Imaging with illumination and detection arrays

Colin Sheppard
Nano-Physics Department
Italian Institute of Technology (IIT), Genoa, Italy
School of Chemistry, University of Wollongong, Australia
colinjrsheppard@gmail.com
Two ways to form an image

Full-field detection

detector array

Scanning system

single element detector

CHAPTER 1

The Generalized Microscope*

C. J. R. SHEPPARD
School of Physics, and Australian Key Centre for Microscopy and Microanalysis, The University of Sydney, NSW 2006, Australia

*This chapter is based upon an invited presentation at the Symposium of the Australian Society for Electron Microscopy, University of Sydney, 1996.
Scanning microscopes of Type 1 (non-confocal)

Detector only measures intensity

Imaging by first lens (objective lens)

Figure 1. Scanning microscopes of Type 1.

C. J. R. SHEPPARD and A. CHOUDHURY
Image formation in the scanning microscope
OPTICA ACTA, 1977, VOL. 24, NO. 10, 1051–1073
Same as ‘single-pixel’ camera!

But single-pixel camera is not new!

- Logie Baird television (1928)
- Flying spot microscope
- Scanning electron microscope (Oatley, 1948)
- Non-descanned detector in 2-photon microscope
  (even allows detection through a scattering medium!)
Scanning and conventional microscopes are equivalent

- Based on Principle of Reciprocity
- Holds even with loss or multiple scattering
  (but not inelastic scattering, e.g. fluorescence)
- First shown for electron microscopes

Zeitler & Thomson, Optik 31 258 (1970)
Welford, J. Microscopy 96 105 (1972)
Barnett, Optik 38 585 (1973)
Engel, Optik 41 117 (1974)

WARNING: Some papers say conventional is better, some say scanning is better!
In fact both are the same.
But not for fluorescence: scanning gives better resolution (Stokes shift)
Scanning vs. conventional microscope

**Conventional**
- Condenser
- Objective
- Image
- Source
- Object

**Conventional with image scanning**
- Condenser
- Objective
- Scanned
- Point detector
- Object

**Scanning**
- Scanned
- Point source
- Objective
- Collector
- Detector

**Confocal**
- Scanned
- Point source
- Objective
- Collector
- Scanned

or CCD detector

Equivalent
Confocal microscopy

• Advantages
  
  Optical sectioning
  – 3D imaging
  – Surface profiling

  Reduced scattered light
  – Imaging through scattering media, e.g. tissue

  Improved resolution (for small pinhole)

• Reflection
  – Industrial applications, surface profiling
  – Scattering media, tissue (non-invasive)

• Fluorescence
  – Autofluorescence or labelled
  – Fixed or living
Confocal reflectance (Oxford 1974-89)

Stereo pair of a pollen grain

Endeavour, 10, 17-19 (and cover) (1986)

Rat brain (cerebellum)

Colour confocal reflection image of a leaf

J. Microsc. 165, 103-117 (1992)

Microtubules labeled with 15nm gold

Confocal Imaging (non-fluorescence)

$h$ is amplitude PSF

\[ I(x_d, y_d) = \left| \int \int h_1(x, y) t(x - x_s, y - y_s) h_2(x_d - x, y_d - y) \, dx \, dy \right|^2 \]

- Pinhole: $x_d, y_d = 0$:
  \[ I = \left| (h_1(x, y) h_2(-x, -y)) \otimes t(x, y) \right|^2 \]

- $h_2$ even:
  \[ I = \left| (h_1 h_2) \otimes t \right|^2 \]

  - Same as coherent microscope, with $h_{\text{eff}} = h_1 h_2$
  - Transfer function is convolution of $c_1$ with $c_2$
OTF for confocal fluorescence

Fig. 1. Transfer function for the confocal fluorescent microscope for various recent wavelengths. The spatial frequency axis is normalised by the in-wavelength.

Even weaker (or negative) for finite-sized pinhole

goes negative!

Fig. 4. Normalized in-focus (2-D) OTF for different radii of the detector. The dashed curve represents the 2-D OTF when \( v_d \rightarrow \infty \).

Effect of Stokes shift

Effect of pinhole size
Plot suggests possibility to use pupil filters to increase the magnitude of the OTF at high frequencies!

Super-resolution by confocal fluorescent microscopy
I. J. Cox, C. J. R. Sheppard and T. Wilson

Confocal fluorescent microscopy with a finite-sized circular detector
Min Gu and C. J. R. Sheppard
3D Spatial Frequency cut-offs

Maximum $4/\lambda$ ($4n/\Lambda$ in medium, e.g. $6/\Lambda$)

Coherent (holography)

Confocal fluorescence or Structured illumination

Maximum possible with propagating waves, sphere radius $4n/\lambda$

no missing cone

Abbe limit (large condenser, or Fluorescence)
Limitations of confocal microscopy

- **Speed**
  - Illuminate only one spot at a time
  - In fluorescence, speed limited by saturation of fluorophore
  - Solution: illuminate by more than one spot
    - Spinning disk
    - Line illumination
    - Structured illumination

- **Signal level**
  - Increasing pinhole size reduces resolution, sectioning

- **Resolution**
  - 4Pi microscopy
  - STED
  - Localization microscopy (PALM/STORM)
  - Structured illumination/Image scanning microscopy

- **Penetration**
  - Coherence gating
  - Two/three photon excitation
  - Focal modulation microscopy (FMM)
Main problem: Finite sized pinhole

- Need finite sized pinhole to get adequate signal
- Then resolution improvement is lost
Illumination and detection arrays

- Structured illumination (Lukosz, 1963; Gustafsson, 2000)
- Tandem scanning (spinning disc), Petrán (1968)
- Singular value decomposition (Bertero & Pike, 1982)
- ‘Type 3’: Maximum signal in detector plane (Reinholz, 1987)
- Pixel reassignment (Sheppard, 1988)
- Subtractive imaging (Wilson, 1984; Cogswell & Sheppard, 1990; + many others)
- Source/detector arrays (Benedetti, 1996)
  - Max image
  - Min image gives crosstalk + background
  - Max-Min, Similar to confocal
  - Superconfocal Max+Min-2 Mean
Illumination and detection arrays (II)

• Programmable array microscope (Hanley 1999, Verveer 1998)
  • Scanned array + Detector array. Conjugate image is confocal $I_{\text{conf}}$
  • Non-conjugate image is $I_{\text{conv}} - I_{\text{conf}}$
  • Random array $I_{\text{conv}} + I_{\text{conf}}$

• Structured illumination + nonlinear (Heintzmann, 2002; Gustafsson)

• Structured detection, J Lu, Concello, Xie, Lichtmann (2009)
  • SPIN Structured illumination pattern written by modulated beam.
    • Harmonics attenuated by the illumination OTF. Can get modulation pattern without DC offset
  • SPADE “patterned detection” Illumination constant, Detector switched on and off

• Structured detection, RW Lu, Biomed Opt Exp (2013)
  • Digital mask
Scanning microscope with partially coherent source and detector

Image formation in scanning microscopes with partially coherent source and detector

C. J. R. SHEPPARD and T. WILSON

Scanning (Type 1)
Confocal with finite pinhole

Image intensity:

\[
I(x_s) = \int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} S(x_1) h_1 \left( \frac{x_0 + x_1/M}{\lambda f} \right) h_1^* \left( \frac{x_0' + x_1/M}{\lambda f} \right) t(x_s - x_0) t^*(x_s - x_0') \times h_2 \left( \frac{x_0 + x_2/M}{\lambda f} \right) h_2^* \left( \frac{x_0' + x_2/M}{\lambda f} \right) D(x_2) \, dx_1 \, dx_0 \, dx_0' \, dx_2.
\]

Transmission cross coefficient (TCC):

\[
C(m; p) = \int_{-\infty}^{+\infty} F_S \left[ \xi_1' - \xi_1 \frac{\xi_1'}{\lambda Mf} \right] F_D \left[ \frac{(p - m)}{M} + \frac{(\xi_1 - \xi_1')}{\lambda Mf} \right] P_1(\xi_1) P_1^*(\xi_1') \times P_2(\lambda f m - \xi_1) P_2^*(\lambda f p - \xi_1') \, d\xi_1 \, d\xi_1'.
\]

\[
F_S(u) = \int_{-\infty}^{+\infty} S(x_1) \exp\{ -2\pi j u x_1 \} \, dx_1,
\]

\[
F_D(v) = \int_{-\infty}^{+\infty} D(x_2) \exp\{ -2\pi j v x_2 \} \, dx_2.
\]

\[
F_{S,D} \text{ are FTs of source, detector:}
\]
General microscope with source/detector arrays

1D theory:

\[
I(x_2, x_8) = \int \int \int_{-\infty}^{+\infty} S(x_1 - Mx_8) \left( \frac{x_1/M - x_0}{\lambda d} \right) h_1 \left( \frac{x_1/M - x_0}{\lambda d} \right) t(x_0) \left( \frac{x_1/M - x_0}{\lambda d} \right) dx_1 \, dx_0 \, dx_0' \times h_2 \left( \frac{x_2/M - x_0}{\lambda d} \right) h_2* \left( \frac{x_2/M - x_0'}{\lambda d} \right) D(x_2 - Mx_8) \, dx_1 \, dx_0 \, dx_0',
\]

• source and detector arrays (scanned)
• reduces to conventional, structured illumination (SIM), scanning, confocal, spinning disk, etc.
• partially coherent system (but can also analyze a fluorescence system)
Fluorescence microscope with source/detector arrays

\[ I(x_2, x_s) = \iint S(x_1 - Mx_s)H_1 \left( \frac{x_1}{M - x_0} \right) T(x_0)H_2 \left( \frac{x_2}{M - x_0} \right) D(x_2 - Mx_s) \, dx_1 \, dx_0. \]

\[ I(x_2, x_s) = \iint S(x_1 - Mx_s)H_1 \left( \frac{x_1}{M - x_0} \right) T(x_0)H_2 \left( \frac{x_2}{M - x_0} \right) D(x_2 - Mx_s) \, dx_1 \, dx_0. \]

\( H \) is intensity PSF \quad \( T \) is intensity object

source array

detector array

Corresponding equation for a fluorescence system
Again, applies for conventional, scanning, spinning disk microscopes

function of 2 (2D) variables
Fluorescence microscope with source/detector arrays

For point source

\[ I(x_1, x_2) = \int H_1(x_1 - x)H_2(x_2 - x)T(x) \, dx \]

Signal at point \( x_2 \) when illuminated at point \( x_1 \)
Scanning microscopes with detector array

Detector replaced by detector array

• general case similar to ptychography
• quadrant detector for differential phase contrast (DPC)

Dekkers & de Lang, Differential phase contrast in a STEM, Optik 41, 452-456 (1974)
Mehta & Sheppard, Quantitative phase-gradient imaging at high resolution with asymmetric illumination-based differential phase contrast (AI-DPC) Optics Letts. 34, 1924 (2009)
Offset pinhole

- Point spread function gets narrower
- Intensity decreases
- But increased side lobes
- And effective psf shifts sideways

\[ I(v) = \left[ \frac{2J_1(v - \overline{v})}{v - \overline{v}} \right]^2 \left[ \frac{2J_1(v + \overline{v})}{v + \overline{v}} \right]^2 \]

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
Gives the image of a shifted object point
Offset pinhole & reassignment

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

conventional given by envelope

offset pinhole

after 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\(_1\) x OTF\(_2\)

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

Fig. 2. Incoherent transfer functions for a fluorescence microscope. The radius of the circular pupils is \(a\).
Pixel reassignment

- Considers fluorescence and partially-coherent systems
- Concept that a detector element gives information about points of the object other than the illuminated point
- Introduces pixel reassignment and summation approach
- Explains why a confocal microscope can give superior resolution compared with a conventional one
Bertero & Pike (from 1982)

Let $f(y)$ be the complex effective transparency (or reflectivity for a reflection microscope) in the object plane; by this we mean that, for a given scanning position, the image $g(x)$ formed by an ideal microscope with a uniformly filled illumination lens is

$$g(x) = \int_{-\infty}^{+\infty} \text{sinc}(x - y) \text{sinc}(y) f(y) dy,$$

(1)

where $\text{sinc}(x) = \sin(\pi x) / (\pi x)$. The basic idea, then, is to record the whole image $g(x)$ at each scanning position and to solve the integral equation (1) for $f(y)$.

i.e. does not solve $g(x,y)$ to give $f(y)$

Analytic inversion formula for confocal scanning microscopy
Eq. (5). A first possibility relies on the exact inversion formula for the imaging equation (28) derived by Bertero et al. (1987b):

\[ M(x, t) = \frac{4\pi}{\Omega} \cos(\Omega x) \delta(t). \] (34)

In this case, the object at point \( t \) is reconstructed only from the data at the same scanning position \( t \) and the multiplication by \((4\pi/\Omega)\cos(\Omega x)\) could also be implemented optically by means of a mask (Bertero et al. 1992). However, besides Eq. (34) and because of the redundancy of the data in Eq. (29), a whole family of reconstruction kernels can be constructed, all yielding Eq. (33) as overall PSF (Defrise and De Mol 1992), including the following one,

\[ M(x, t) = \frac{\pi}{\Omega} \delta\left(t - \frac{x}{2}\right) \] (35)

first proposed by Sheppard (1988). In principle, these reconstruction formulas

Super-resolution in confocal scanning microscopy: generalized inversion formulae

Image scanning microscopy

Claus B. Müller and Jörg Enderlein

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.
Optical sectioning

But, for $v_{d\text{max}} \rightarrow \infty$, no optical sectioning!
Need to limit size of array

$v = 2.747$
(0.72 AU)
magic number

points on detector array $> 0.72$ AU, image regions away from the focal plane

Locus of $u_{\text{max}} (v)$

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

peak intensity goes above 1!

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

Peak of point spread function for large array is $4(1 - 16/3\pi^2) = 1.84$

(4 elements gives ~1.4)
Unnormalized OTF for confocal and ISM

(a) Confocal with finite pinhole
(b) ISM with finite detector array

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

Unnormalized OTF for confocal and ISM

Dashed curves: 
confocal with finite pinhole

Solid curves: 
ISM with finite detector array

Unnormalized takes account of signal level

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

Colin J. R. Sheppard,1,7 Stephan Roth,2,3 Rainer Heintzmann,2,3 Marco Castello,1,4 Giuseppe Vicidomini,1 Rui Chen,5 Xudong Chen,5 and Alberto Diaspro1,4,6

Fig. 5. A logarithmic plot of the unnormalized OTFs for a confocal microscope (dashed lines) and ISM (solid lines) with different pinhole/array sizes. The first positive lobe only of the confocal OTF is shown. The behavior for subtracting images from two pinhole sizes \( \left( I_{0.5AU} - \frac{1}{4} I_{1AU} \right) \), or using a matched filter with two ring detectors is also shown.

Optical microscopy with extended depth of field

By C. J. R. Sheppard, D. K. Hamilton and I. J. Cox

Unnormalized transfer function proposed in:

Printed in Great Britain

Vol. 24, No. 24 | 28 Nov 2016 | OPTICS EXPRESS 27280
Images of two points

\( v_0 = 1.92 (~2) \) corresponds to Rayleigh separation (blue curves)

(a) Coherent

(b) Full illumination

(c) Incoherent

(d) Confocal reflection

(e) Confocal fluorescence

(f) Fluorescence ISM

(g) Reflection, maximum detection
Annular ring detector array

Normalized OTFs

For confocal, a ring detector gives a lower cut-off frequency

A large array gives a narrower PSF than a small array, so why not miss out the central part

Dashed black line: conventional
Solid black line: ISM, large array
Zeiss Airyscan

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 (4-8×) increase in SNR in the final image

Joseph Huff

Carl Zeiss Microscopy, LLC, Thornwood, New York, USA. Correspondence should be addressed to J.H. (joseph.huff@zeiss.com).

Doing it optically

Optical photon reassignment microscopy (OPRA)

Stephan Roth, Colin JR Sheppard, Kai Wicker and Rainer Heintzmann

Roth et al. Optical Nanoscopy 2013, 2:5
http://www.optnano.com/content/2/1/5

Original Article

Open Access
Sampling considerations

• Need to sample image at Nyquist rate
• Bandwidth is doubled for pixel reassignment
• If sampling in the detector plane is equal to the sampling of the object illumination, the reconstructed image will exhibit double the sampling rate
• Sampling of the illumination can be at conventional Nyquist rate $\nu = \pi / 2 = 1.57$, rather than at confocal Nyquist rate
  – Speed advantage over confocal
• This does not contradict information capacity, as multiple images are detected
• Redundancy, so can use compressive sensing
General microscope with source/detector arrays

1D theory:

\[ I(x_2, x_0) = \int \int \int_{-\infty}^{+\infty} S(x_1 - Mx_0) h_1 \left( \frac{x_1/M - x_0}{\lambda d} \right) h_1^* \left( \frac{x_1/M - x_0'}{\lambda d} \right) \ t(x_0) \ t^*(x_0') \]

\[ \times h_2 \left( \frac{x_2/M - x_0}{\lambda d} \right) h_2^* \left( \frac{x_2/M - x_0'}{\lambda d} \right) D(x_2 - Mx_0) \ dx_1 \ dx_0 \ dx_0', \]

Fluorescence (incoherent):

\[ I(x_1, x_2) = \int H_1(x_1 - x)H_2(x_2 - x)T(x)dx \]
General Fluorescence case

2D (or really 4D) image

\[ I(x_1, x_2) = \int H_1(x_1 - x)H_2(x_2 - x)T(x)\,dx \]

2D (or 4D) Fourier transform

\[ \tilde{I}(m_1, m_2) = \mathcal{F}_1(m_1)\mathcal{F}_2(m_2)\mathcal{F}(m_1 + m_2) \]

Central and difference coordinates

\[ m = \frac{m_1 + m_2}{2}, m' = \frac{m_1 - m_2}{2} \]

\[ \tilde{I}(m, m') = \mathcal{F}_1\left(m + \frac{m'}{2}\right)\mathcal{F}_2\left(m - \frac{m'}{2}\right)\mathcal{F}(2m) \]

Conventional: \( \tilde{H}_1 = \delta\left(m + \frac{m'}{2}\right) \)

Scanning: \( \tilde{H}_2 = \delta\left(m - \frac{m'}{2}\right) \)

Confocal: \( \int dm' \rightarrow \mathcal{F}_1 \otimes \mathcal{F}_2 \)

Pixel reassignment:

\[ m' = 0 \]

\[ \mathcal{F}(m, m') = \mathcal{F}_1(m)\mathcal{F}_2(m)\mathcal{F}(2m) \]
Works for any reassignment factor $a$

- Can use different reassignment factors $a$
- For a large array, OTF is

$$C_{\text{eff}}(l) = C_1[(1-a)l]C_2(al).$$

$a = \frac{1}{2}$ is highest for all spatial frequencies

---

Fig. 4. The OTF for 1PE fluorescence ISM with no Stokes shift, for different values of reassignment factor $a$. 

---

Superresolution by image scanning microscopy using pixel reassignment

Colin J. R. Sheppard,$^{1,*}$ Shalin B. Mehta,$^*$ and Rainer Heintzmann$^{1,**}$
Effect of changing $a$

- Changing $a$ changes the slope of a line through the origin.
- $a=0$ is scanning, $a=1$ is conventional.

with Stokes shift

$C(m,m')$

Image formation in image scanning microscopy, including the case of two-photon excitation

Colin J. R. Sheppard, Marco Castello, Giorgio Tortarolo, Giuseppe Vicidomini, and Alberto Diaspro.
With Stokes shift, large array

- scanning is better than conventional
- true confocal is better
- ISM for optimum $a$ is even better
- ISM for $a=1/2$ is better than confocal for Stokes ratio $<1.4$
- $a=1/2$ is fine for Stokes ratio of 1.1

Normalized to scanning, no Stokes shift

**Stokes ratio = 1.5**

**Stokes ratio = 1.1**

**Research Article**

Image formation in image scanning microscopy, including the case of two-photon excitation

Colin J. R. Sheppard, Marco Castello, Giorgio Tortarolo, Giuseppe Vicidomini, and Alberto Diaspro
Effect of array size and Stokes shift

Curves normalized by FWHM for conventional fluorescence microscope

- For Stokes ratio of 1.1, \( a = 1/2 \) is OK
- As Stokes ratio increases, the improvement relative to conventional improves
- For small arrays, value of \( a \) doesn’t matter

Fig. 9. The FWHM of the PSF, normalized by the FWHM for conventional 1PE fluorescence microscopy, for ISM as a function of detector array size, with Stokes ratio \( \beta \) as parameter. The solid curves show the results for reassignment factor \( a = 1/(1 + \beta) \), and the dashed curves for \( a = 1/2 \).
Bessel beam, 1977-1980

• \( J_0 \) beam is propagationally invariant (1978):

The radial distribution of amplitude for a \( \delta \) ring is given by a zero-order Bessel function in any plane (in the region of validity) perpendicular to the optic axis. That this is so is not surprising because such a wave is the circularly symmetric mode of free space. We are acquainted with modes of this form in circular waveguides, and we can consider free space as the limiting case of a waveguide of very large diameter. Such an overmoded waveguide has an infinity of circularly symmetric modes, that is the scale of the Bessel functions may be chosen at will. A wave with zero-order Bessel-function radial distribution propagates without change.


Nonparaxial electromagnetic Bessel beam (1978):

\[ \varepsilon = 0.9 \]

low contrast!

C. J. R. Sheppard
The use of lenses with annular aperture in scanning optical microscopy

OPTICA ACTA, 1977, VOL. 24, NO. 10, 1051–1073

First paper to use term “confocal microscope” (1977)

Image formation in the scanning microscope

C. J. R. SHEPPARD and A. CHOUDHURY
Department of Engineering Science, Parks Road, Oxford, England

(Received 22 December 1976)

Abstract. Fourier imaging in the scanning microscope is considered. It is shown that there are two geometries of the microscope, which have been designated Type 1 and Type 2. Those of Type 1 exhibit identical imaging to the conventional microscope, whereas those of Type 2 (confocal microscopes) display various differences. Imaging of a single point object, two-point resolution and response to a straight edge are also considered. The effect of various arrangements using lenses with annular pupil functions is also discussed. It is found that Type 2 microscopes have improved imaging properties over conventional microscopes and that these may be further improved by use of one or two lenses with annular pupils.

Confocal microscope with Bessel beam (1980):

United States Patent [19]

Sheppard

4,198,571


[54] SCANNING MICROSCOPES

[75] Inventor: Colin J. R. Sheppard,
Bessel beam in confocal

- Normalized OTFs
- But confocal has very low signal level
- What about ISM with Bessel beam?

Fig. 17. The OTFs for a confocal microscope with illumination by a Bessel beam, a confocal microscope with two circular pupils, and ISM with two circular pupils and a large array. The OTFs are normalized to unity at zero spatial frequency.
ISM with pupil filters

Bessel beam (annular filter) gives poor response at mid-frequencies because

\[ C_{\text{eff}}(l) = C_1[(1 - a)l]C_2(al). \]

Parabolic amplitude filter is better

Optimization of pupil filters for maximal signal concentration factor

C. J. R. Sheppard
Optimum $a$ varies with spatial frequency

Fig. 16. A contour plot of $C(m, m')$ for 1PE fluorescence with a parabolic filter $b = 1$ and no Stokes shift. The approximate locus for $a(m)$ to maximize $C(m)$ is shown (dashed line).
Multiphoton microscopy

Proposal of different types of scanning nonlinear microscopy based on the high intensity in the focused spot, including two-photon fluorescence and CARS (1978)

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

C. J. R. Sheppard and R. Kompfner


First published scanning SHG images (1978)

KD*P crystal, SHG images CW NdYAG laser 1064nm

Demonstrates optical sectioning

Second-harmonic imaging in the scanning optical microscope

J. N. Gannaway, C. J. R. Sheppard

Radial polarization (2007)

3D SHG with fs pulses (1998)

Rat tail tendon

Second harmonic generation polarization microscopy with tightly focused linearly and radially polarized beams

E.Y.K. Yew, C.J.R. Sheppard

Optics Communications 275 (2007) 453–457

Image formation in two-photon fluorescence microscopy

C. J. R. Sheppard, M. Gu
Two-photon fluorescence ISM

Fig.12. Plot of $C(m,m')$ for 2PE fluorescence, and no Stokes shift.
Two-photon fluorescence ISM

Fig. 1. The OTF for two-photon fluorescence with different reassignment factors, $a$. A value of zero gives a 2PE fluorescence microscopy with a large detector. A value of unity gives an image identical to that in a conventional 1PE fluorescence microscope. The

- Can alter reassignment factor $a$
- OTF is

$$C_{\text{eff}}(l) = C_1[(1-a)l]C_2(al).$$

- Resolution improved compared with two-photon fluorescence with a large single-element detector

Fig. 2. The useful cut-off frequency $m$, as a function of the noise level, for pixel reassignment with the optimum value of reassignment factor $a$ (green curve). The optimum value of $a$ is also shown (blue curve). The useful cut-off frequencies for conventional 1PE and 2PE are shown for comparison (purple and red curves, respectively).

Research Article

Image formation in image scanning microscopy, including the case of two-photon excitation

Colin J. R. Sheppard, Marco Castello, Giorgio Tortarolo, Giuseppe Vicedomini, and Alberto Diaspro
Medusa

Mauro Buttafava & Alberto Tosi
DEIB, Politecnico di Milano,
Via Ponzio 34, Milan, Italy

Marco Castello, Giorgio Tortarolo,
Giuseppe Vicidomini, Alberto Diaspro,
IIT, Genova, Italy
SPAD array

The first APD array designed for microscopy.

Features:
- 5 x 5 matrix
- 75 µm pitch
- 50 µm x 50 µm active area
- fill factor $= \frac{50^2}{75^2} \approx 44\%$
  (future improvement: microlenses array)
- 25 TTL signals + (3 ch.) communication bus

Mauro Buttafava & Alberto Tosi
DEIB, Politecnico di Milano, Via Ponzio 34, Milan, Italy

Marco Castello, Giorgio Tortarolo, Giuseppe Vicidomini, Alberto Diaspro,
IIT, Genova, Italy

<table>
<thead>
<tr>
<th>SPADs</th>
<th>Crosstalk probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>First neighbors - orthogonal</td>
<td>&lt; 1%</td>
</tr>
<tr>
<td>First neighbors - diagonal</td>
<td>&lt; 0.2%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hold-OFF Time</th>
<th>Afterpulsing probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 ns</td>
<td>6.5%</td>
</tr>
<tr>
<td>100 ns</td>
<td>2.4%</td>
</tr>
<tr>
<td>200 ns</td>
<td>1.4%</td>
</tr>
</tbody>
</table>

Uniformity:
Biological sample: Tubuline

Confocal (open pinhole)  ISM (pixel reassignment 5x5)  ISM (deconvolution)

Mauro Buttafava & Alberto Tosi
DEIB, Politecnico di Milano,
Via Ponzio 34, Milan, Italy

Marco Castello, Giorgio Tortarolo,
Giuseppe Vicidomini, Alberto Diaspro,
IIT, Genova, Italy
Discussion

• Structured illumination can give improved resolution (x2)
• Confocal microscopy gives improved resolution but spatial frequency response at high spatial frequencies is low ($\times \sqrt{2}$ in PSF)
• But signal is also low, so must open pinhole, giving almost no improvement in resolution
• Pixel reassignment increases signal collection efficiency
• Also gives improved resolution, better than confocal
• And speed is increased
• ISM with 2 photon excitation improves resolution
• ISM with pupil filters can improve high frequency response
Single-pixel camera

256x256 pixels

1300 random measurements with compressive sensing

32x32 = 1296 pixels bicubic upsampled

Larkin
http://www.nontrivialzeros.net/Hype _&_Spin/Misleading%20Results%20in%20Single%20Pixel%20Camera-v1.02.pdf

2041 citations