3D Imaging

Colin Sheppard
Nano-Physics Department
Italian Institute of Technology (IIT)
Genoa, Italy
colinjrsheppard@gmail.com
3-D point spread function $h$ is 3-D Fourier transform of 3-D pupil
(cap of spherical shell, Ewald sphere, paraboloid in paraxial approx.)
• Want to image as many values of $\mathbf{K}$ as possible
Coherent imaging (also holography)

transmission

\[ k = \frac{2\pi}{\lambda} \]

\[ |k_1| = |k_2| \]

Grating vector \( \mathbf{K} \) lies on a sphere through the origin

\[
\frac{1 - \cos \alpha}{\lambda} = \frac{2}{\lambda} \sin^2 \frac{\alpha}{2}
\]

only image frequencies on cap of sphere
Reflection

\[
\frac{1 - \cos \alpha}{\lambda} = 2 \frac{\sin^2 \alpha}{\lambda} \]

\[
\frac{\sin \alpha}{\lambda}
\]
Problem with conventional imaging

3-D Optical Transfer Function (OTF) for incoherent imaging (e.g. fluorescence)

Cut off corresponds to Grating period of $\lambda/2n\sin\alpha$ in a medium

Want to image as big a range of spatial frequencies as possible

Spatial frequencies imaged

Transverse spatial frequency

Axial spatial frequency

Missing cone

NB This OTF is also the 3D FT of the intensity in a coherent image

Mertz, Transformations in Optics (1965)

Frieden, JOSA 57, 58-66 (1967)
Confocal 3D CTF

CTF is convolution of pupil functions $P_1 \otimes P_2$
Confocal reflection

\[ \frac{4}{\lambda} \sin^2 \left( \frac{\alpha}{2} \right) \]

The spatial frequency cut-off in three-dimensional imaging II
C. J. R. Sheppard
3D Coherent Transfer Function (CTF)
Confocal Reflection, Aplanatic

\[ \alpha = 60^\circ \]

\[ \alpha = 90^\circ \]

C. J. R. Sheppard and Min Gu, Y. Kawata and S. Kawata
Cross sections through the CTF

Sloping surface:
Section through CTF at an angle
(projection-slice theorem)
So amplitude of image of a sloping surface is FT of section through CTF

C. J. R. Sheppard and K. G. Larkin
Effect of numerical aperture on interference fringe spacing
1995 / Vol. 34, No. 22 / APPLIED OPTICS 4731

Fig. 6. Cross sections through the coherent transfer function for a system satisfying sine condition $NA = 0.95$; $K$ is a normalized radial spatial frequency.
3-D OTF confocal fluorescence

The spatial frequency cut-off in three-dimensional imaging II

C. J. R. Sheppard
3-D OTF, confocal fluorescence

Fig. 5 The OTF in confocal fluorescence for a point detector ($v_d = 0$). There is no missing cone, so that 3-D imaging performance is good.

Fig. 6 The OTF in confocal fluorescence for a finite-sized detector ($v_d = 10$). The response is poorer than that for a point detector (Fig. 5), and the missing cone is beginning to appear.
Source/Detector arrays

- Singular value decomposition (Bertero & Pike 1982)
- Type 3: Maximum signal in detector plane (Reinholz 1987)
- Pixel reassignment 1988
- Source/detector arrays (Benedetti 1996)
- Programmable array microscope (PAM) (Hanley 1998)
- Structured illumination (Gustafsson 2000)
- Structured illumination + nonlinear
  (Heinzmann 2002, Gustafsson)
Offset pinhole

Fig. 2. Normalized intensity distribution in the direction of offset against normalized distance from the optic axis of a point image for a confocal microscope with various values of pinhole displacement.

Fig. 4. Two-point resolution against pinhole offset for a nearly confocal microscope.

Improvement in resolution by nearly confocal microscopy

APPLIED OPTICS, Vol. 21, page 778, March 1, 1982

I. J. Cox, C. J. R. Sheppard, and T. Wilson
Pixel reassignment

Abstract

A new explanation for the imaging improvement of confocal microscopy is presented. A method of further increasing the imaging performance is also discussed.

Optical transfer function

\[ I(x_s) = \{|h_1|^2 \otimes |h_2|^2\} (2x_s) \]

\[ C(m) = \{(P_1 \otimes P_1^*) (P_2 \otimes P_2^*)\} (m\lambda f/2) \]

OTF_1 \times OTF_2

product of rescaled OTFs
(not convolution of OTFs as for confocal)

high spatial frequencies enhanced

Fig. 2. Incoherent transfer functions for a fluorescence microscope. The radius of the circular pupils is \( a \).
How it works
Offset pinhole & reassignment

- Integrate without reassignment: same as conventional
- Integrate with reassignment (to centre of illumination and detection): PSF sharpened and signal improved
Image scanning microscopy

**FIG. 1** (color online). ISM Setup, (1) Excitation with super-continuum white light source and acousto-optic tunable filter, (2) 90/10 nonpolarizing beam splitter cube, (3) major dichroic mirror, (4) piezo scan mirror, (5) 4f telescope, (6) UPL APO 60x W microscope objective, (7) beam diagnostic camera, (8) confocal aperture, and (9) EM CCD detection camera system.

**FIG. 2** (color online). Image of a single fluorescent bead of 100 nm diameter. Left panel: CLSM image; middle panel: ISM image; right panel: Fourier-weighted ISM image. The horizontal bar in the left panel has a length of 1 μm.
Blurry vision belongs to history

Hans Blom and Jerker Widengren

Making simple modifications to laser-scanning microscopes—like those found in many laboratories—can beat the classical diffraction limit by a factor of 2.

When a wide area of the sample is illuminated, the structured illumination approach is conceptually clear and well established [2]. Müller and Enderlein’s insight was to realize that a diffraction-limited laser focus is itself a special type of structured illumination, containing all possible Fourier modes allowed by the confocal microscope. In order to image the entire spatial pattern of the fluorescence generated by such a laser excitation, that is, the convoluted image of the laser focus and the sample, Müller and Enderlein use a CCD camera in place of the point detector found in conventional confocal-laser microscopes.
In-focus PSF for a 9x9 detector array

The 9x9 array assumed to span just beyond the first zero of airy disc (4 \( \lambda/NA \))
In-focus PSF for a 9x9 detector array

PSF after reassignment
3D PSF for the same array
Optical sectioning

But, for $v_{\text{dmax}} \rightarrow \infty$, no optical sectioning!

$v = 2.747$

Figure 4. The intensity in the confocal image of a single point. The locus of the auto-focus scan of the image is also shown. The cross-hatched region is that in which the intensity is greater than 0.01. The corresponding region for a conventional system is shown shaded.


The extended-focus, auto-focus and surface-profiling techniques of confocal microscopy

C. J. R. SHEPPARD and H. J. MATTHEWS
Integration over finite detector array

Resolution and signal strength improve as size of array ($v_{d_{\text{max}}}$) increases.

Peak of point spread function for large $v_{d_{\text{max}}}$ is

$$I_{\text{peak}} = 4\left(1 - \frac{16}{3\pi^2}\right) = 1.84$$

(4 elements gives ~1.4)

Peak is >1!
- Super-concentration
- Beats classical limit of étendue
Image of a point object: Effect of array size

- large array, I decays slowly
- $I/I_0$ increases
- 0.72 AU
- 1 AU
- 1st zero

Conventional: reassign fluorescence 1 AU
Confocal: reassign coherent
Unnormalized OTF for confocal and ISM

Interpretation of the optical transfer function: Significance for image scanning microscopy

Colin J. R. Sheppard, Stephan Roth, Rainer Heintzmann, Marco Castello, Giuseppe Vicidomini, Rui Chen, Xudong Chen, and Alberto Diaspro

Stokes’ shift

• Actually, reassignment works for ANY value of shift.

• Shift of $\frac{1}{2}$ gives product of 2 matched OTFs.

• Can also do a similar thing if there is a Stokes’ shift. Then need to shift to $\beta/(1+\beta)$, where $\beta$ is $\lambda_{fl}/\lambda_{exc}$.
  Cut off increased a factor $(1+\beta)$, compared with a conventional microscope.

• With Stokes’ shift, cut off
  -for scanning, non-confocal is a factor $\beta$ better than conventional
  -for confocal it is factor $(1+\beta)$ better.
Over the past 25 years, confocal imaging has become the standard technique for most fluorescence microscopy applications. The increased use of confocal imaging systems in basic biomedical research can be attributed to their ability to produce high-contrast, optically sectioned images while providing enough acquisition versatility to address many sample and application demands.

substantial (4-8x) increase in SNR in the final image

Summary

• Both confocal and structured illumination give factor of two improvement in spatial frequency cut off
• Confocal only gives ~1.4x resolution improvement because OTF drops off
• In practice finite pinhole size means no improvement over conventional
• Pixel reassignment gives resolution improvement coupled with increased collection efficiency
• Integrating all the signal results in no optical sectioning
• Limit detector array to $v_{dmax} = 2.747$.
  - resolution improvement factor = 1.45,
  - $I_{peak} \sim 130\%$,
  - collection efficiency $\sim 80\%$. 
Fringe projection, or
Structured illumination

Optical reconstruction using a second grating

Also Near-field Microscope with a grating

It is known that diffraction sets an upper limit to the resolving power of an optical system or, put more precisely, to the band-width of spatial frequencies that the system can transmit.

A new method is described for obtaining optical images with a resolution exceeding the limits set by diffraction. The optical system itself is not modified, but a mask, or the image of a mask formed by projection is introduced in or near to the object plane. This mask has a variable transmission (for example a grating), and is movable in the object field. A second similar mask is introduced in or near to the image plane, or the plane of an intermediate image, and is moved conjugately with the object plane mask. The image obtained during the scanning by the masks is integrated in time by a receptor of suitable inertia (for example, the eye, or a photographic emulsion).

There results an image of the object with enhanced resolution and contrast (the bandwidth of the transmitted spatial frequencies is increased, and the frequency response is raised).

The method may be used with coherent, partially coherent or incoherent illumination. Its method of operation and imaging properties are described.

Near-field, EH Synge *Phil. Mag.* **6**, 356-362 (1928)
Structured illumination microscopy

Mats Gustafsson

The actin cytoskeleton of a human neutrophil, visualized in 3D by structured illumination microscopy.
(a) top view (XY projection),
(b) side view (XZ projection).
Structured illumination microscopy


4 Pi microscope (Hell)

4Pi (a) Fluorescence Microscope

Fig. 3 The cut-off of the three-dimensional fluorescence transfer function: (a) conventional, (b) confocal, (c) illuminated coherently from both sides.

Reflection and transmission confocal microscopy
C.J.R. Sheppard and C.J. Cogswell

Optics in Medicine, Biology and Environmental Research
Editors: Gert von Bally Shyam Khanna

Selected Contributions to the First International Conference on Optics Within Life Sciences (OWLS I)
Garmisch-Partenkirchen, Germany, 12-16 August 1990
4Pi (c) Fluorescence Microscope

\[ \alpha = \pi / 3 \]

M. Gu and C. J. R. Sheppard

Three-dimensional transfer functions in 4Pi confocal microscopes

Two-photon 4Pi (c) Confocal Fluorescence Microscope

\[ \alpha = \pi / 3 \]

9 peaks

Min Gu, C.J.R. Sheppard
Optical transfer function analysis for two-photon 4Pi confocal fluorescence microscopy
Optics Communications 114 (1995) 45–49
$I^5M$: 3D widefield light microscopy with better than 100 nm axial resolution

M. G. L. Gustafsson,* D. A. Agard*,† & J. W. Sedat*
Comparison of different imaging methods

Extended resolution fluorescence microscopy
Mats GL Gustafsson
Comparison of 4Pi and I$^5$M
3D Spatial Frequencies

Maximum \(\frac{4}{\lambda}(4n/\lambda\text{ in medium, e.g. } 6/\lambda)\)

- **Coherent**
  - Confocal fluorescence
  - or
  - Structured illumination

- **Abbe (incoherent)**
  - no missing cone

Maximum possible with propagating waves, sphere radius \(4n/\lambda\)
Nonlinear imaging

• Incoherent versus coherent imaging (square law)
• Saturable absorber sharpens point spread function (Choudhury, 1977)
• Nonlinearity of detection in optical storage (Braat, 1980s)
• Nonlinear effects in lithography
• Multiphoton imaging (SHG, 2-photon fluorescence)
• Stimulated emission depletion microscopy (STED) (Hell)
• Saturation in structured illumination (Heintzmann, Gustafsson)
• PALM, STORM
Multiphoton microscopy

- Proposes different types of scanning nonlinear microscopy based on the high intensity in the focused spot, including two-photon fluorescence and CARS.

In the scanning optical microscope\cite{1,2}, nonlinear interactions are expected to occur between the object and a highly focused beam of light, which we hope will open new ways of studying matter in microscopic detail hitherto not available. Nonlinear interactions\cite{3-5} include the generation of sum frequencies, Raman scattering, two-photon fluorescence, and others. We feel

C. J. R. Sheppard and R. Kompfner


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Optical sectioning in SHG Microscopy

KD*P crystal, second harmonic images - cw
NdYAG laser 1064nm

Second-harmonic imaging in the scanning optical microscope

J. N. GANNAWAY, C. J. R. SHEPPARD
Nonlinear structured illumination microscopy


Saturated patterned excitation microscopy—a concept for optical resolution improvement

- Gustafsson (2005)
Structured illumination microscopy with nonlinearity from saturation

Fig. 6. A field of 50 nm fluorescent beads, imaged by (a) conventional microscopy, (b) conventional microscopy plus filtering, (c) linear structured illumination, and (d) saturated structured illumination using illumination pulses with 5.3 mJ/cm² energy density, taking into account three harmonic orders in the processing. As no scanning is

Stimulated emission depletion microscopy (STED)
Localization microscopy

The future?

- Confocal microscopy is still the “gold standard”
- Image scanning microscopy for improved signal
- Focal modulation for penetration
- 2-photon fluorescence for live cell microscopy
- Light sheet for large objects
- FF-OCT for medical screening and industrial applications
- Structured illumination (fringe projection or array illumination) for speed and resolution
- Digital holographic microscope for fast imaging (single shot)
- All with digital deconvolution