Confocal Microscopy

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Schematic diagram of confocal microscope
Depth discrimination
Depth-discrimination

Fig. 3. (a) Conventional scanning microscope image. (b) Confocal-scanning microscope image.

Hamilton DK, Wilson T, Sheppard CJR

Experimental observations of the depth-discrimination properties of scanning microscopes

Properties of confocal

• Optical sectioning
• Reduced scattered light
• Improved resolution
Confocal microscopy

– Reflection
  • Industrial applications, surface profiling
  • Scattering media, tissue

– Fluorescence
  • Autofluorescence or labelled
  • Fixed or living
Confocal profiling

- Form 3-D image
- Find axial position of maximum intensity, $z_{\text{max}}(x, y)$
Sampling
Autofocus and surface profile
Isometric view
Goldman, 1940

Slit-scanning confocal with angular gating

Spaltlampenphotographie und -photometrie, Ophthalmologica 98, 257-270 (1940).
ある条件で鍍金した試料を用いて、光の透過性と反射性を検証する実験を行った。特に、試料の表面処理とその反射特性に注目し、より優れた光学特性を示す材料の開発を目的としている。

症例1: （a）は反射型の像をレンズCに投げて、垂直な光学系でDの上に反射させ、再びレンズL_1によって反射型の像をDの上に投げて、その結果を確認した。これにより、反射型の光学系における反射特性を把握することができた。

症例2: （b）は透過型の像をレンズCに投げて、垂直な光学系でDの上に透過させ、再びレンズL_1によって透過型の像をDの上に投げて、その結果を確認した。これにより、透過型の光学系における透過特性を把握することができた。
Tandem scanning microscope
(Nipkow disc microscope)
Petráň 1968

FIGURE 1. Tandem scanning reflected light microscope (Petráň et al., 1968).

Petráň, M. & Hadtrvský, M. (1966) Způsob a zarizení pro omezení rozptylu světla v mikroskopu pro osvětlení 
Oxford microscope, 1975

This short review of the development of Scanning Optical Microscopy at Oxford was originally prepared for MICRO 89, but was lost in the post. It contains some of the early micrographs, none of which has been published before.

15 Years of Scanning Optical Microscopy at Oxford
C. J. R. SHEPPARD

First image

Beam-scanning
Confocal reflectance

Stereo pair of a pollen grain


Rat brain (cerebellum)

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

Colour confocal reflection image of a leaf

Microtubules labeled with 15nm gold

Confocal microscope with computer


Conventional confocal autofocus surface profile
Commercialization of confocal microscope

- Oxford Optoelectronics 1982
- LaserSharp SOM100, 1984
- BioRad MRC500, 1987
Methods of scanning

Beam scanning
- Galvo-mirrors
- Feed-back stabilized
- Resonant
- Nipkow disc
- Polygon mirrors
- Acousto-optic

Object scanning
- Electro-mechanical
- Stepper motors
- Piezoelectric

Lens scanning

Light also needs to be descanned on the way out
Scanning arrangements
(b) Close-coupled mirrors
(c) Relay system
Pinholes

Two pinholes are different:

• 1 is spatial filter (pinhole is bigger than the spot)
  (Don’t really need this if using a laser)
• 2 is smaller than the spot.
Fibre optical confocal
(single-mode fibre used as pinhole)

MR Harris, US patent 5120953,
filed 25 June 1990
Acousto-optic scanning + slit (a: Noran)

For fluorescence cannot descan with acousto-optic device (Stokes’ shift)
Acousto-optic scanning + linear CCD (b: Lasertec)
Two slits + CCD (c: Meridian)
Two slits + linear CCD (d: Zeiss 5 Live)

Note that the Fourier transform of a line in $x$ is a line in $y$
Scanning vs. conventional microscope

- Conventional
- Conventional with image scanning or CCD detector

Scanning

- Scanning microscope (Type 1)
- Confocal microscope (Type 2)
Coherent Imaging

\[ t(x, y) \quad (t \otimes h) \]
Confocal Imaging
(not fluorescence)

After sample

\( x_s, y_s \) are scan coordinates

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

- Pinhole: $x_d, y_d = 0$

\[
I(0,0) = \left| \int \int h_1(x, y)h_2(-x,-y)t(x-x_s,y-y_s) dx dy \right|^2
\]

\[
= \left| (h_1(x, y)h_2(-x,-y)) \otimes t(x, y) \right|^2
\]

$h_2$ even:

\[
I = \left| (h_1h_2) \otimes t \right|^2
\]

- Coherent microscope, with $h_{\text{eff}} = h_1h_2$
\[ I(x_d, y_d) = \left| \iint U(x', y') \exp\left\{ -\frac{ik}{f} (x'x_d + y'y_d) \right\} dx'dy' \right|^2 \]

\[ I(0,0) = \left| \iint U(x', y') dx'dy' \right|^2 \]

The pinhole integrates **amplitude** over the back focal plane.
The pinhole integrates amplitude over the back focal plane.

Put

\[ U(x', y') = \left( \iint h_1(x, y) t(x - x_s, y - y_s) \exp\left\{-\frac{ik}{f} (xx' + yy')\right\} dx dy \right) P_2(x', y') \]

Then

\[ I(0, 0) = \left| \iint \iint h_1(x, y) t(x - x_s, y - y_s) \exp\left\{-\frac{ik}{f} (xx' + yy')\right\} P_2(x', y') dx' dy' dx dy \right|^2 \]

as before
Scanning acoustic microscope is confocal

Figure 2. Scanning microscopes of Type 2.

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Image formation in the scanning microscope

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

So confocal imaging is coherent imaging with an effective amplitude point spread function

\[ h_{\text{eff}} = h_1 \, h_2. \]
Image of a point object

Normalized intensity $I$

Normalized lateral position, $\nu$

Sharpened up by a factor $\sim \sqrt{2} = 1.4$
Images of two points

(a) Coherent

(b) Full illumination

(c) Incoherent

(d) Confocal reflection

(e) Confocal fluorescence

Blue lines correspond to Rayleigh separation
Two-point resolution
For a coherent system, the coherent transfer function is given by a scaled pupil function:

\[ c(m, n) = P(\lambda mf, \lambda nf) \]

As the pupil function is the Fourier transform of the point spread function, for confocal \( c = F\{h_1 h_2\} \)

\[ c(m, n) = P_1(\lambda mf, \lambda nf) \otimes P_2(\lambda mf, \lambda nf) \]

convolution of psfs for objective and collector
$C(m;p)$ (confocal)

Conventional: full, complete, or matched illumination ($S = 1$)

$$I(x_s, y_s) = \text{const.} \int \int \int_{-\infty}^{+\infty} C(m, n; p, q) T_0(m, n) T_0^*(p, q) \exp(2\pi j \{(m-p)x_s + (n-q)y_s\}) \, dm \, dn \, dp \, dq,$$

Maximum frequency in image is when $m = 1$, $p = -1$

C. J. R. SHEPPARD and A. CHOUDHURY
Image formation in the scanning microscope
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Transmission and reflection

For defocused systems, we must distinguish between transmission and reflection systems. For transmission the defocus of the two lenses are equal and opposite

\[ P_2 = P_1^* \]

so that the coherent transfer function \( c \) is purely real, but for a reflection system

\[ P_2 = P_1 \]

and \( c \) is in general complex.
Intensity in focal region

Fig. 8.41. Isophotes [contour lines of the intensity $I(u, v)$] in a meridional plane near focus of a converging spherical wave diffracted at a circular aperture. The intensity is normalized to unity at focus. The dotted lines represent the boundary of the geometrical shadow. When the figure is rotated about the $u$-axis, the minima on the $v$-axis generate the Airy dark rings.

3-D image of a point object

(a) Conventional

(b) Confocal
Axial signal from a mirror

Normalized intensity, $I$

\[ I(z) = \left| \int_0^\theta P^2(\theta) \exp(2ikz \cos \theta) \sin \theta \cos \theta \, d\theta \right|^2 \]

\[ = \left| \int_0^\theta [P(\theta) \cos^{1/2} \theta]^2 \exp(2ikz \cos \theta) \sin \theta \, d\theta \right|^2 \]
Optical sectioning in line illumination or aperture array microscopes

• Confocal, decays as $1/z^2$
• Line illumination, decays as $1/z$
• Aperture array, tends to a constant (cross-talk)

C. J. R. SHEPPARD and X. Q. MAO
Confocal microscopes with slit apertures
JOURNAL OF MODERN OPTICS, 1988, VOL. 35, NO. 7, 1169-1185