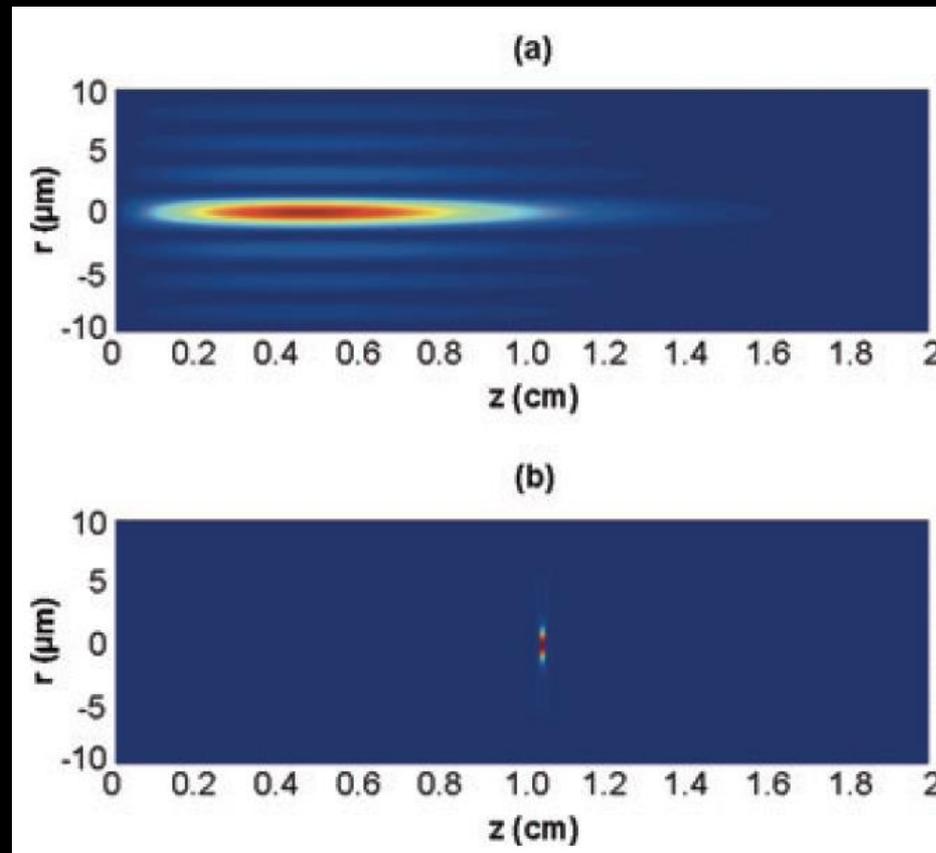
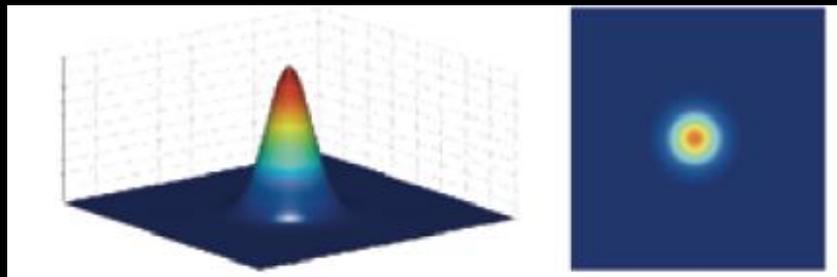
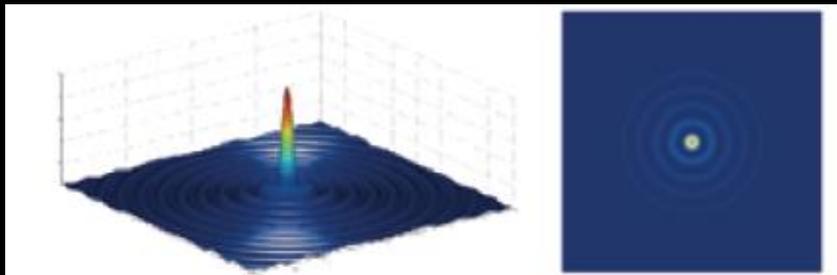


$$E(r, \psi, z) = A \exp(ik_z z) J_0(k_r r)$$

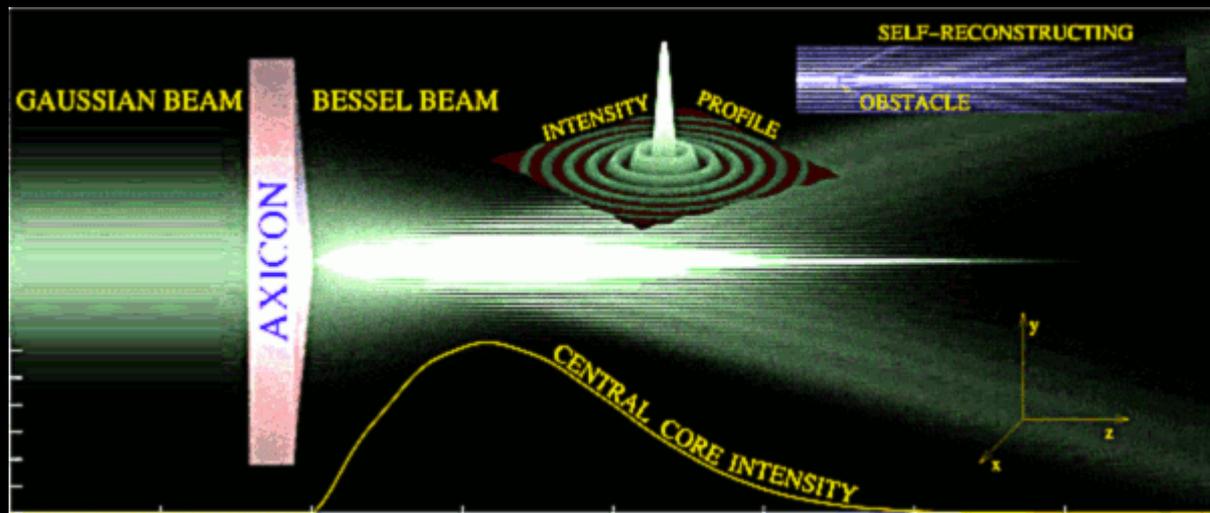
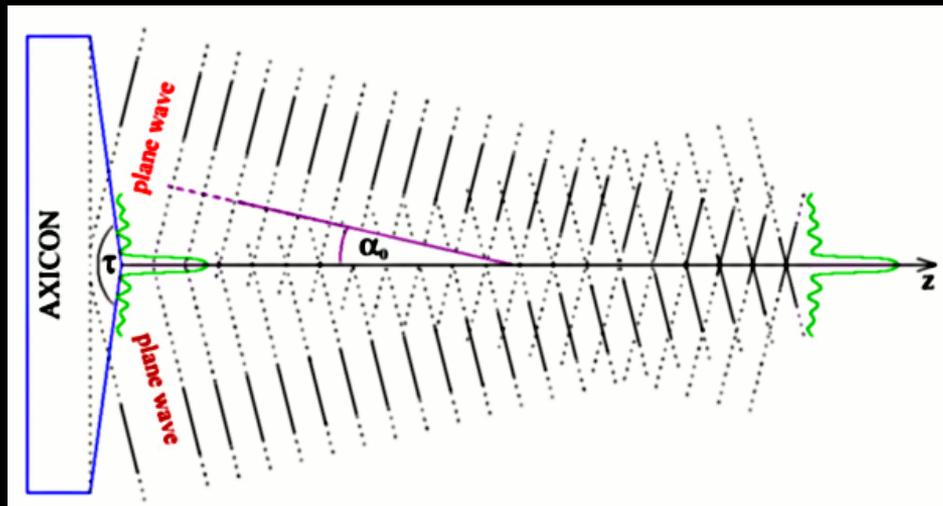
$$J_\alpha(x) = \sum_{m=0}^{\infty} \frac{(-1)^m}{m! \Gamma(m + \alpha + 1)} \left(\frac{x}{2}\right)^{2m + \alpha}$$



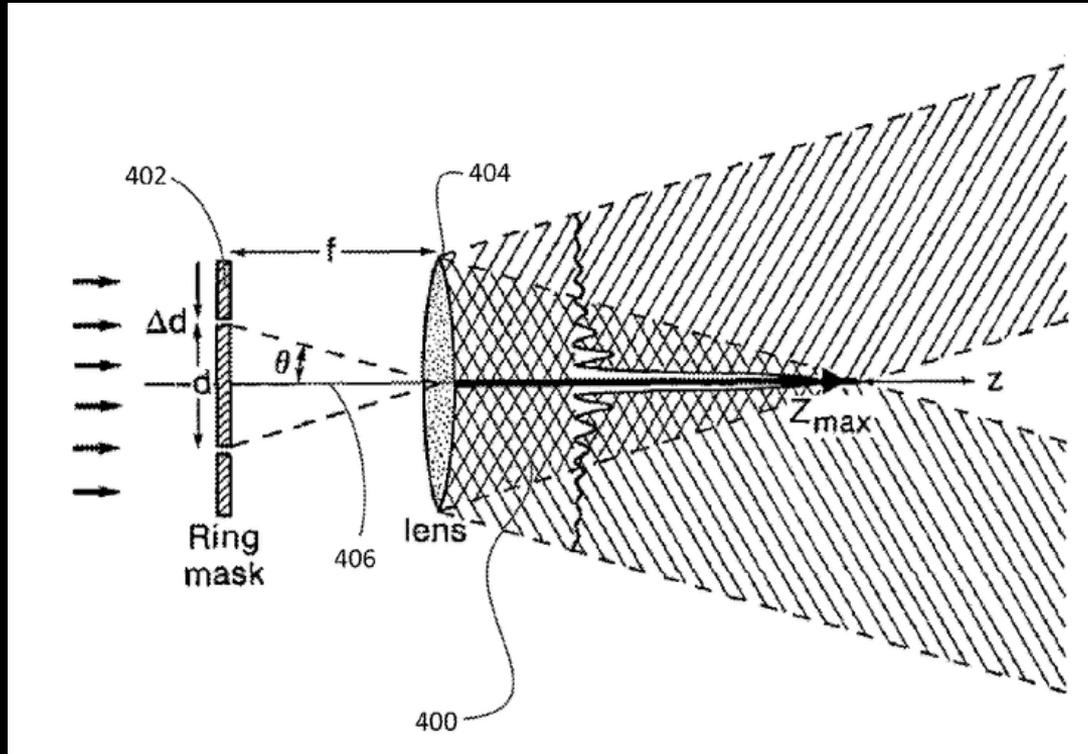
# Bessel beam



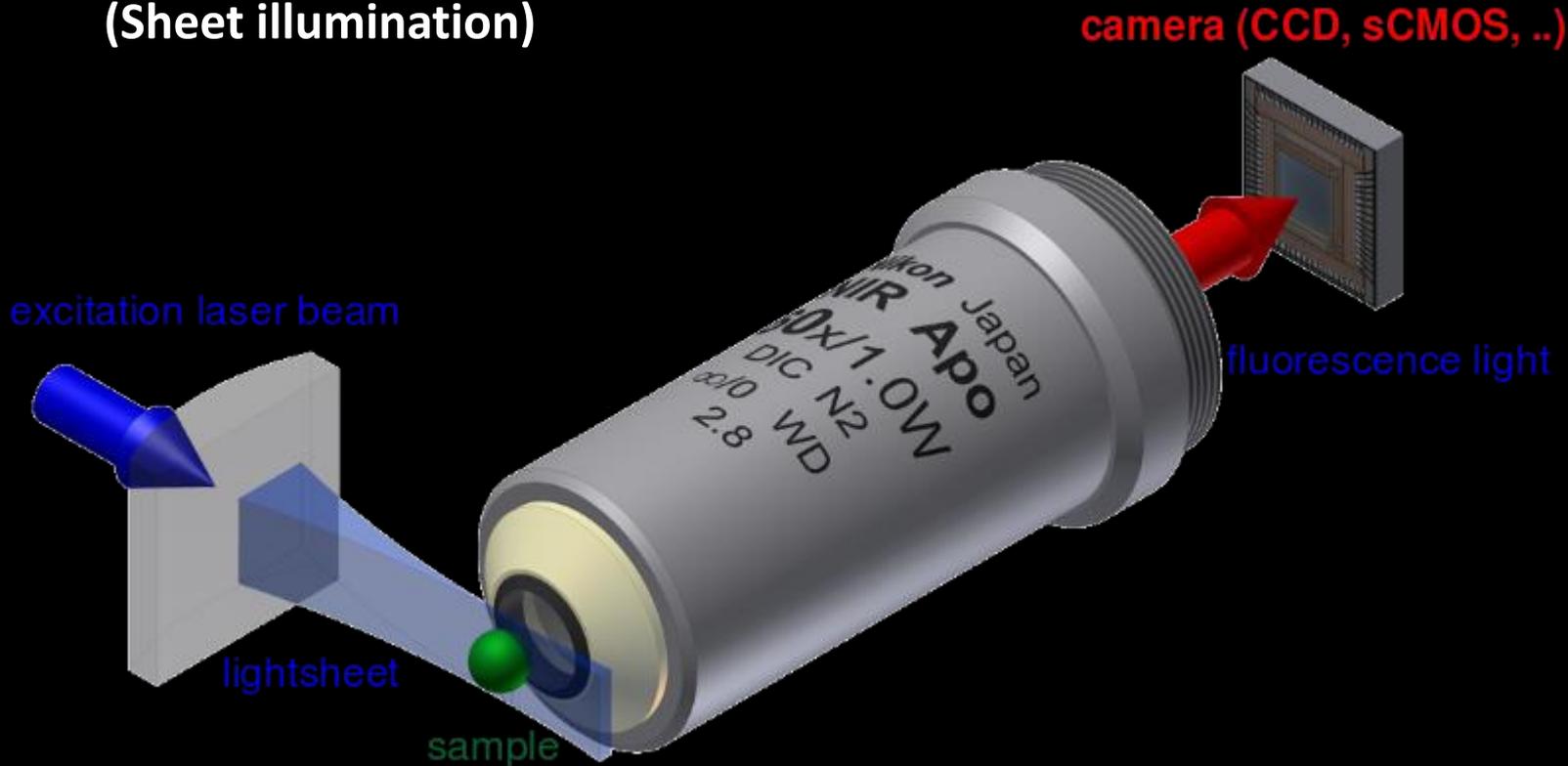
# Making Bessel beam



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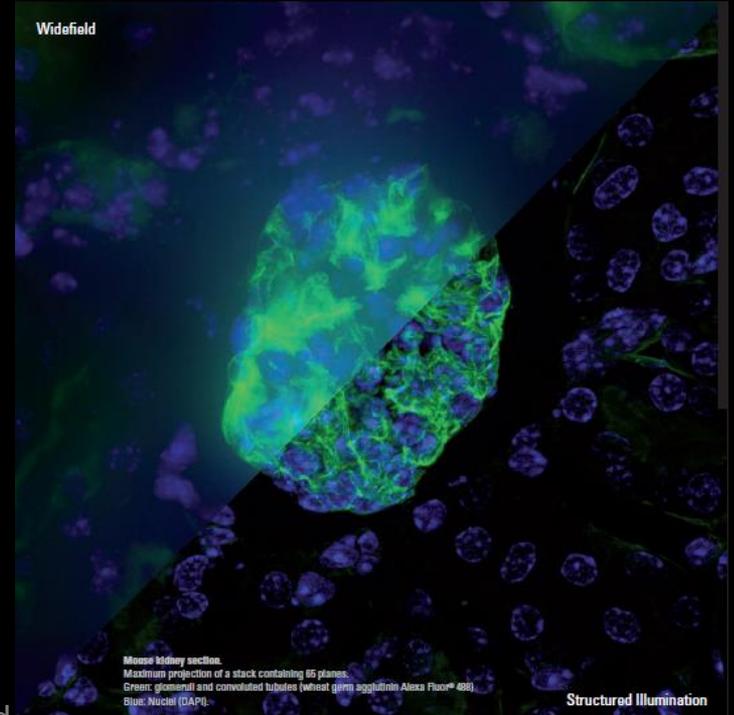
# Plane illumination Microscopy (Sheet illumination)



[http://en.wikipedia.org/wiki/Light\\_sheet\\_fluorescence\\_microscopy](http://en.wikipedia.org/wiki/Light_sheet_fluorescence_microscopy)

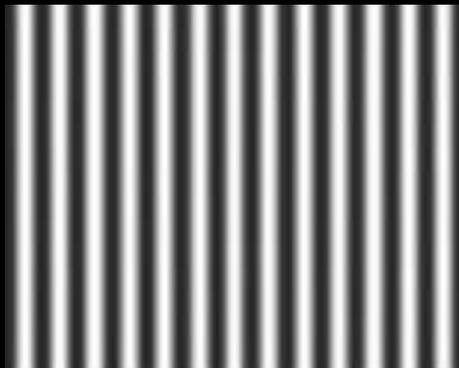
# Structured illumination microscopy

[http://www.leica-microsystems.com/fileadmin/downloads/Leica%20Structured%20Illumination/Brochures/Leica\\_Structured\\_Illumination-Brochure\\_en.pdf](http://www.leica-microsystems.com/fileadmin/downloads/Leica%20Structured%20Illumination/Brochures/Leica_Structured_Illumination-Brochure_en.pdf)



<http://zeiss-campus.magnet.fsu.edu/tutorials/superresolution/hrsim/index.html>

# Structured illumination microscopy



$$I = I_o + I_p$$

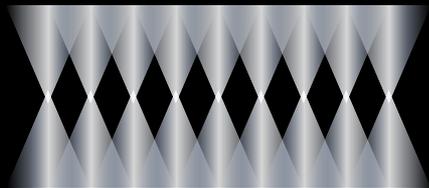
$$I = I_o + I_c \cos \phi + I_s \sin \phi$$

$$\text{set } \phi = 0, \frac{2}{3}\pi, \frac{4}{3}\pi$$

$$I_i = I_o + I_c \cos \phi + I_s \sin \left( \frac{2(i-1)}{3} \pi \right)$$

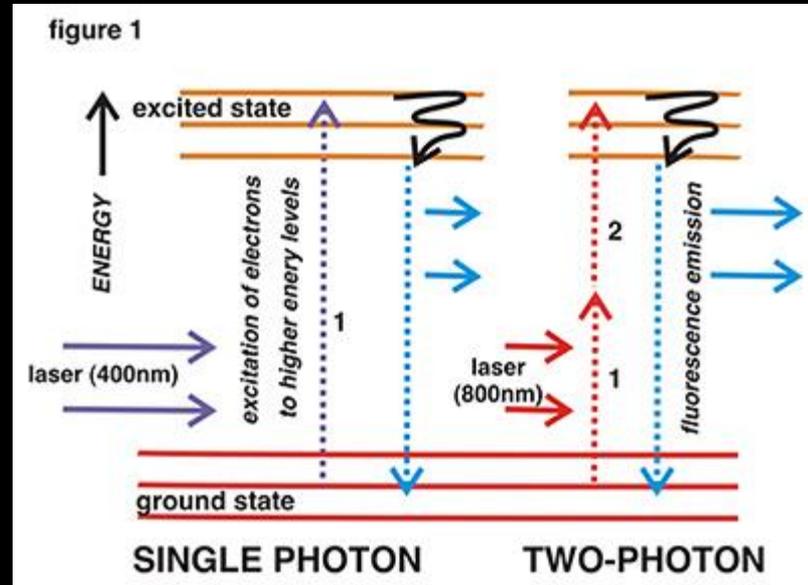
$$I_o = I_1 + I_2 + I_3$$

$$I_p = \left[ (I_1 - I_2)^2 + (I_1 - I_3)^2 + (I_2 - I_3)^2 \right]^{1/2}$$



# Two photon excitation

<http://www.ncl.ac.uk/bioimaging/assets/photos/2photon.jpg>



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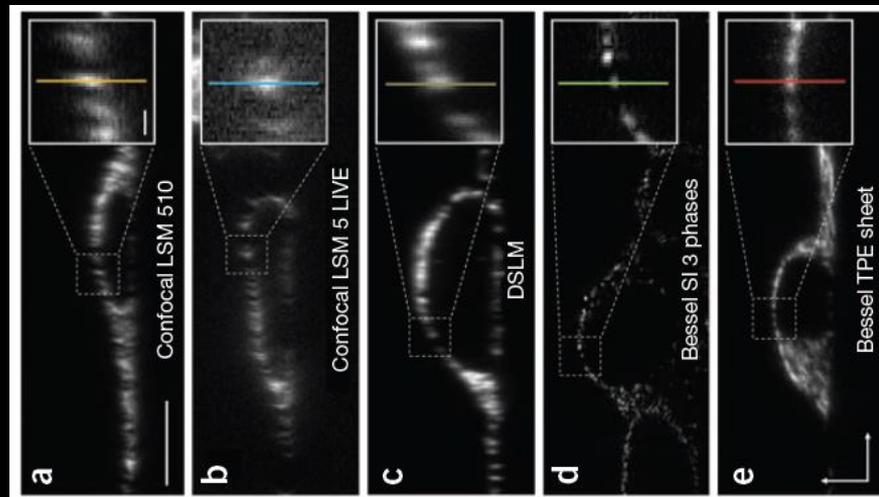
the entire sample thickness to the illuminating radiation. As high-resolution information is obtained only from the vicinity of the focal plane of the detection objective, with these methods the photon budget is increasingly wasted for increasingly thick samples. For live cells, the phototoxic effects of whole-cell illumination can prove even more limiting to physiological studies than photobleaching itself<sup>4</sup>.

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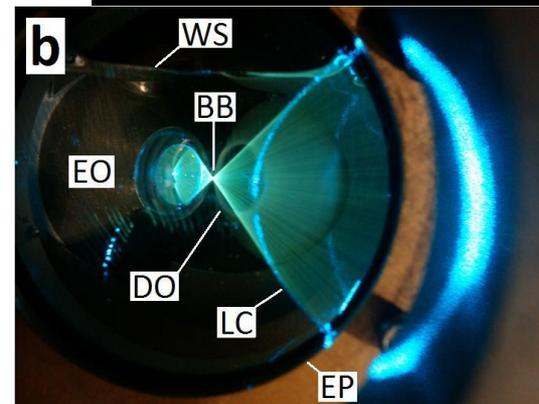
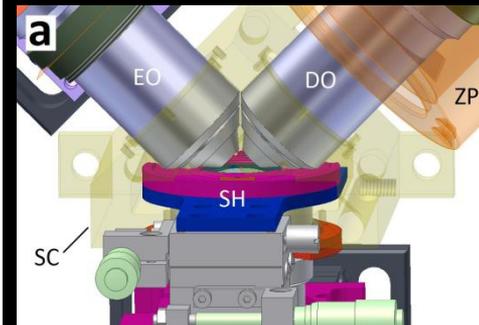
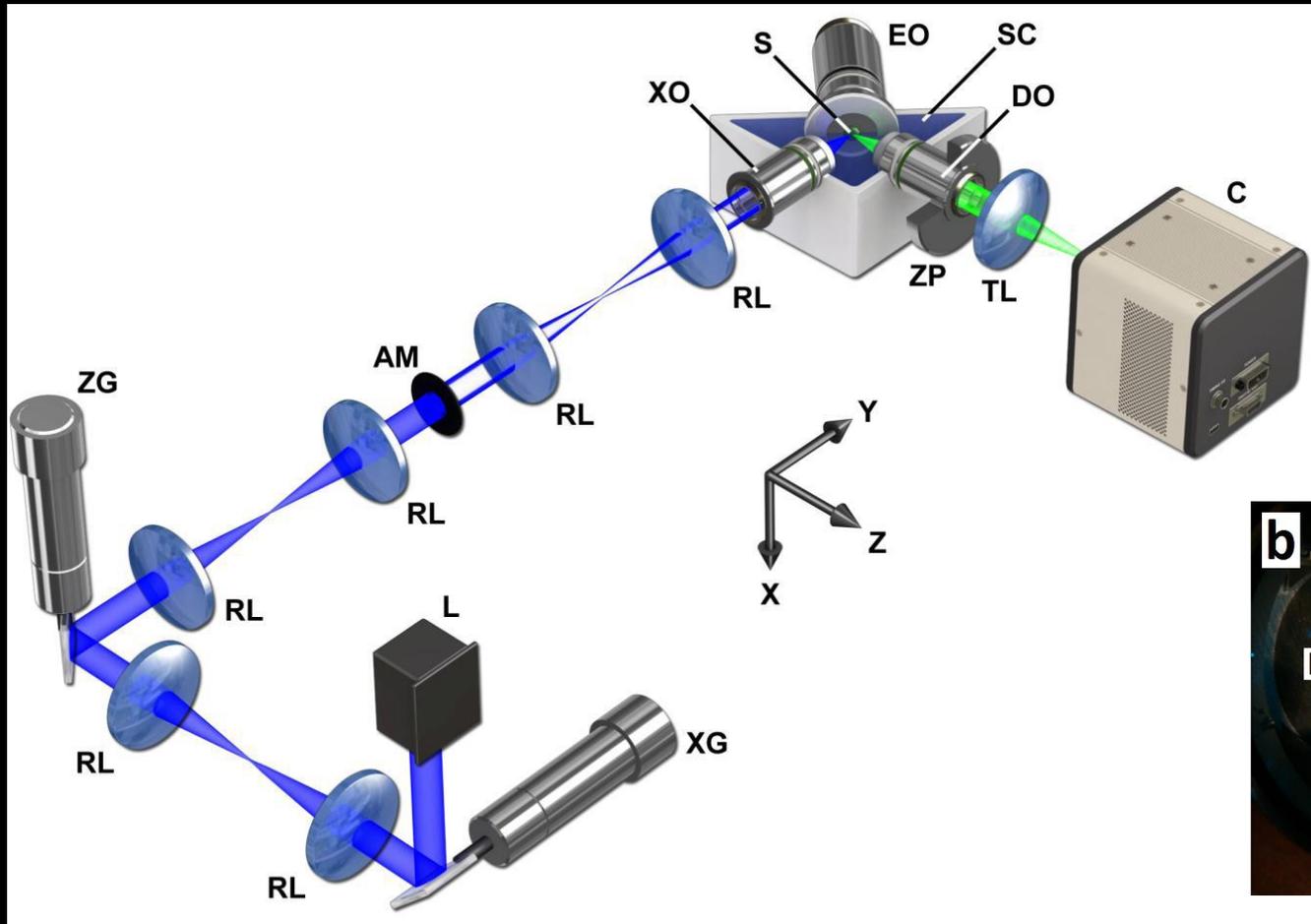
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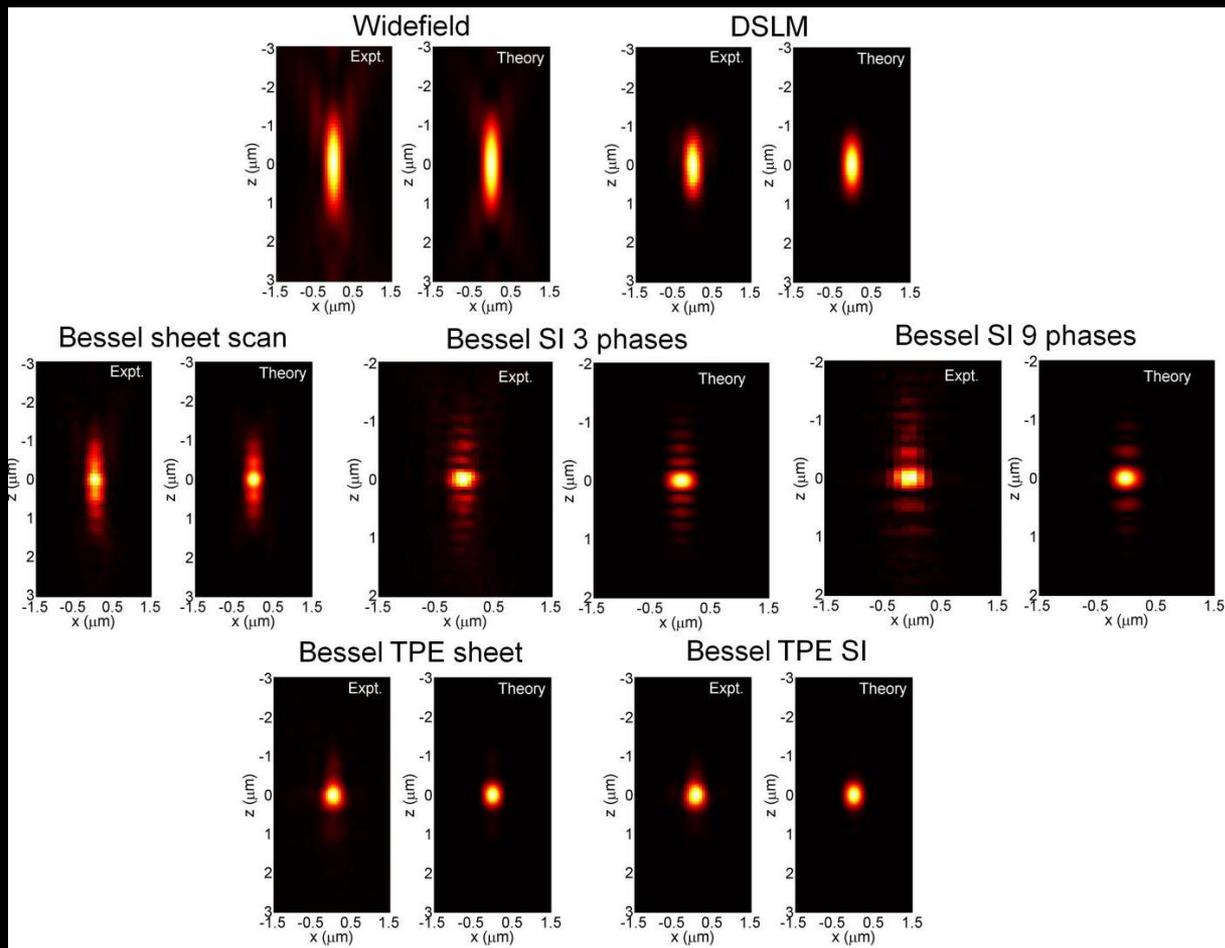
<sup>1</sup>Jaxia Farm Research Campus, Howard Hughes Medical Institute, Ashburn, Virginia, USA. <sup>2</sup>Coleman Technologies, Inc., Chadds Ford, Pennsylvania, USA. <sup>3</sup>National High Magnetic Field Laboratory and Department of Biological Science, Florida State University, Tallahassee, Florida, USA. <sup>4</sup>National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland, USA. <sup>5</sup>National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland, USA. <sup>6</sup>These authors contributed equally to this work. Correspondence should be addressed to E.B. (betzig@jaxia.hhi.org).

RECEIVED 14 SEPTEMBER 2010; ACCEPTED 27 FEBRUARY 2011; PUBLISHED ONLINE 4 MARCH 2011; DOI:10.1038/nature10788

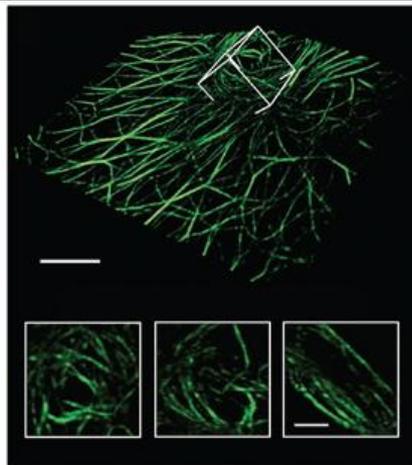
# The Bessel beam plane illumination microscopy



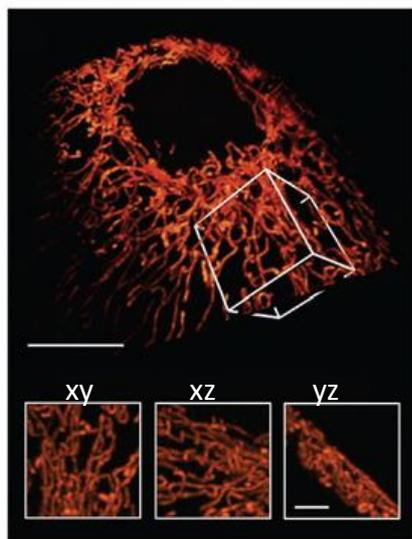
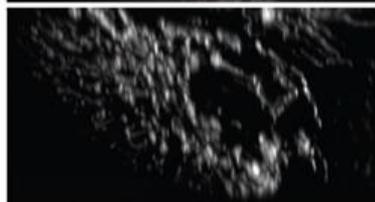
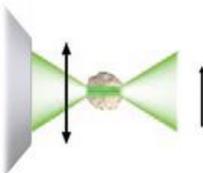
# Point spread functions



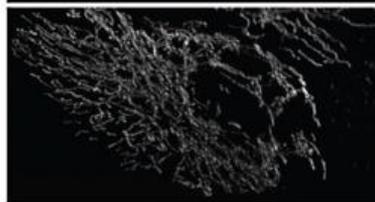
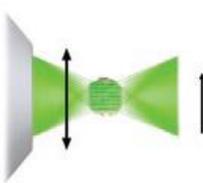
Wide field



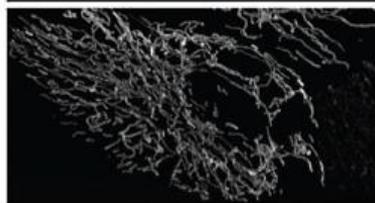
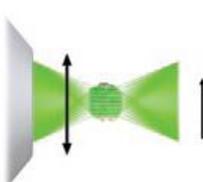
Bessel sheet



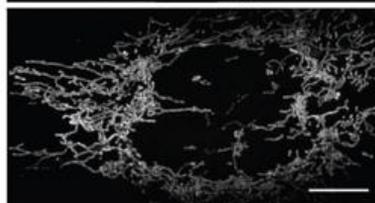
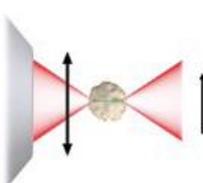
Bessel Si 3 phases



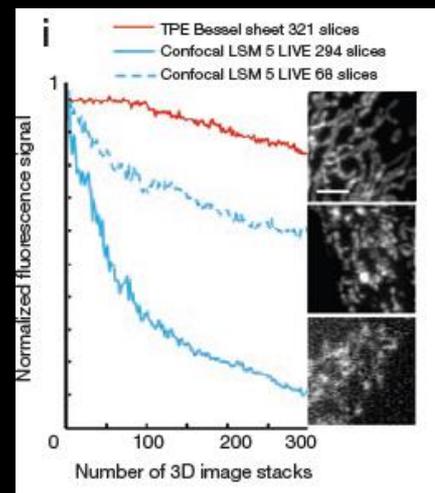
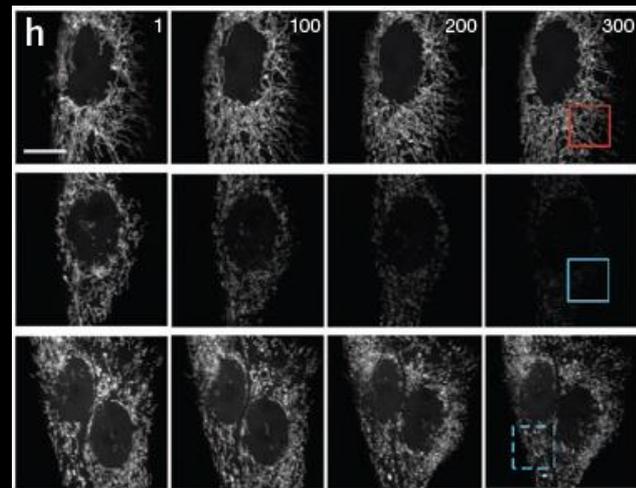
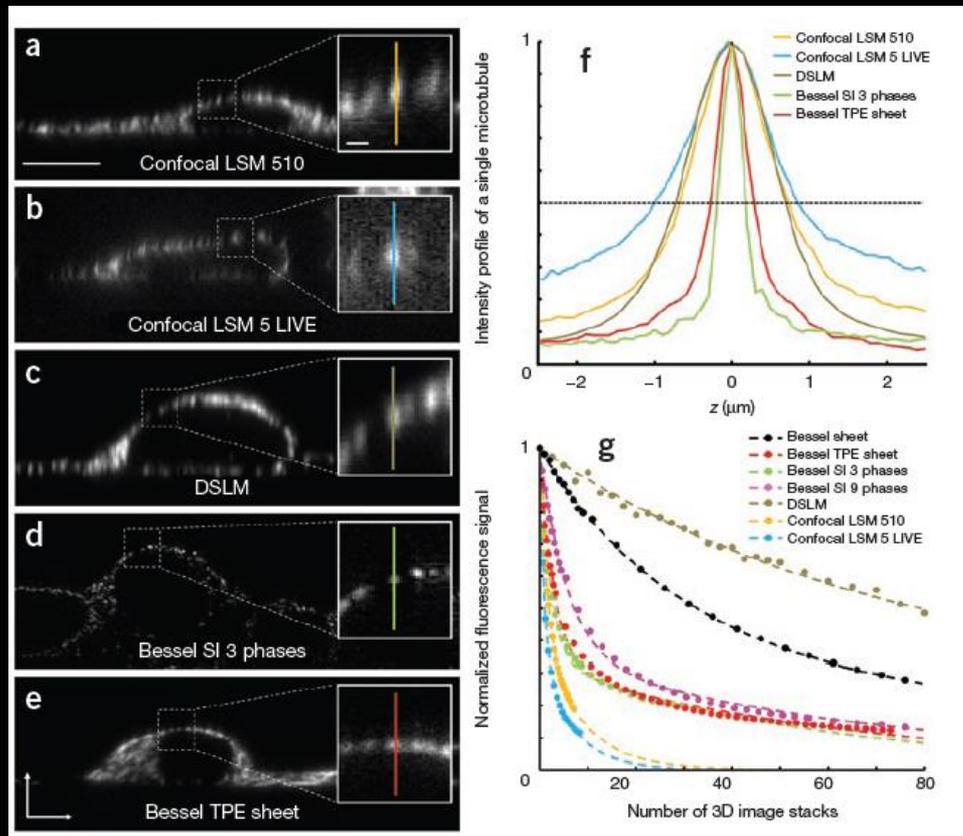
Bessel Si 9 phases



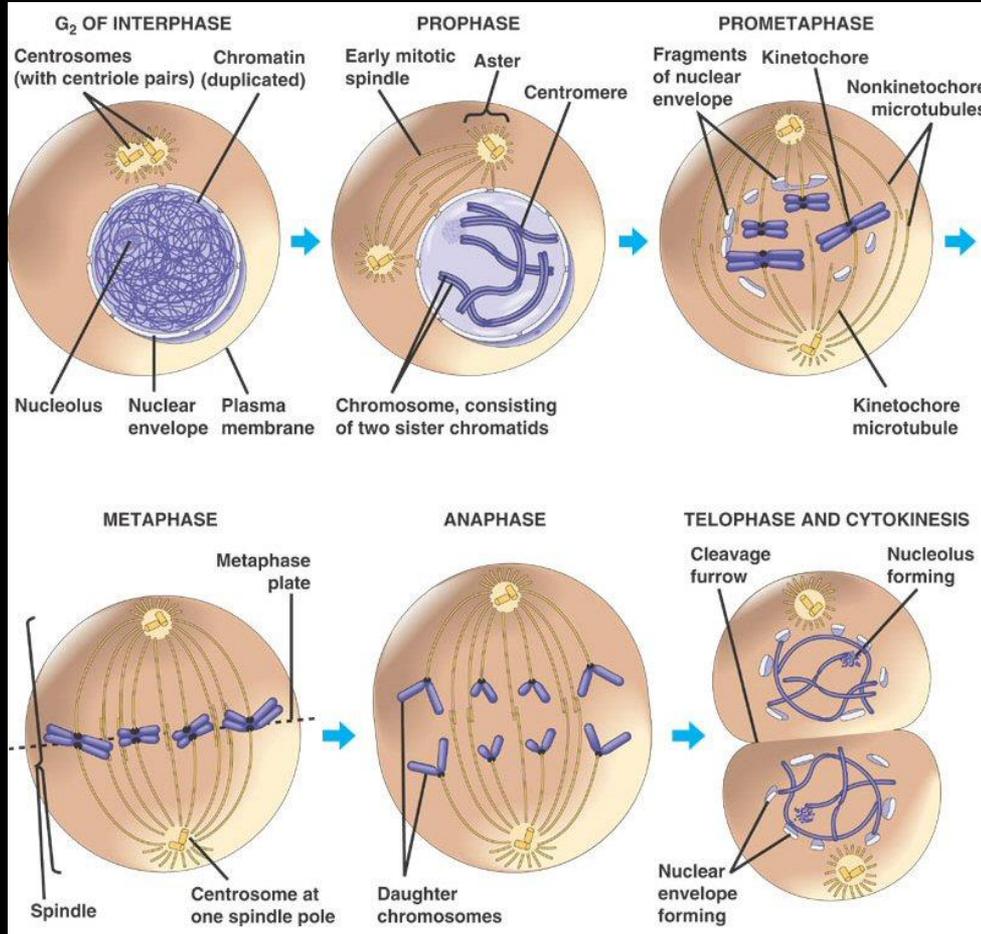
Bessel TPE sheet



# Saving the photon budget



# Imaging of Cell division process



# Fast 3D image acquisition

