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WINTER COLLEGE ON OPTICS

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Confocal Scanning Microscopy

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Confocal Scanning Microscopy

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Confocal Scanning Microscopy

This short course is divided into three lectures.

(i) Introduction to confocal microscopy

In this lecture the confocal optical system is introduced and the origins of the resolution enhancement and the optical sectioning or depth discrimination property are explained. The consequences of this unique imaging mode are illustrated with many examples.

(ii) Imaging modes in Confocal microscopy

The three-dimensional imaging of the confocal microscope arises because of the use of a point, pinhole, detector. The image formation becomes more versatile if optical fibres rather than pinholes are used to detect the image signal. In this way differential amplitude and differential phase contrast images may be obtained in addition to the confocal image. Finally, various interference and optoelectronic implementations will be described.

(iii) Real time Confocal microscopy with white light

The most common configuration of confocal microscope uses a laser as light source and does not usually form an image in real time. These drawbacks may be alleviated to some extent by constructing a number of parallel confocal systems which image adjacent parts of the object. This is the basis of the tandem scanning or Nipkow disc based microscopes which permit real time imaging with white light sources. However these systems are light inefficient and so three relatively new approaches will be discussed. They are based on the use of microlens arrays and aperture correlation techniques.

Books on Confocal Microscopy

T. Wilson and C.J.R. Sheppard. Theory and Practice of Confocal Microscopy, Academic Press, 1984. A little dated now but contains the basic optical theory.

T. Wilson (Editor). Confocal Microscop, Academic Press, 1990. A multi-author volume covering many aspects and applications of confocal microscopy.

J.B. Pawley (Editor). Handbook of Biological Confocal Microscopy, Plenum, 1995. A multi-author volume concentrating on biological applications. The book also has details of many commercially available systems.

Min Gu. Principles of three-dimensional imaging in confocal microscopes, World Scientific, 1996. An extremely thorough theoretical discussion based on the use of the three dimensional transfer function approach.

T.R. Corle and G.S. Kino. Confocal Scanning Optical Microscopy and Related Imaging Systems, Academic Press, 1996. A useful book concentrating on the non-biological aspects and applications of confocal microscopy.

These are the main book references. The main source of journal articles is probably the following:

Applied Optics
Optics Letters
Journal of Optical Society of America
Optics Communications
Journal of Modern Optics
Journal of Microscopy
Bioimaging
Scanning

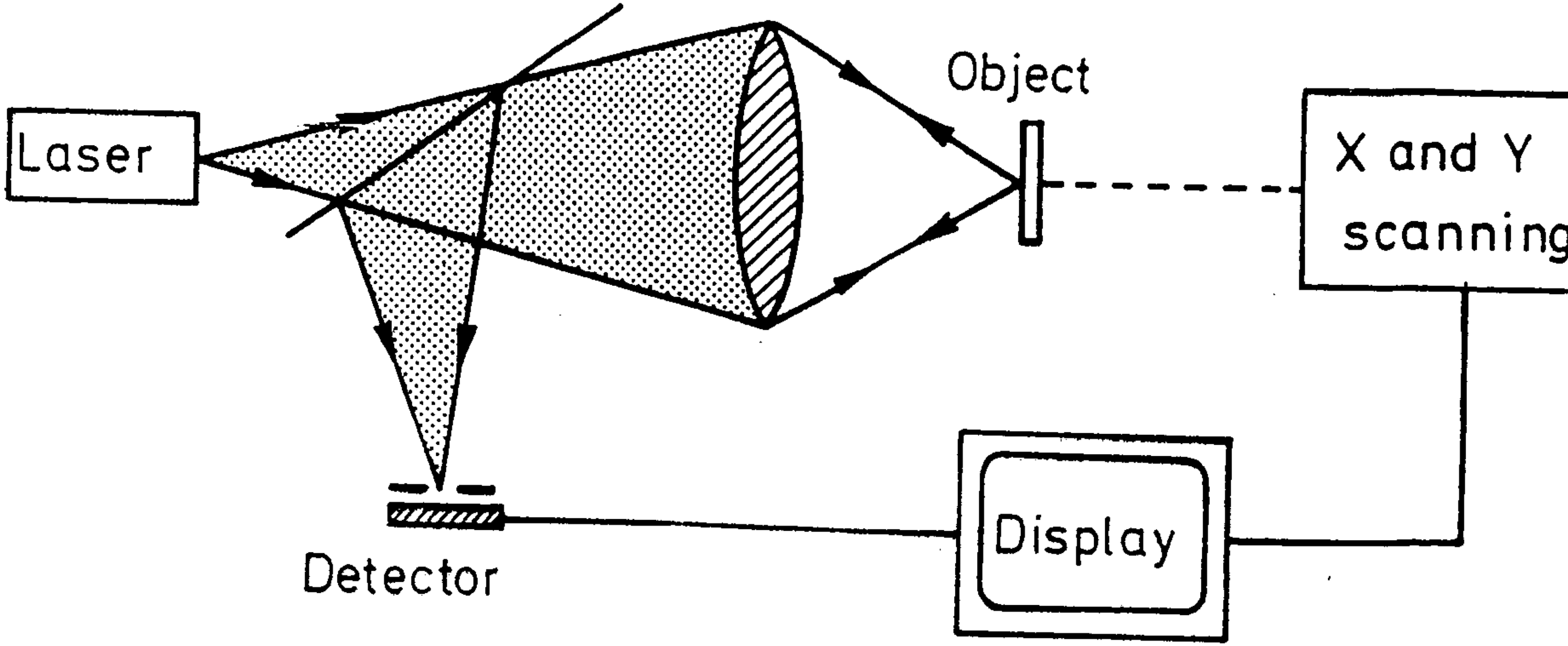
Confocal Microscopy

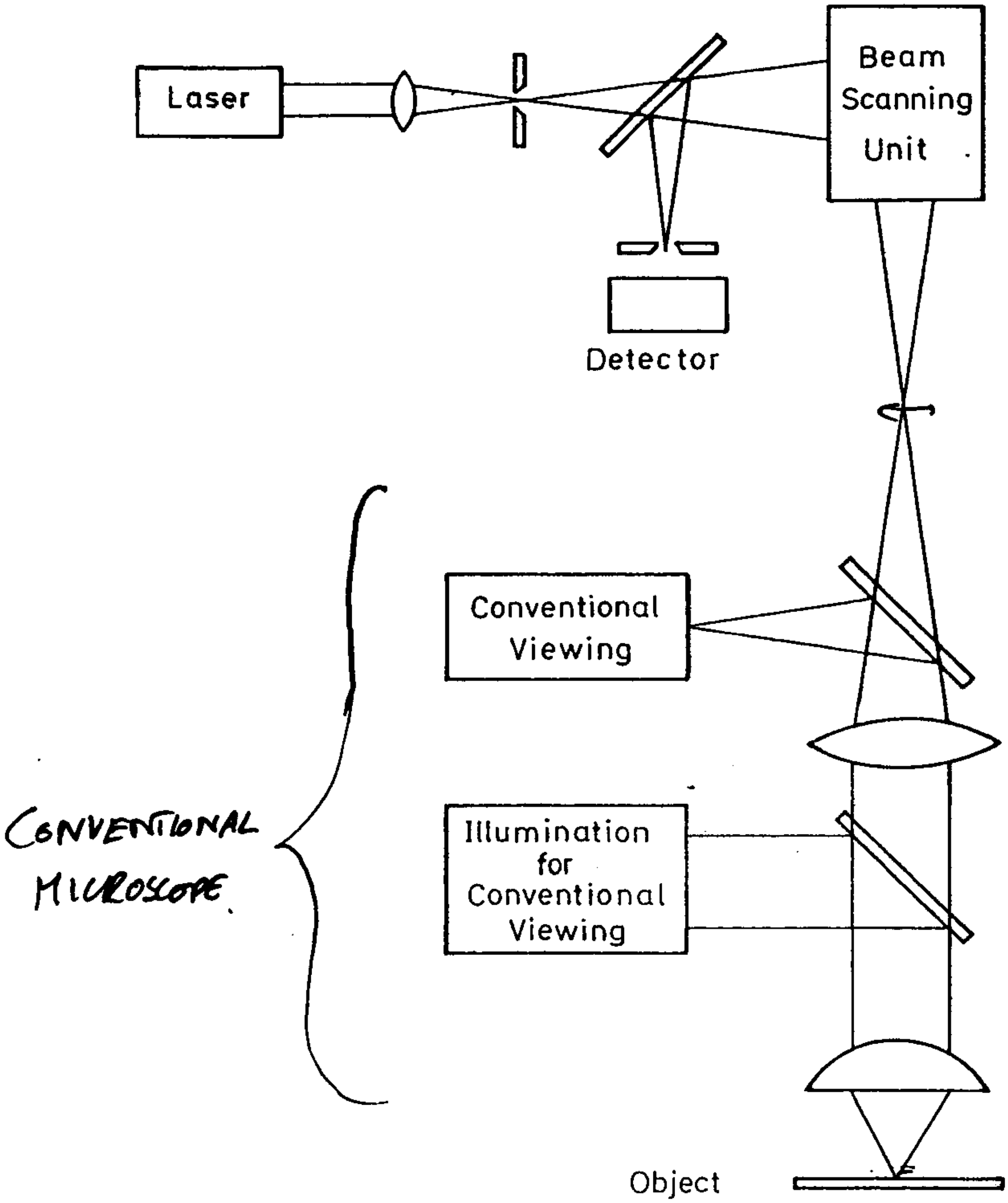
- Implementations
- Lateral Resolution
- Axial Resolution

↓

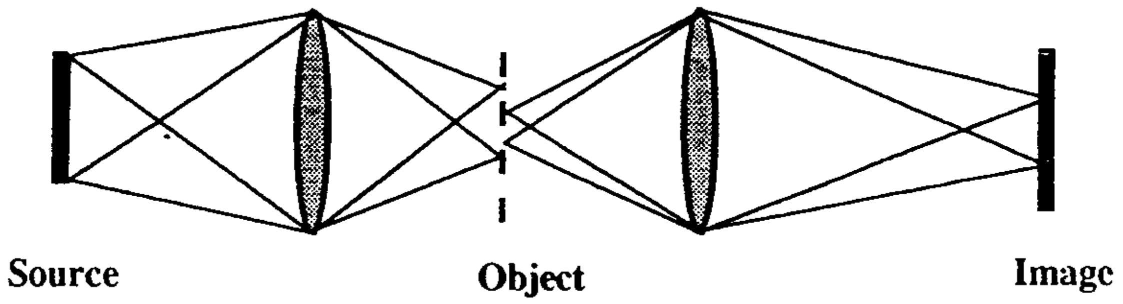
"3-D" imaging

Examples.

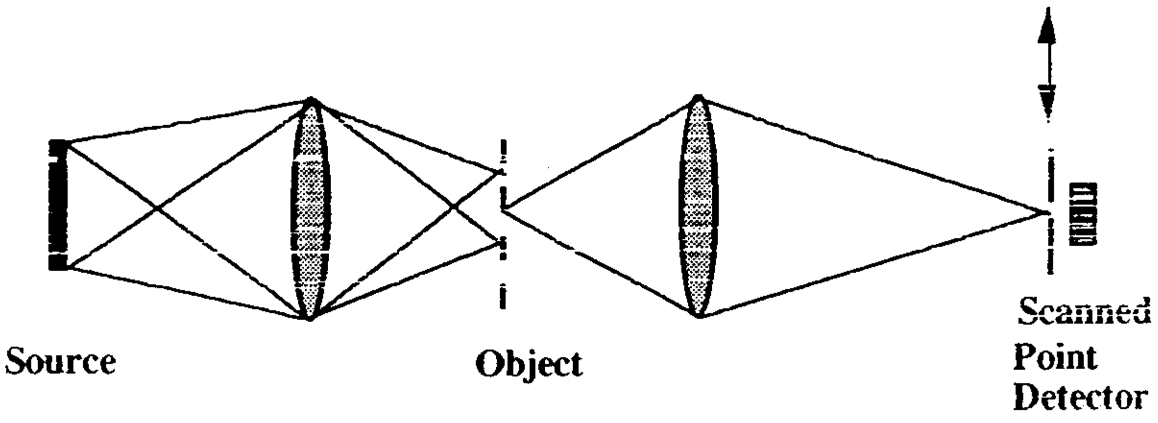

Laser
Object
X and Y
scanning
Detector
Display

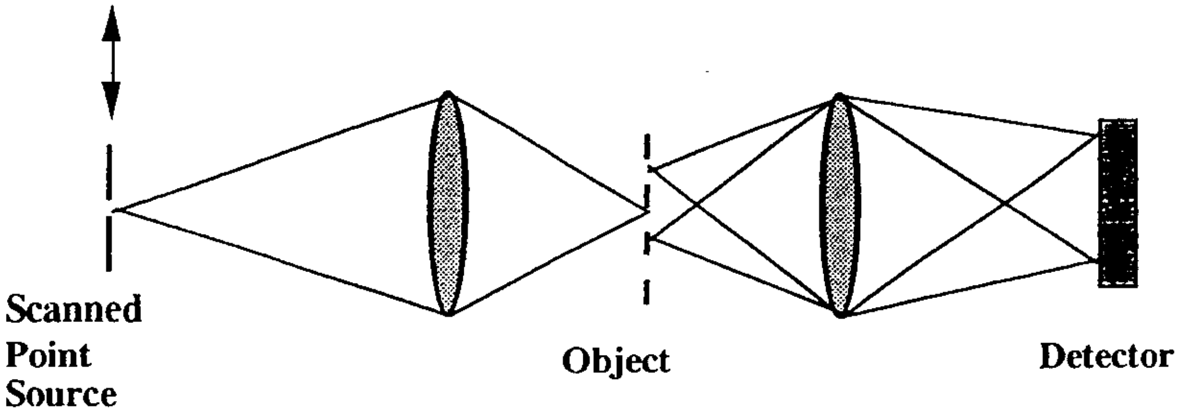

Laser
Beam Scanning Unit
Detector
Conventional Viewing
Illumination for Conventional Viewing
Object
CONVENTIONAL MICROSCOPE

Conventional Microscope

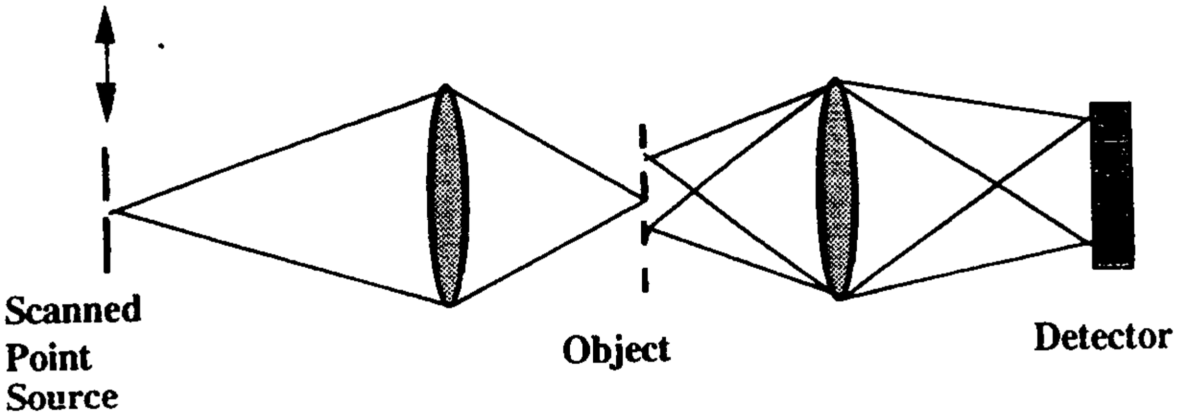


Scanning Microscope

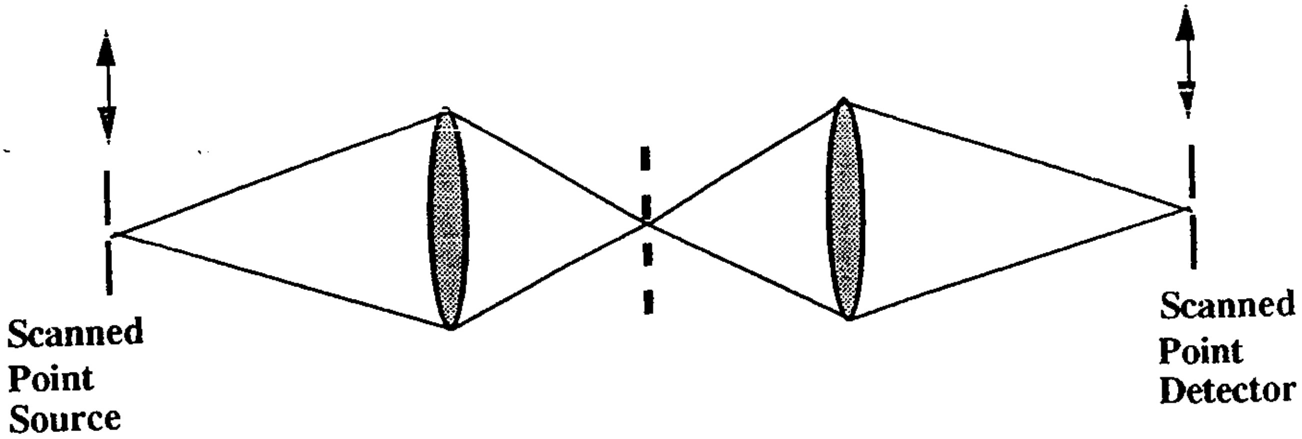




Scanning Microscope



Confocal Microscope



Confocal Imaging

①. Coherent optical system

$$I = \left| h_1 h_2 \underset{\substack{\uparrow \\ \text{correlation}}}{\otimes} t \right|^2$$

h is point spread function (amplitude) of lens

$$h \approx \mathcal{F}\{P\}$$

Eg point object

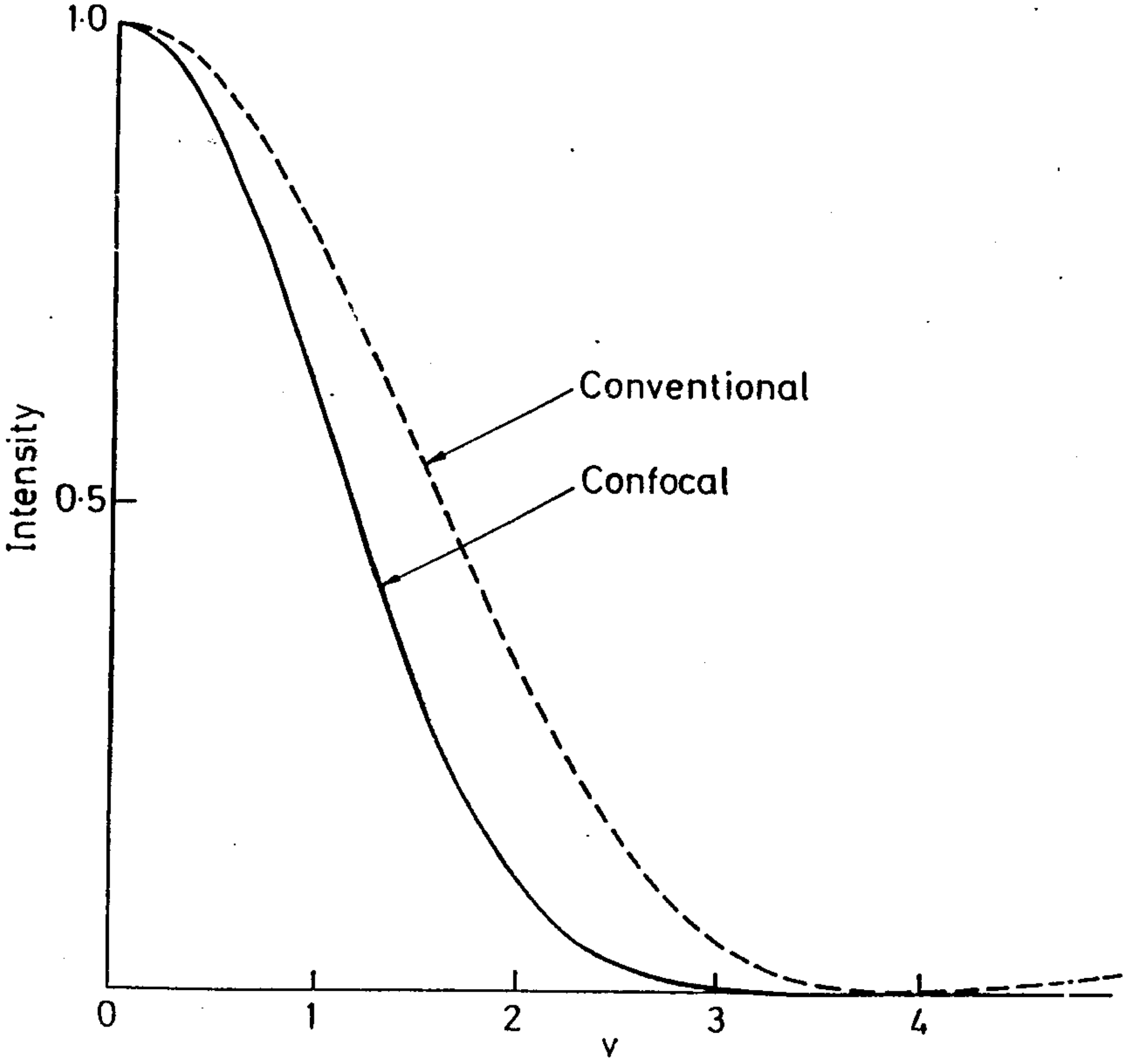
$$I = |h_1 h_2|^2 = \left(\frac{2 J_1(v)}{v} \right)^4$$

conventional

$$I = |h_1|^2 = \left(\frac{2 J_1(v)}{v} \right)^2$$

$$v = \frac{2\pi}{\lambda} \cdot r \cdot \underset{\substack{\uparrow \\ \text{N.A. of lens}}}{\sin \alpha}$$

Image of a Point Object – Confocal / Conventional.



Point Detector - Coherent Optical System

$$I = \left| h_1 h_2 \otimes t \right|^2$$

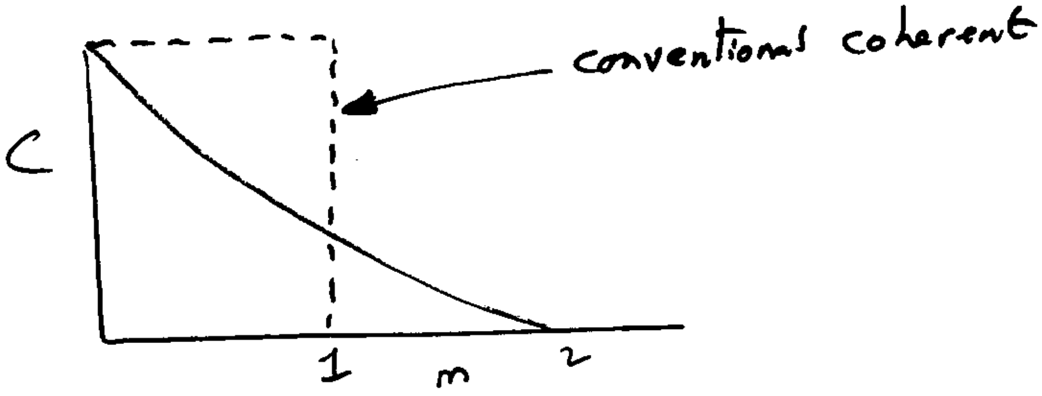
Point Object

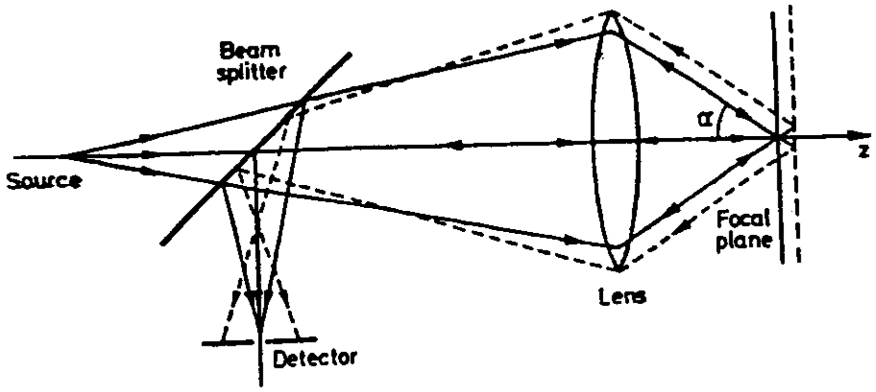
$$I = \left[\frac{2 J_1(v)}{v} \right]^4$$

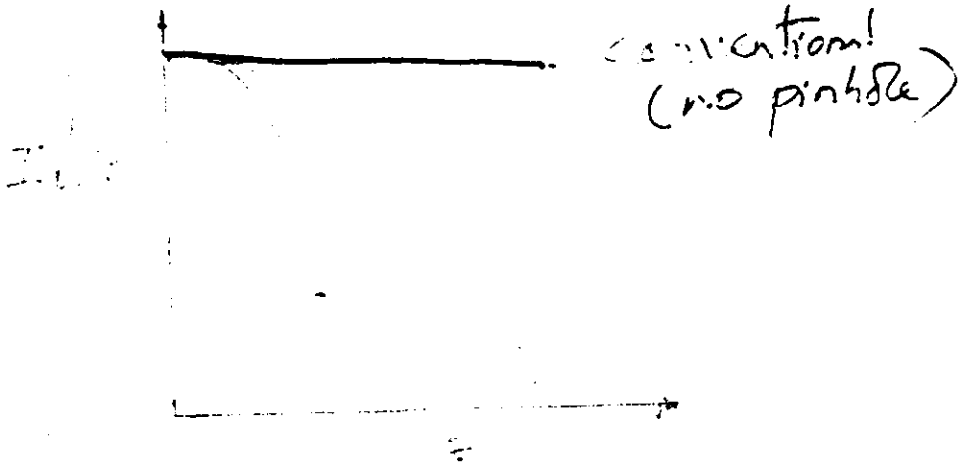
compare conventional microscope $\left[\frac{2 J_1(v)}{v} \right]^2$

Transfer function

$$c(m,n) = P_1 \otimes P_2$$

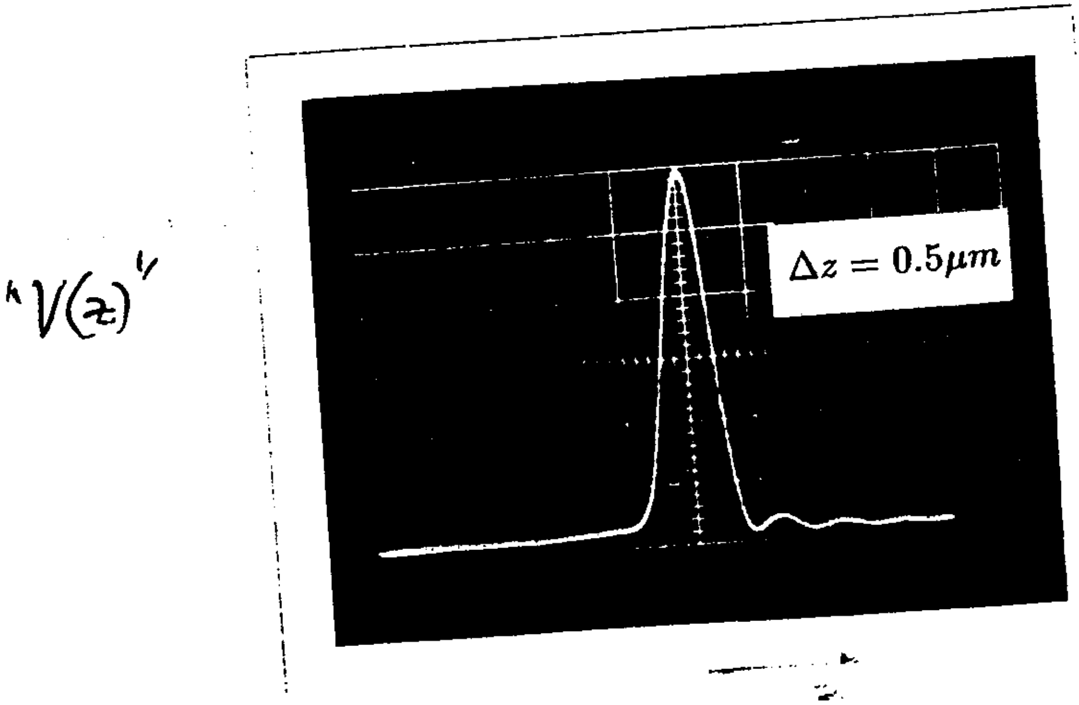


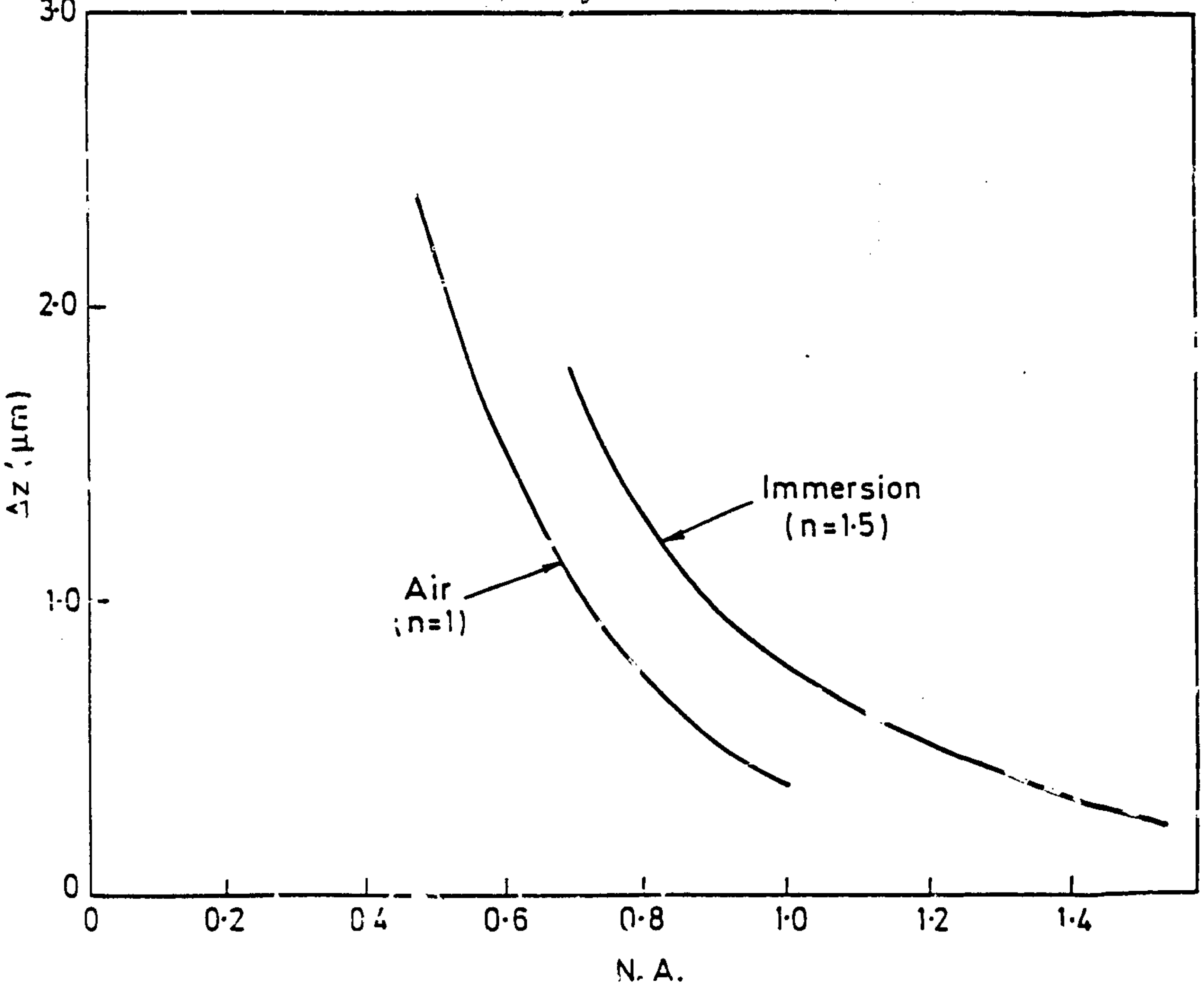




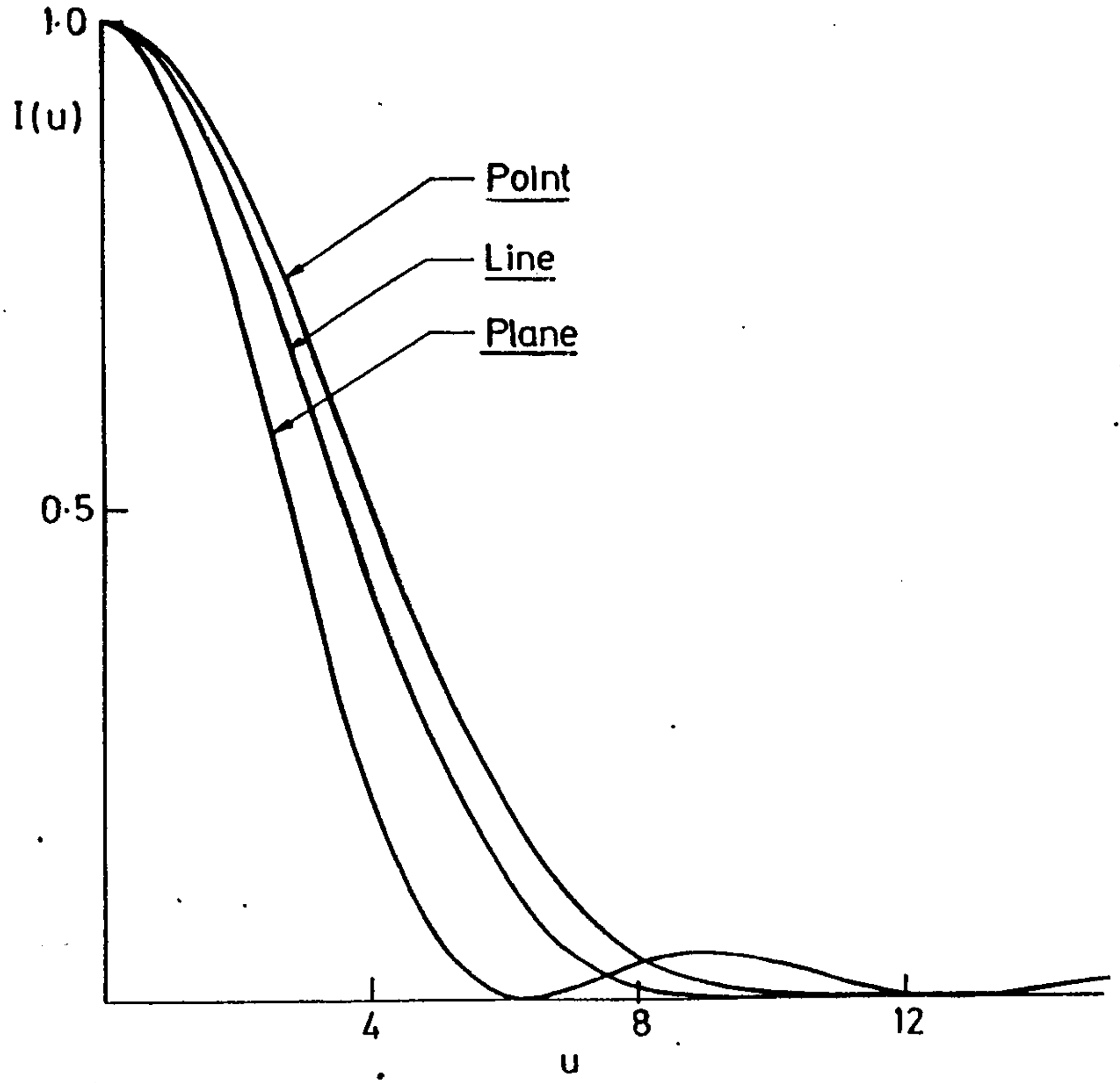
∴ possibility of through focus series of images

Use plane reflector as metric of sectioning.




3·0
2·0
1·0
0
Δz (μm)
0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
N. A.
Immersion
(n=1·5)
Air
(n=1)

Sectioning Strength – object dependent.



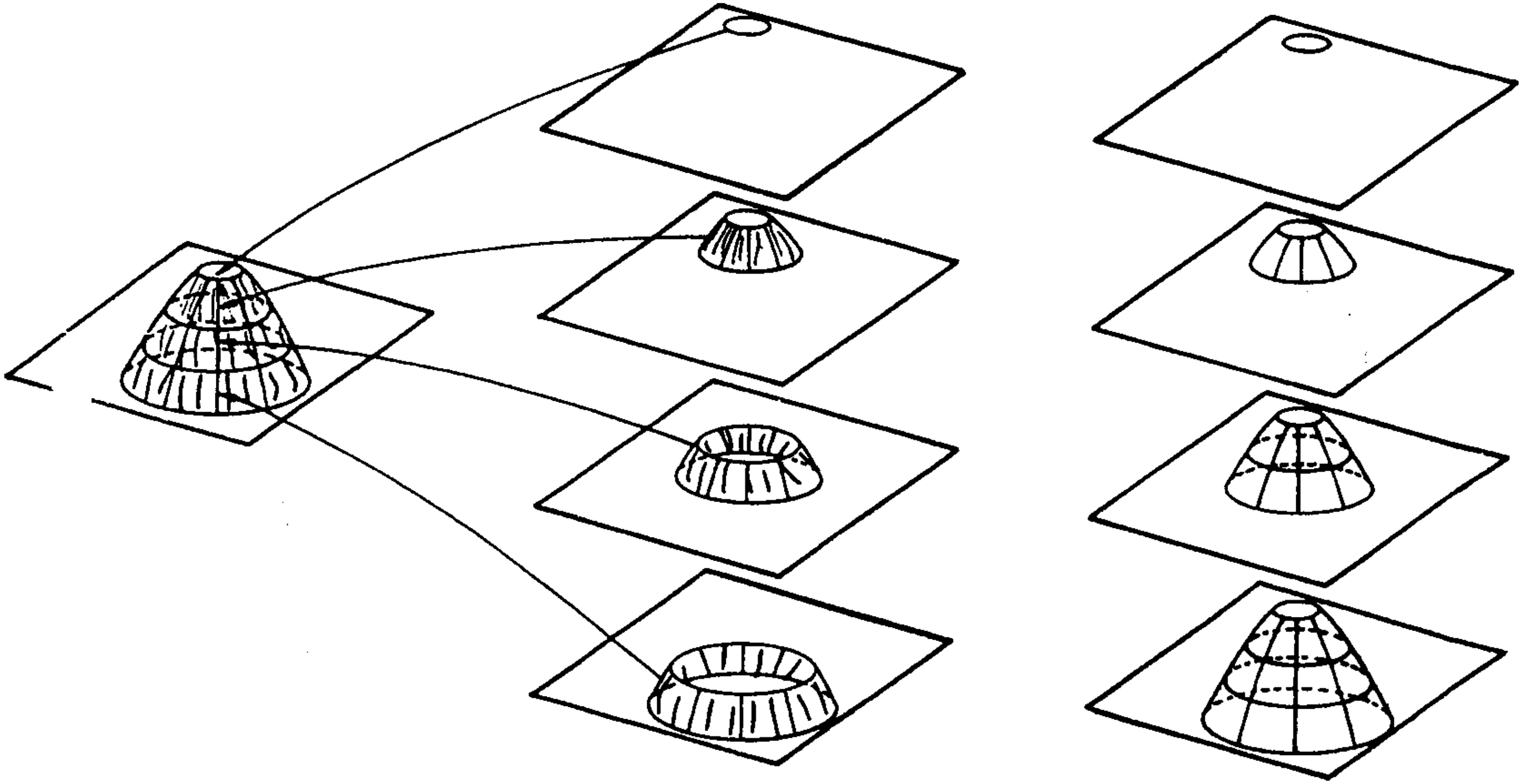
$u \sim z$

plane

$$I(u) = \left(\frac{\sin u/2}{u/2}\right)^2$$

$$\left[u = \frac{8\pi}{\lambda} . z . \sin^2 \alpha/2\right]$$

"optical sections"



LIMITS OF RESOLUTION

LATERAL RESOLUTION (Full width half maximum) (confocal)

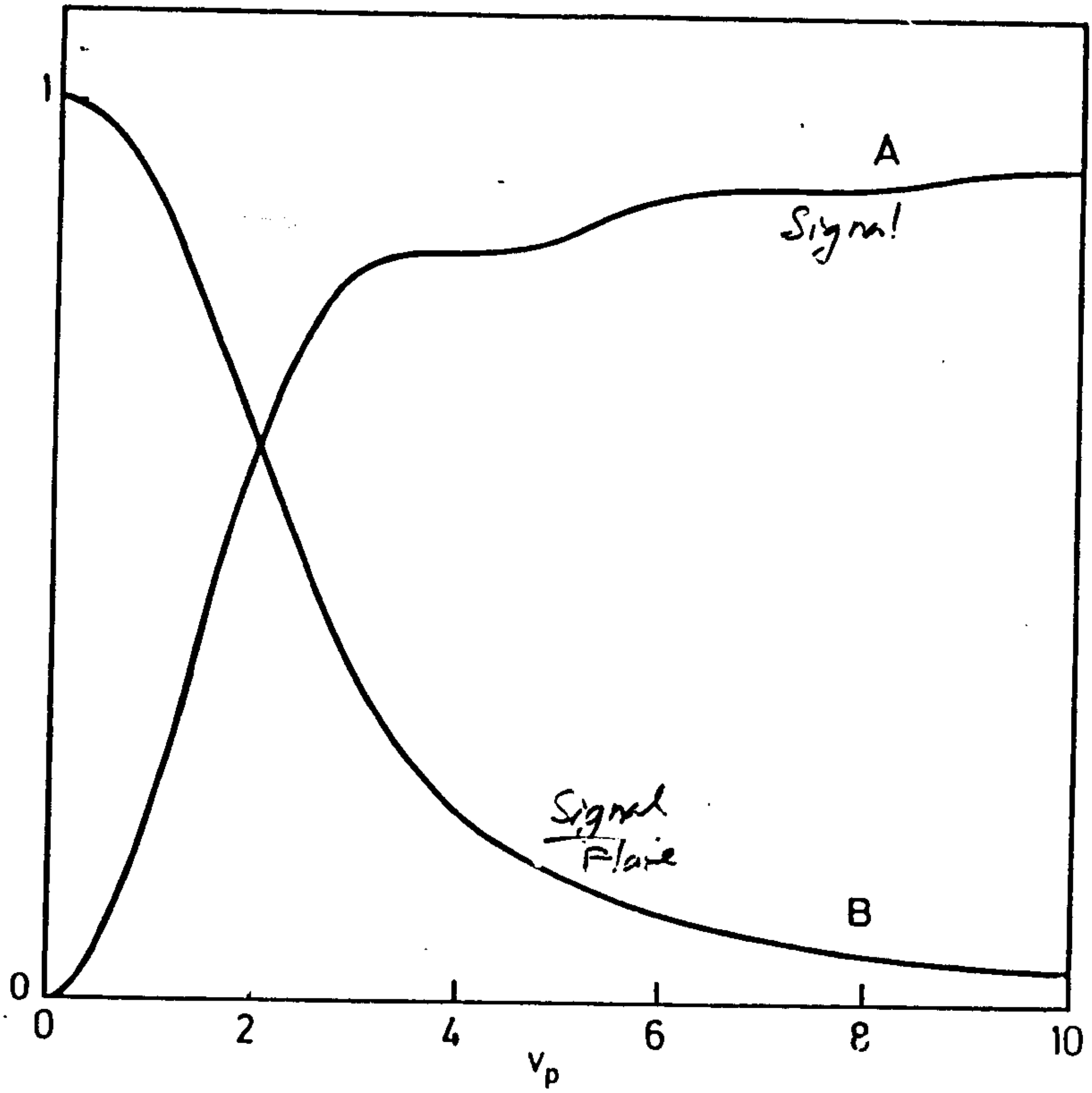
	Wavelength	
NA	442 nm	193 nm
.95	.20	.09 µm
1.4 (immersion)	.14 µm	.06 µm

DEPTH RESOLUTION (FWHM) (Confocal)

	Wavelength	
NA	442 nm	193 nm
.95	.34 µm	.15 µm
1.4 (immersion)	.23 µm	.10 µm

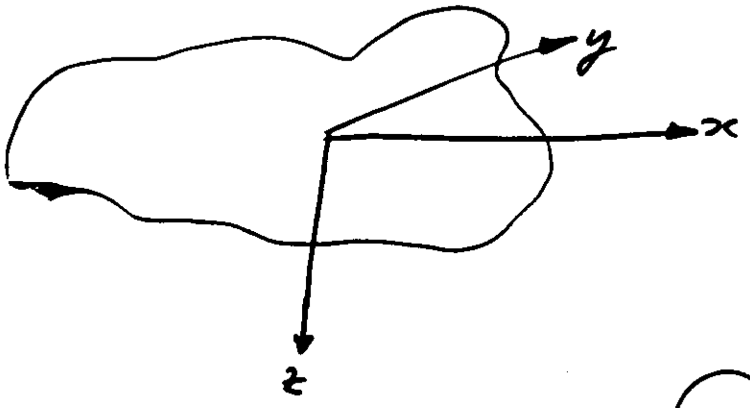
N.B. Depth sensitivity is better than 1 Å (interference methods)

Flare and Scatter



$$V_p = \frac{2\pi}{\lambda} r_p \, n \sin\alpha$$

Pinhole rejects light from everywhere but the object.



choose by "focus"

Confocal section $I(x, y, z)$ (x-z image)

sectioning stronger than depth of focus.

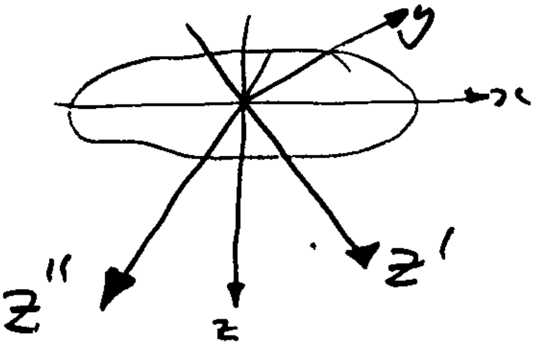
Extended focus $I_{EF}(x,y) = \int_{-\infty}^{+\infty} I(x,y,z)\,dz$

introduce axial scan

Autofocus $I_{AF}(x,y) = I_{max}(x,y)$

Profile z_{max} corresponding to $I_{max}(x,y)$

Stereo stack sections at angles to give L.H and R.H. vi

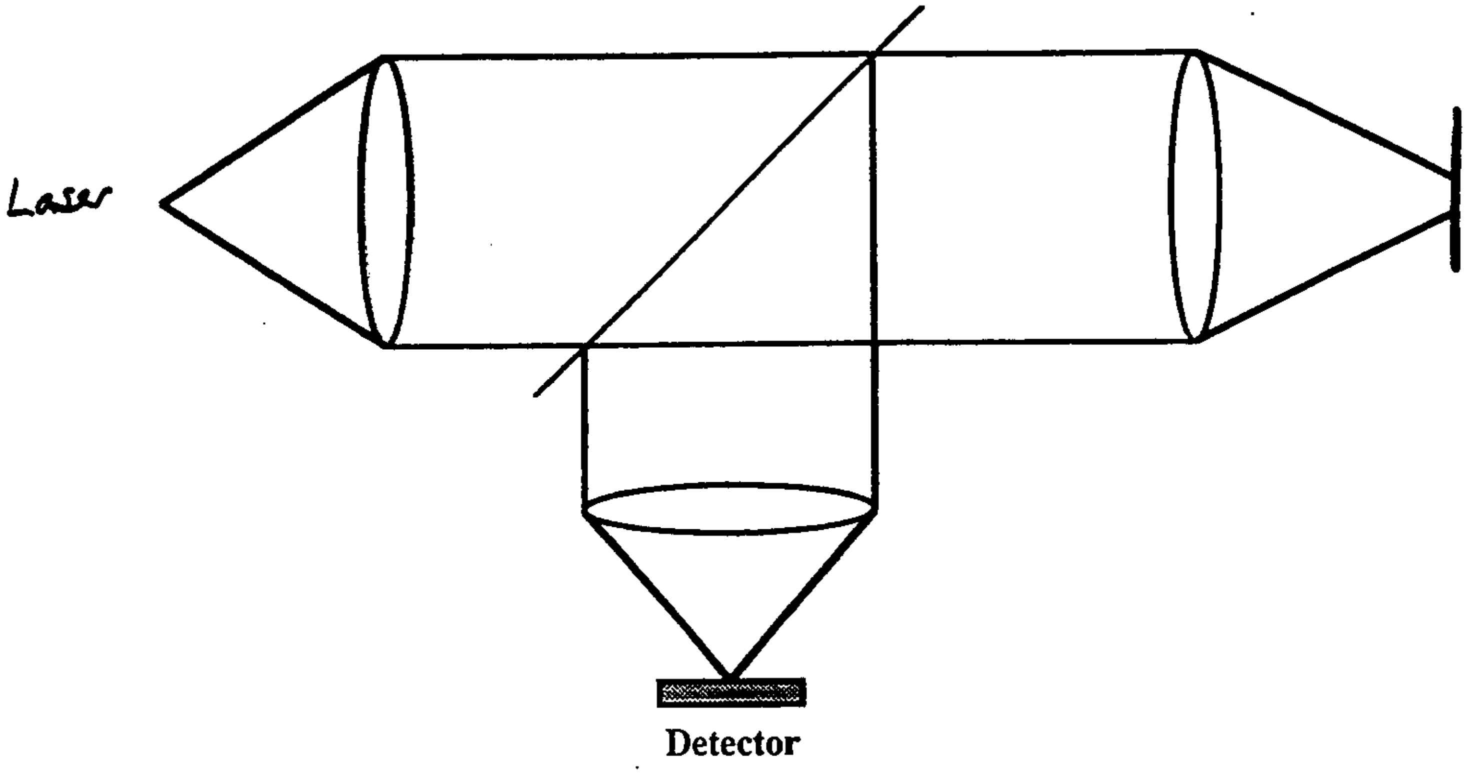


Confocal Microscopy

Coherent Detection

- Optical fibres
  - single mode
  - two mode
- Interference microscopy
- Laser Feedback microscopy

Coherent Detection



Incoherent Detector

$$I = \int |U|^2 \, dS$$

Coherent Detector

$$I = \left| \int U \, dS \right|^2$$

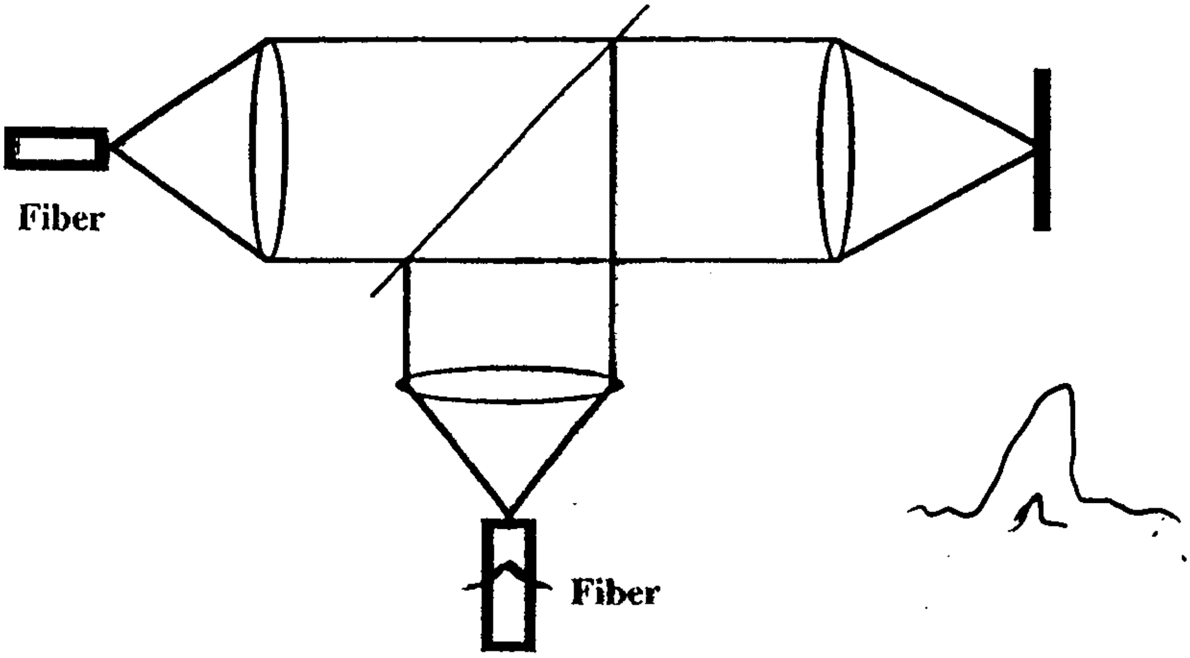
Examples: **Mimic optically**

Single mode optical fibre

Multi-mode optical fibres
Differential imaging

Optical Fiber Detection

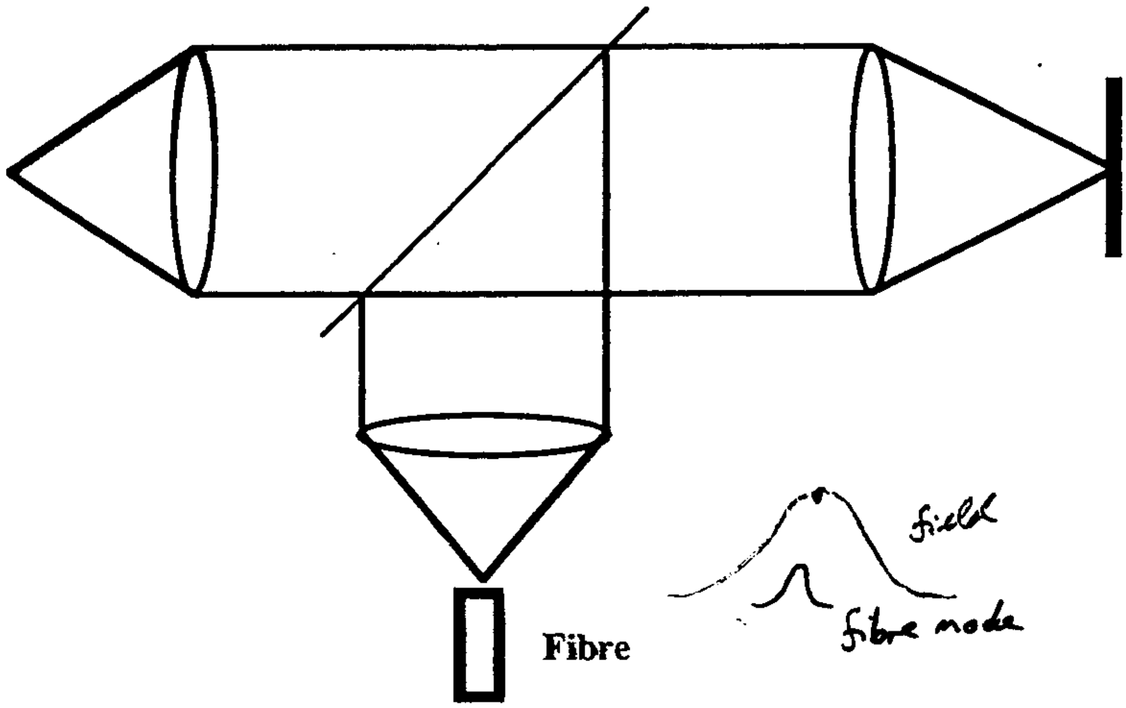
Replace pinhole with fiber



Monomode fiber -- confocal

Two - mode fiber -- field amplitude and
differential amplitude detected
Confocal and differential contrast
Height Contrast

Coherent Detection -- Optical Fibres.



If field at fibre tip is U then modes are excited as

$$a_i = \int U\, e_i\, dS$$

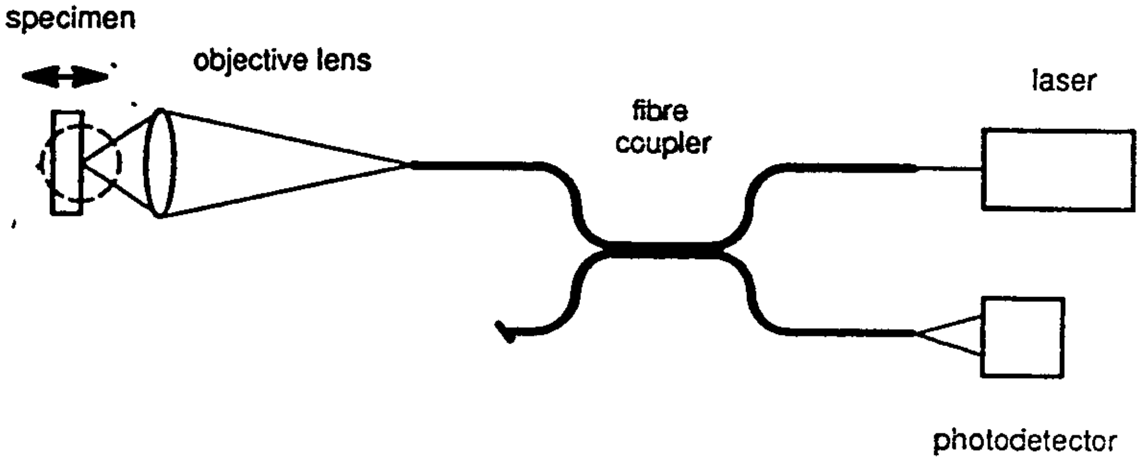
If one mode only excited we detect

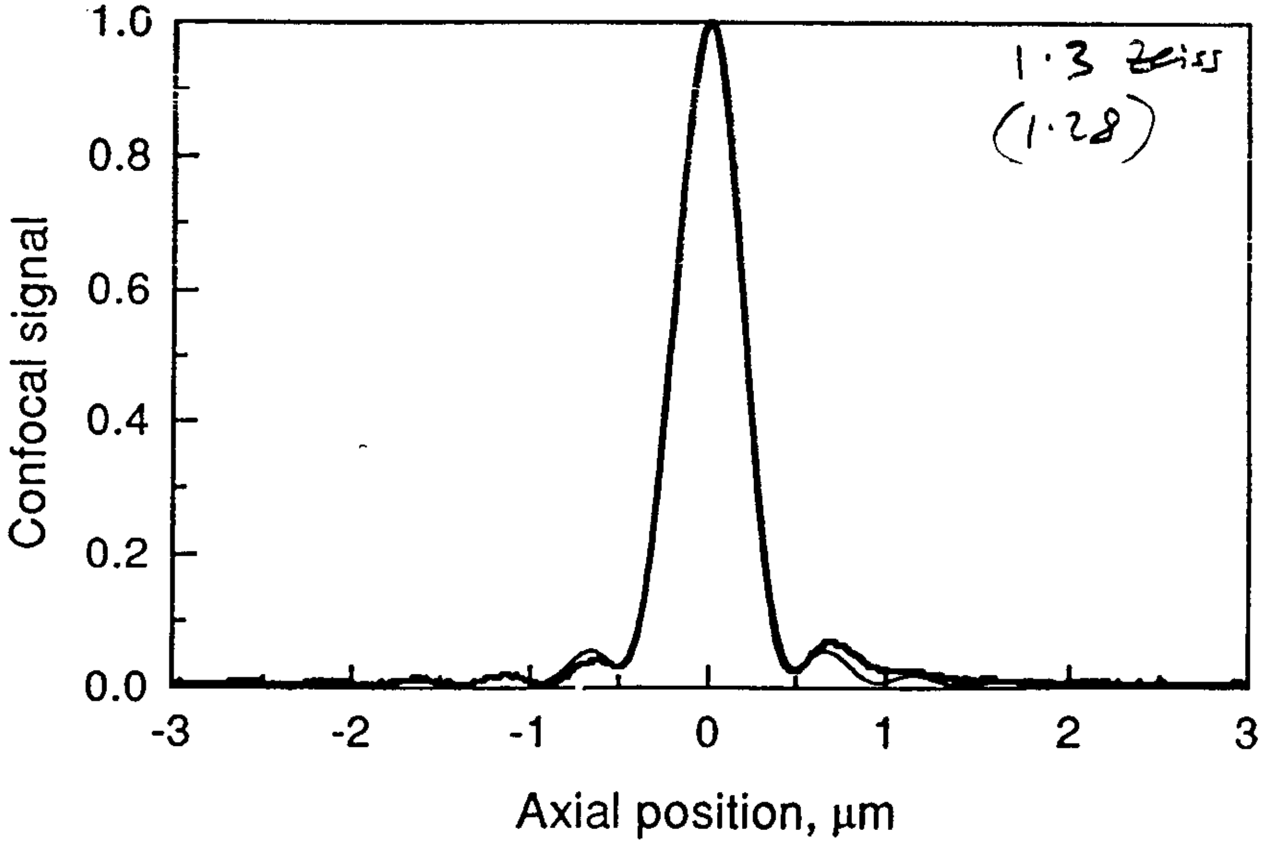
$$\underline{|a_1|^2}$$

where

$$\underline{a_1 = h\; h_{eff} \otimes t}$$

$$\underline{h_{eff} = h \otimes e_1} \quad \boxed{P_{eff} = P\, E_1}$$


specimen
objective lens
fibre
coupler
laser
photodetector


1.0
0.8
0.6
0.4
0.2
0.0
Confocal signal
-3
-2
-1
0
1
2
3
Axial position, µm

1·3 ~~Zeiss~~
(1·28)

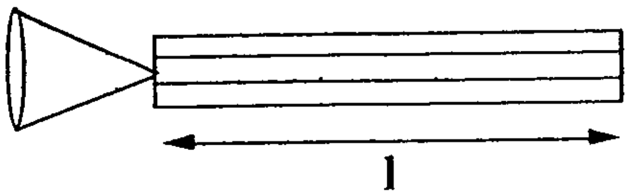
Two mode fiber detection

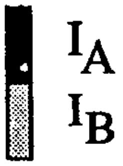
Mode 1 'detects' field $a1\ \underline{e1}\ \exp -j\beta 1l$

Mode 2 'detects' differential of field $a2\ \underline{e2}\ \exp -j\beta 2l$

$$\text{Ufiber} = a1\ \underline{e1}\ \exp -j\beta 1l + a2\ \underline{e2}\ \exp -j\beta 2l$$

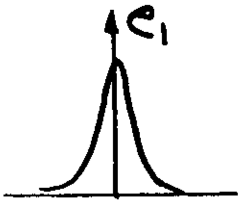
phase difference is tunable by varying fiber length

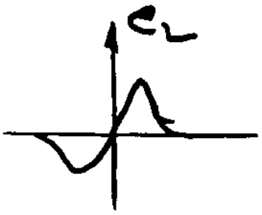




eigenmode e1 is an even function

eigenmode e2 is an odd function





$$I+ + I- = |a1|^2 + |a2|^2$$

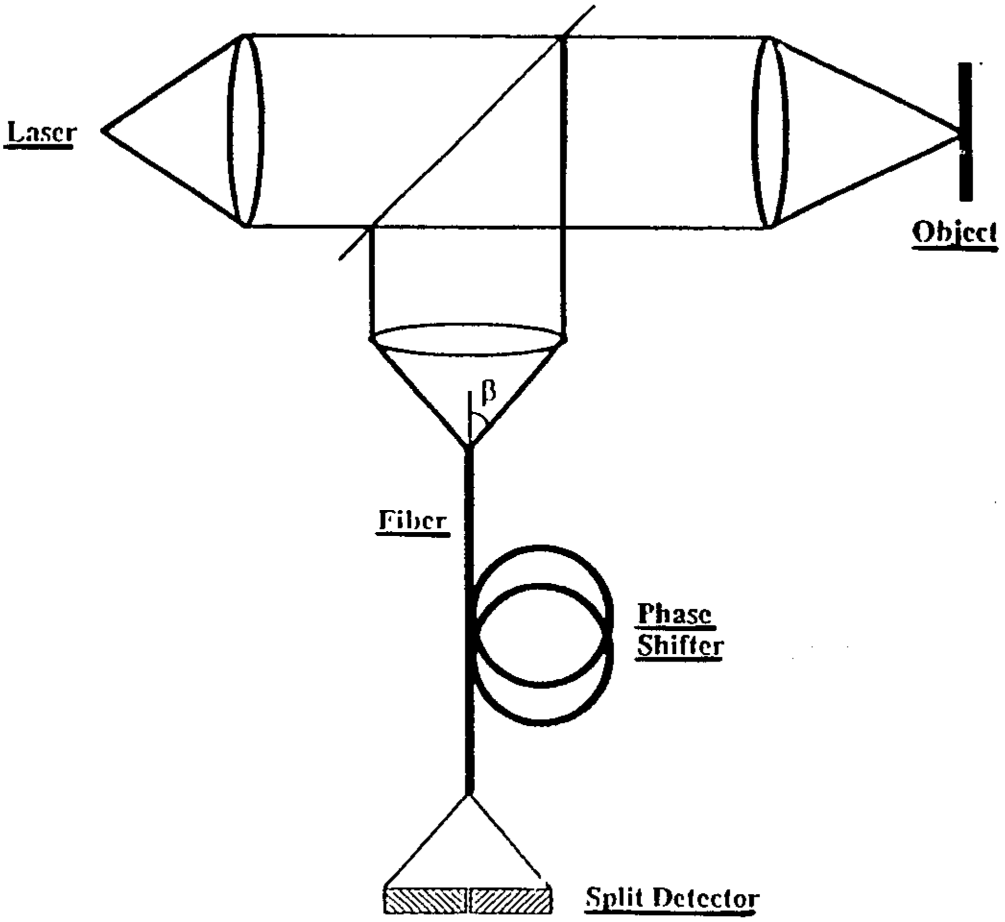
$$I+ - I- = 2\ \mathrm{Re}\{a1a2^* \exp -j\Delta\beta l\}$$

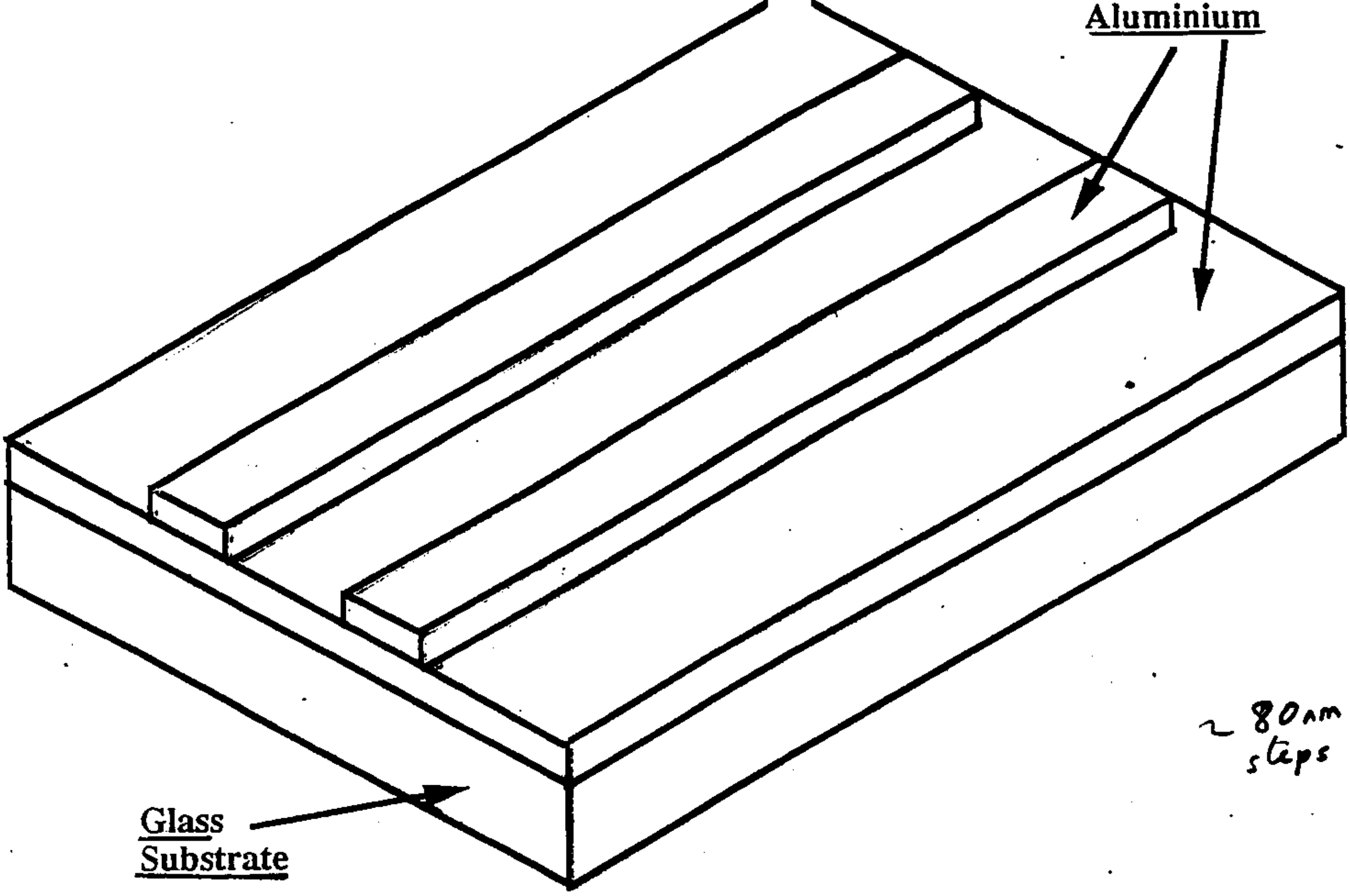
$$\boxed{a2 \sim \frac{da1}{dx}} \qquad \text{and let} \qquad \underline{a1 = b \exp j\phi}$$

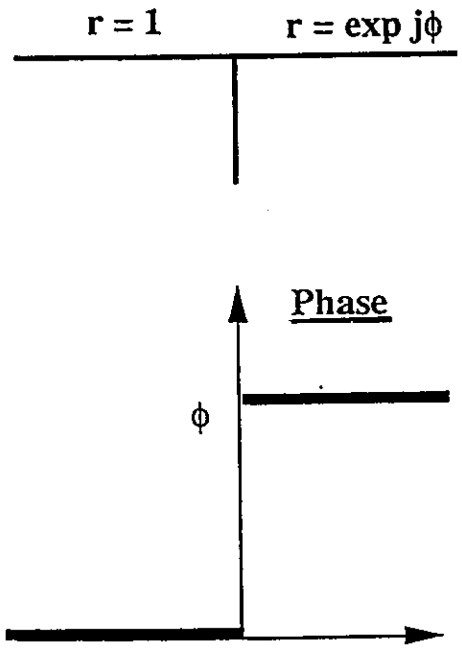
$$\boxed{I+ - I- = \frac{db^2}{dx}\sin(\Delta\beta l) + 2\, b^2 \frac{d\phi}{dx}\cos(\Delta\beta l)}$$

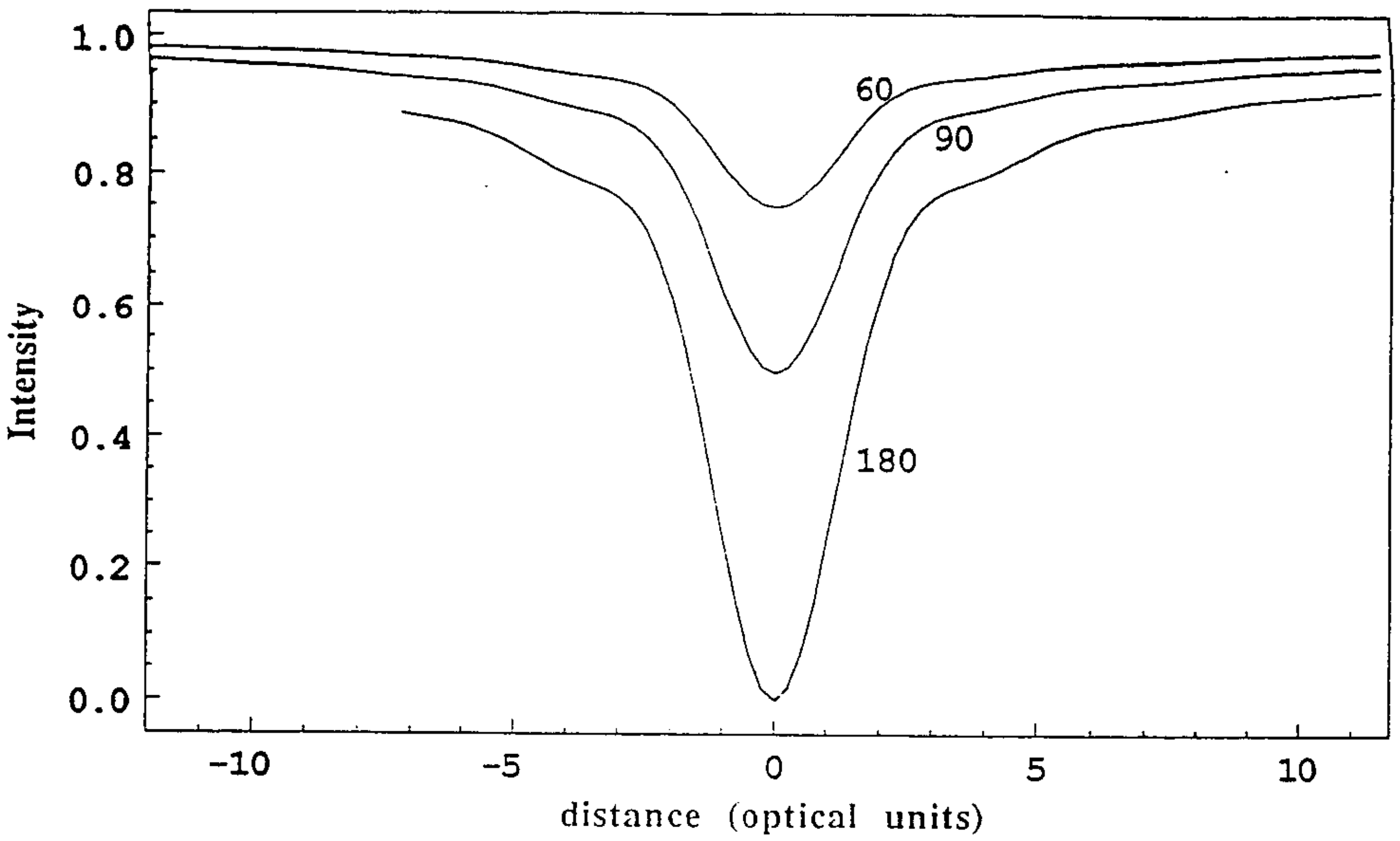
Differential amplitude contrast

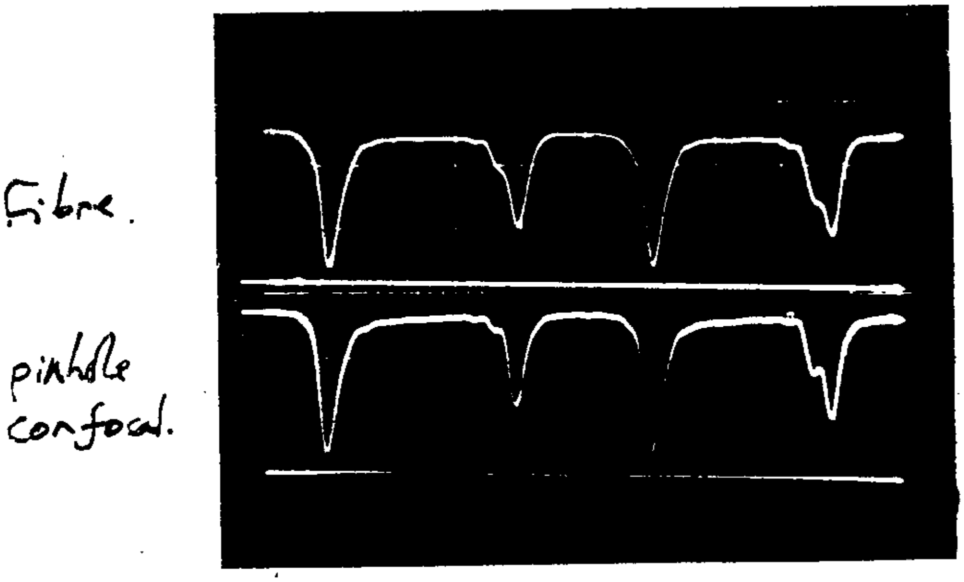
Differential phase contrast



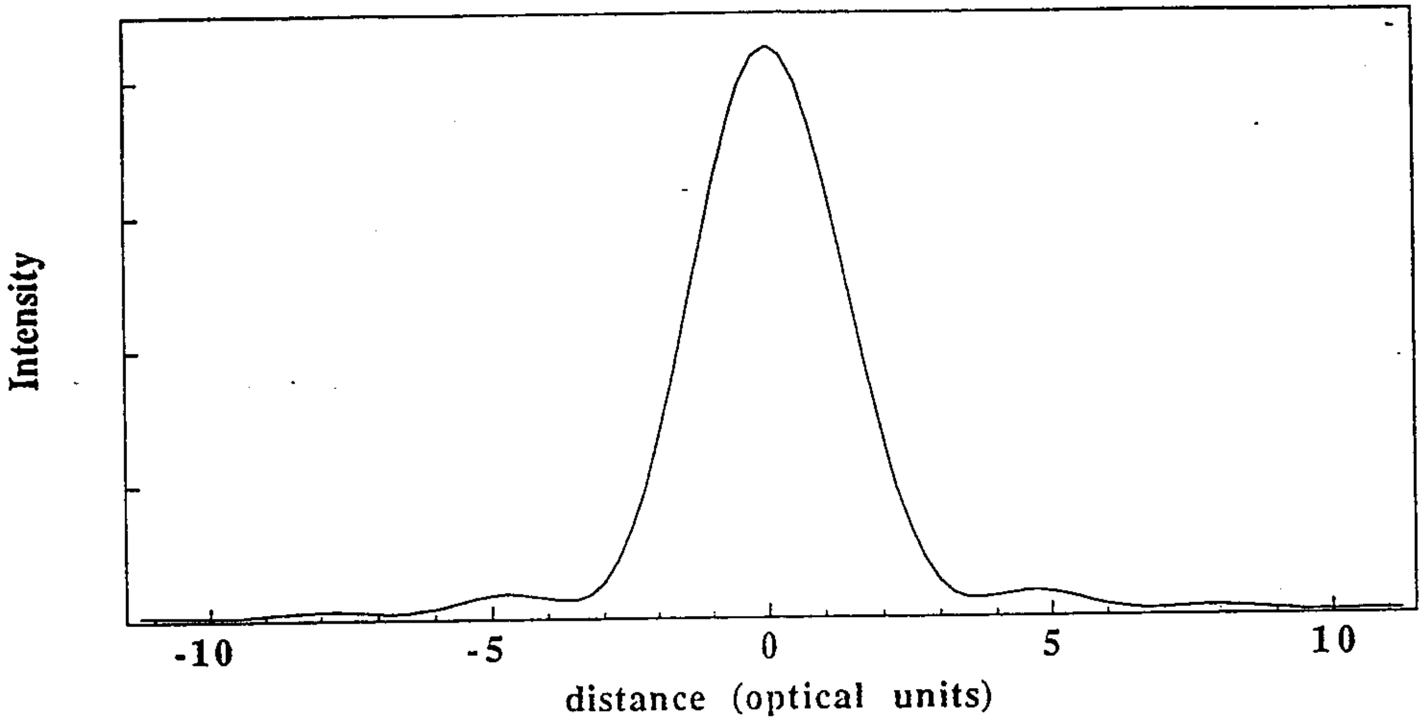

Aluminium
Glass Substrate
~ 80nm steps

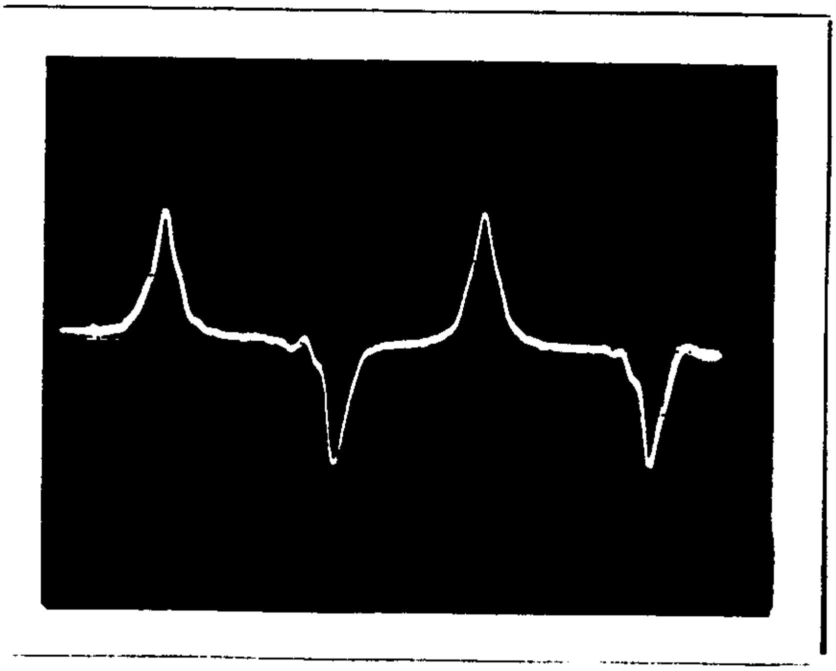

r = 1
r = exp jϕ
Phase
ϕ

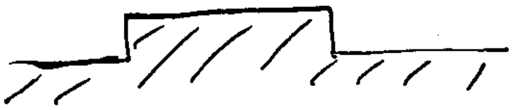

Intensity
1.0
0.8
0.6
0.4
0.2
0.0
-10
-5
0
5
10
distance (optical units)
60
90
180


fibre.
pinhole confocal.

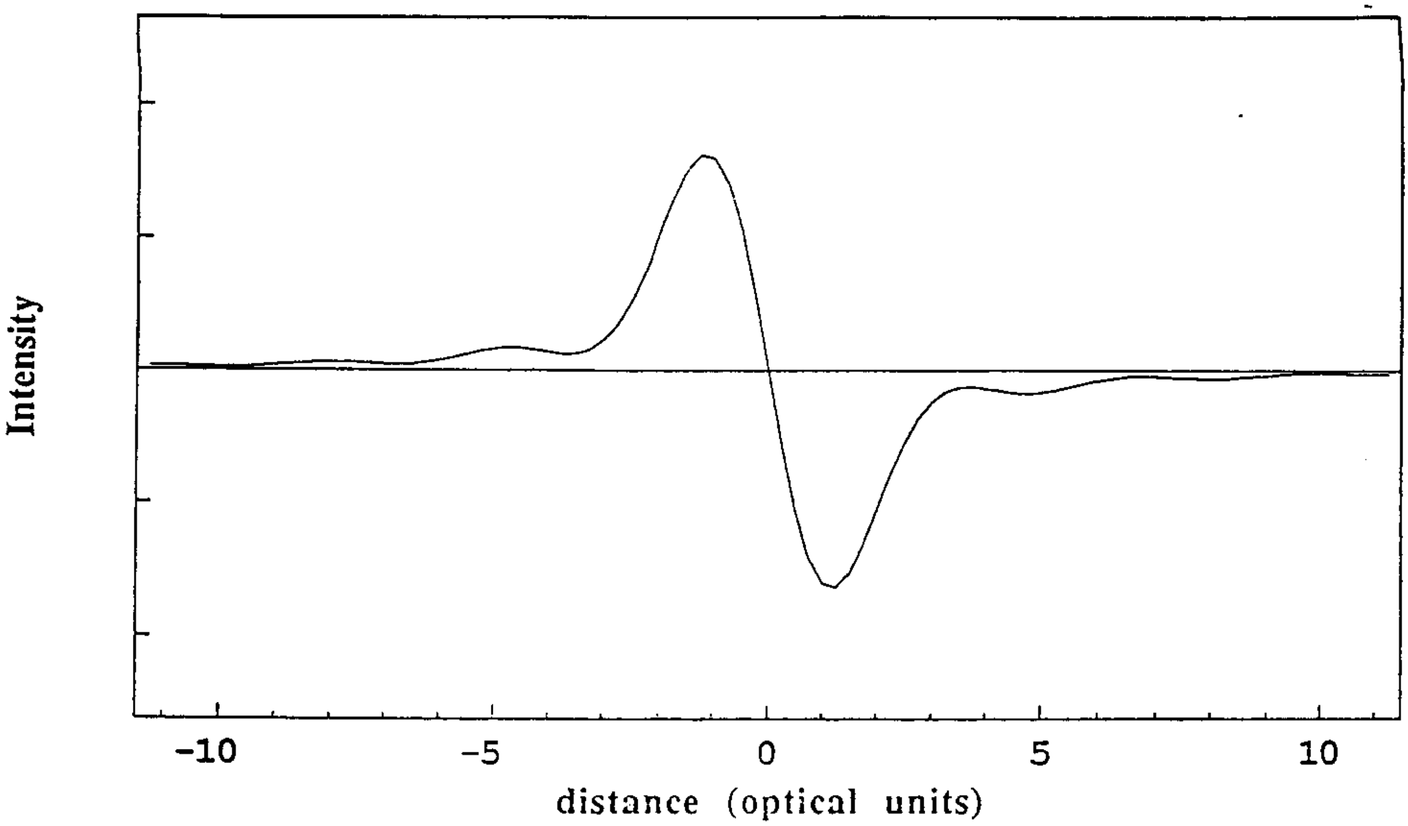
[illegible]

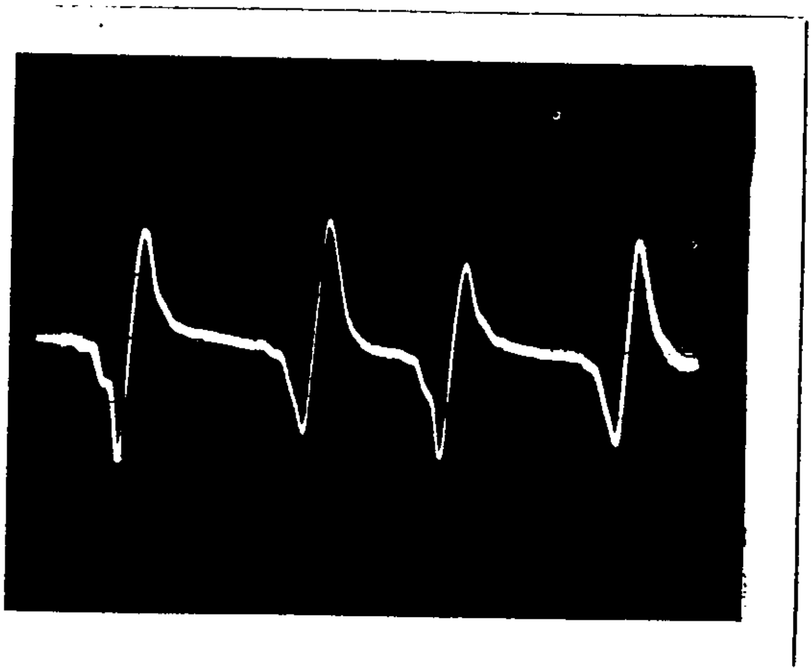

Intensity
-10
-5
0
5
10
distance (optical units)

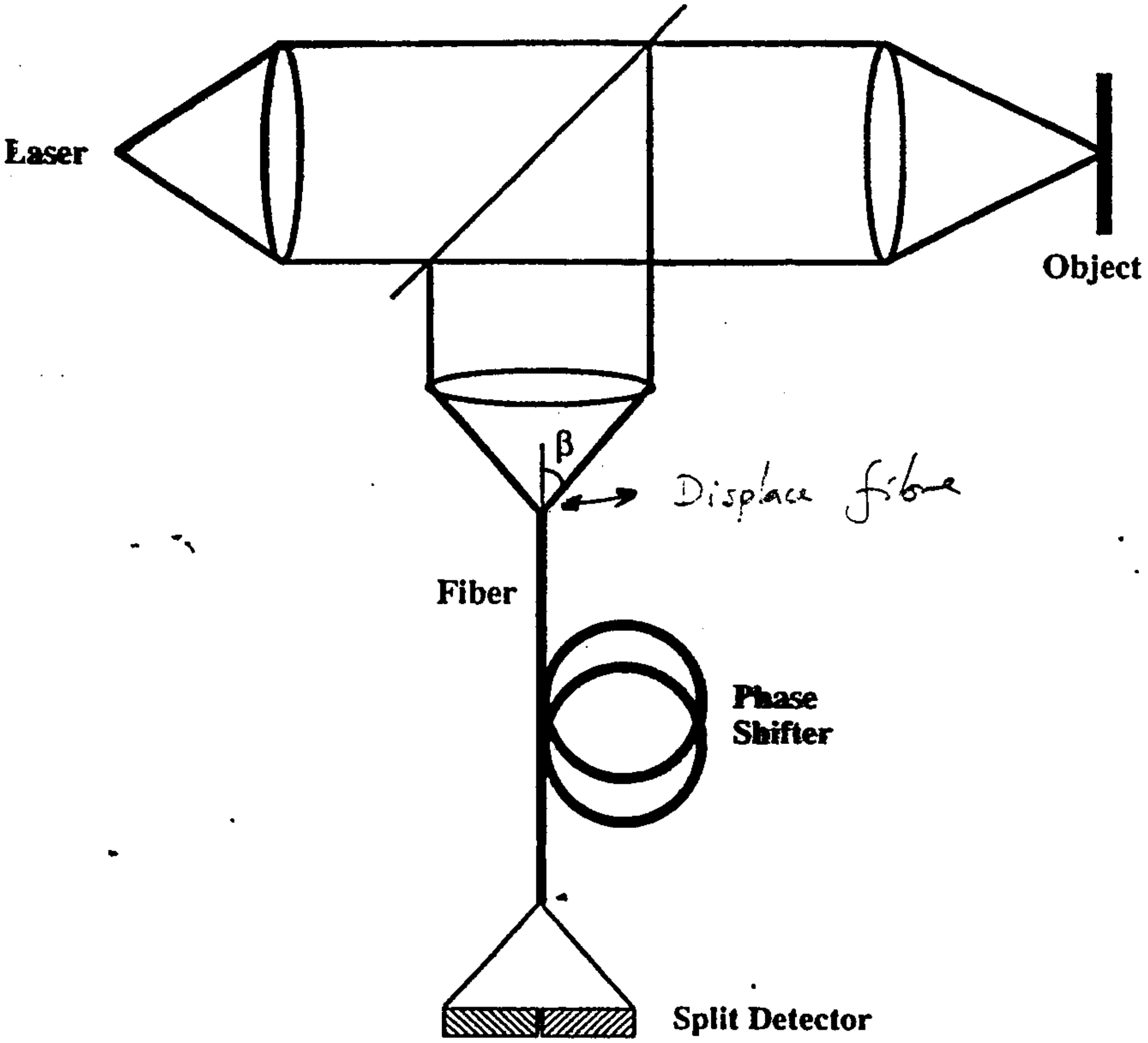




Differential amplitude plot



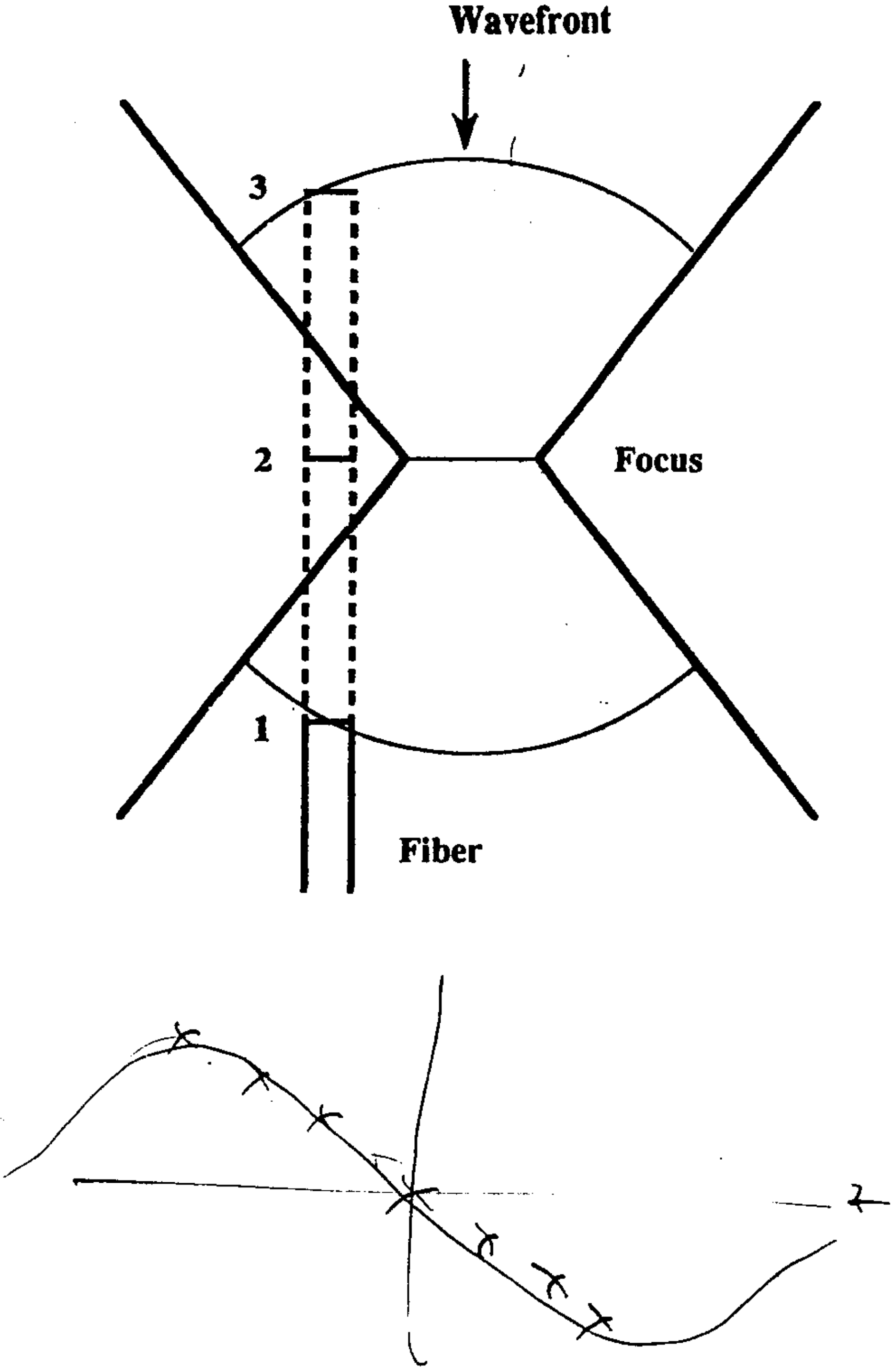




Displace fibre

work in diff phase mode

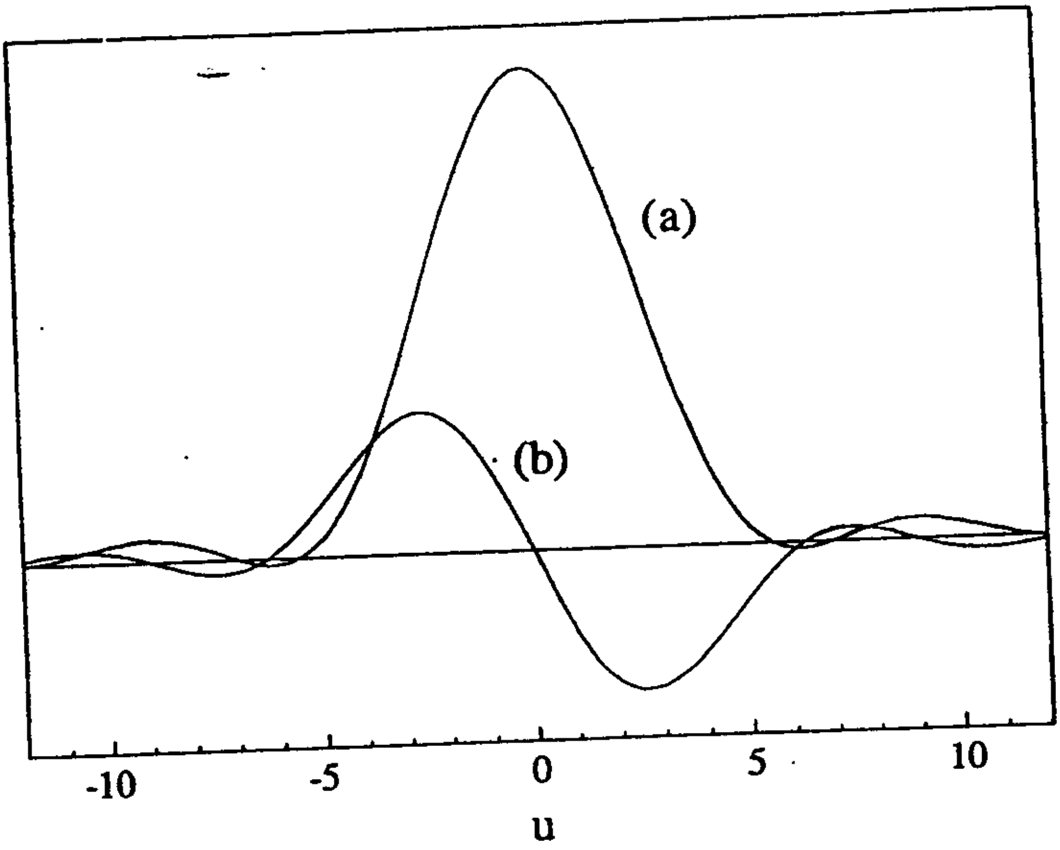
? Axial Response

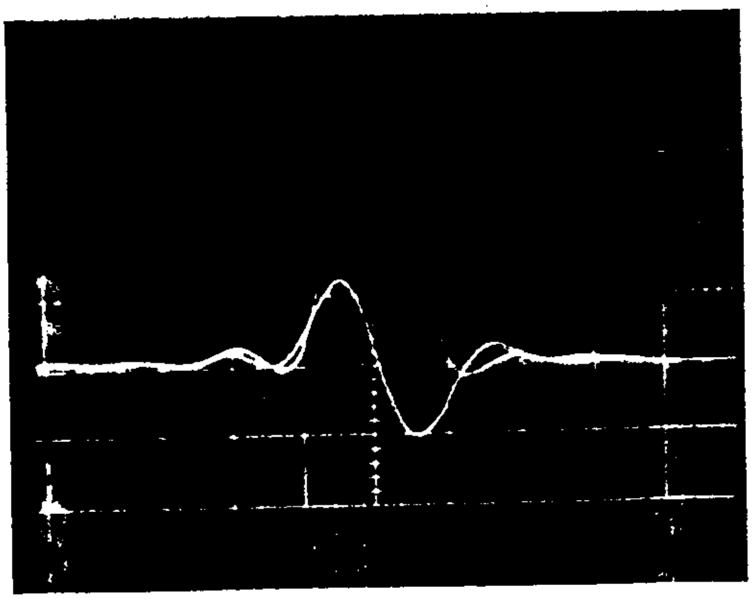


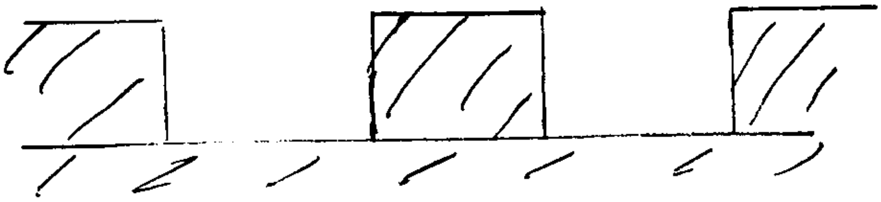
DPC

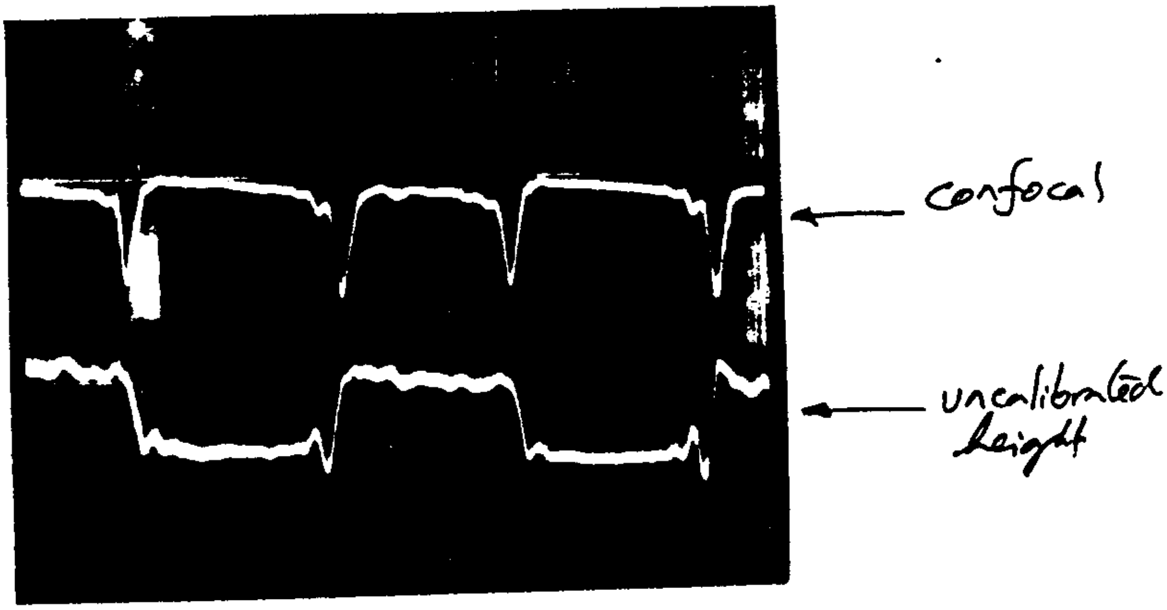
Fig. 2

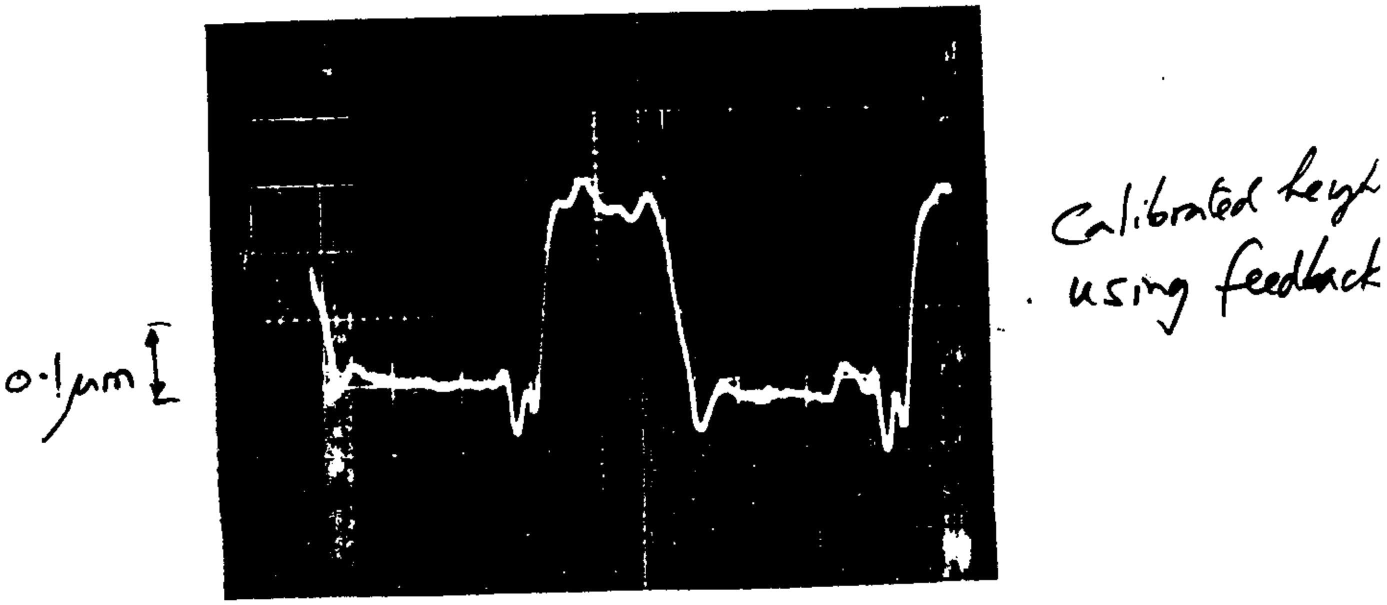
Axial Responce – displacement [illegible]





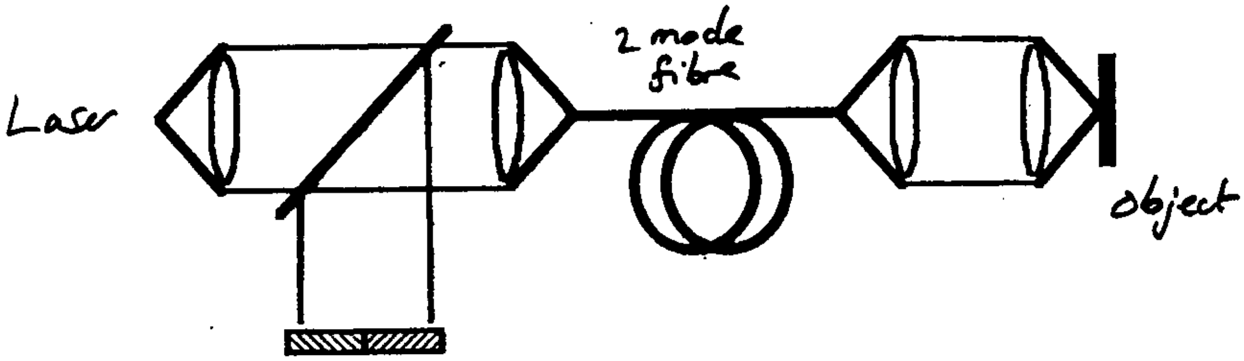



confocal
uncalibrated height


Calibrated height using feedback
0·1μm

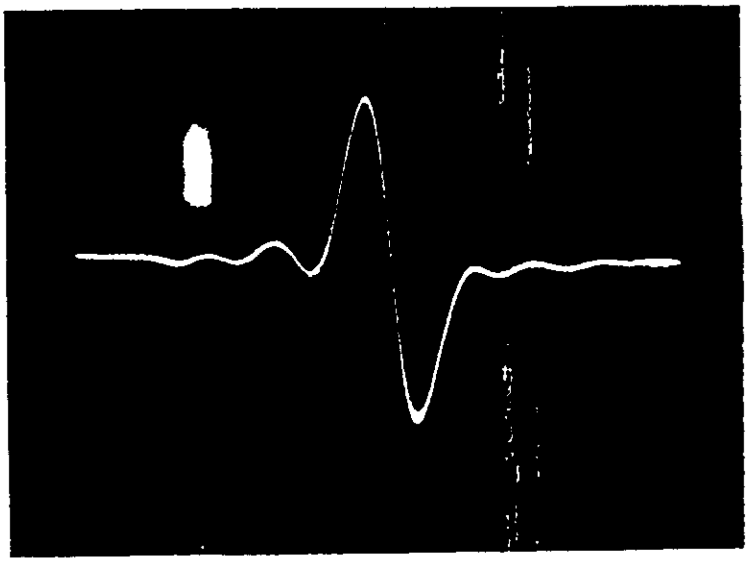
Reciprocal Imaging

Launch and detect BOTH modes

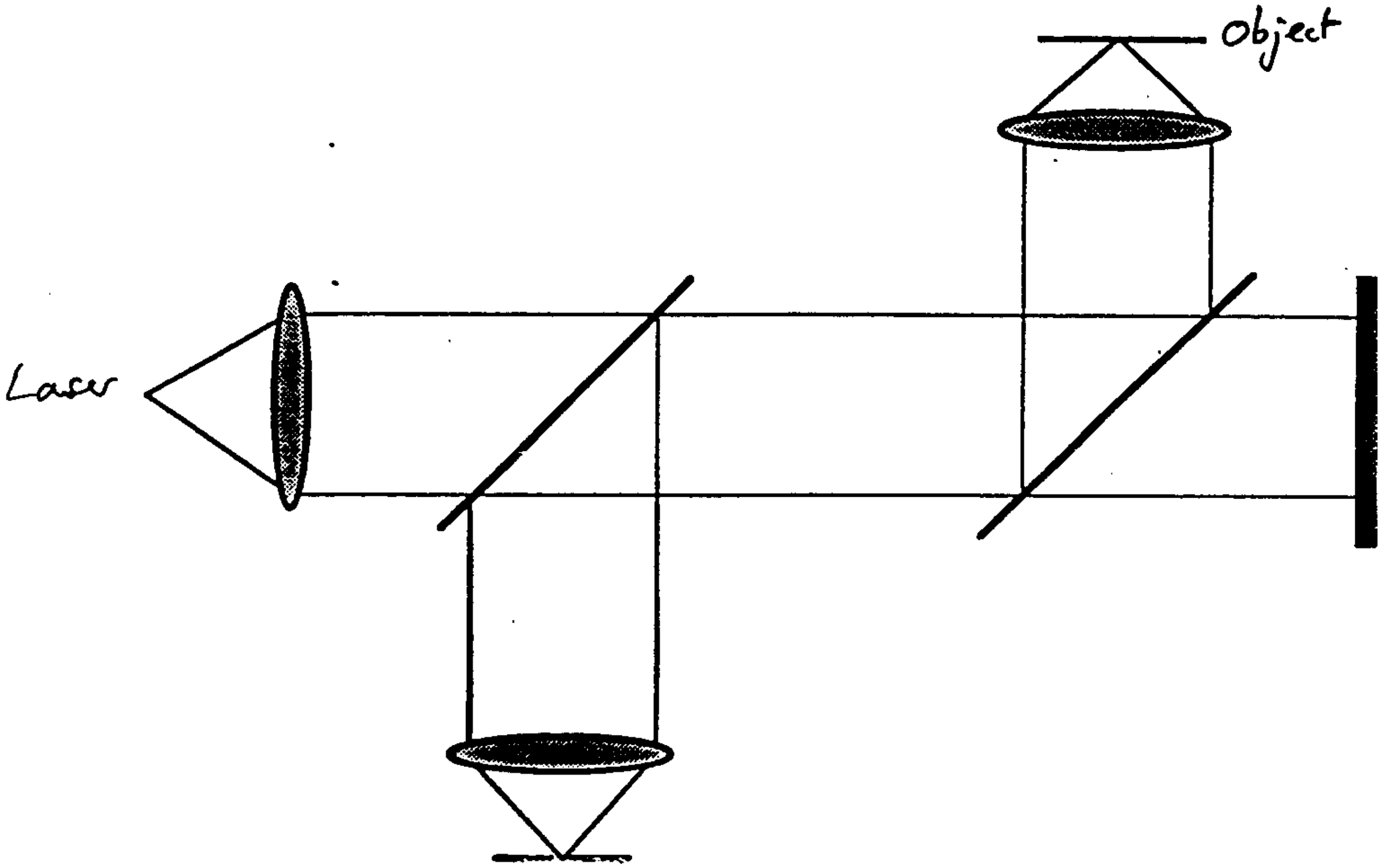


Differential axial scan

Profilometry without lateral shift



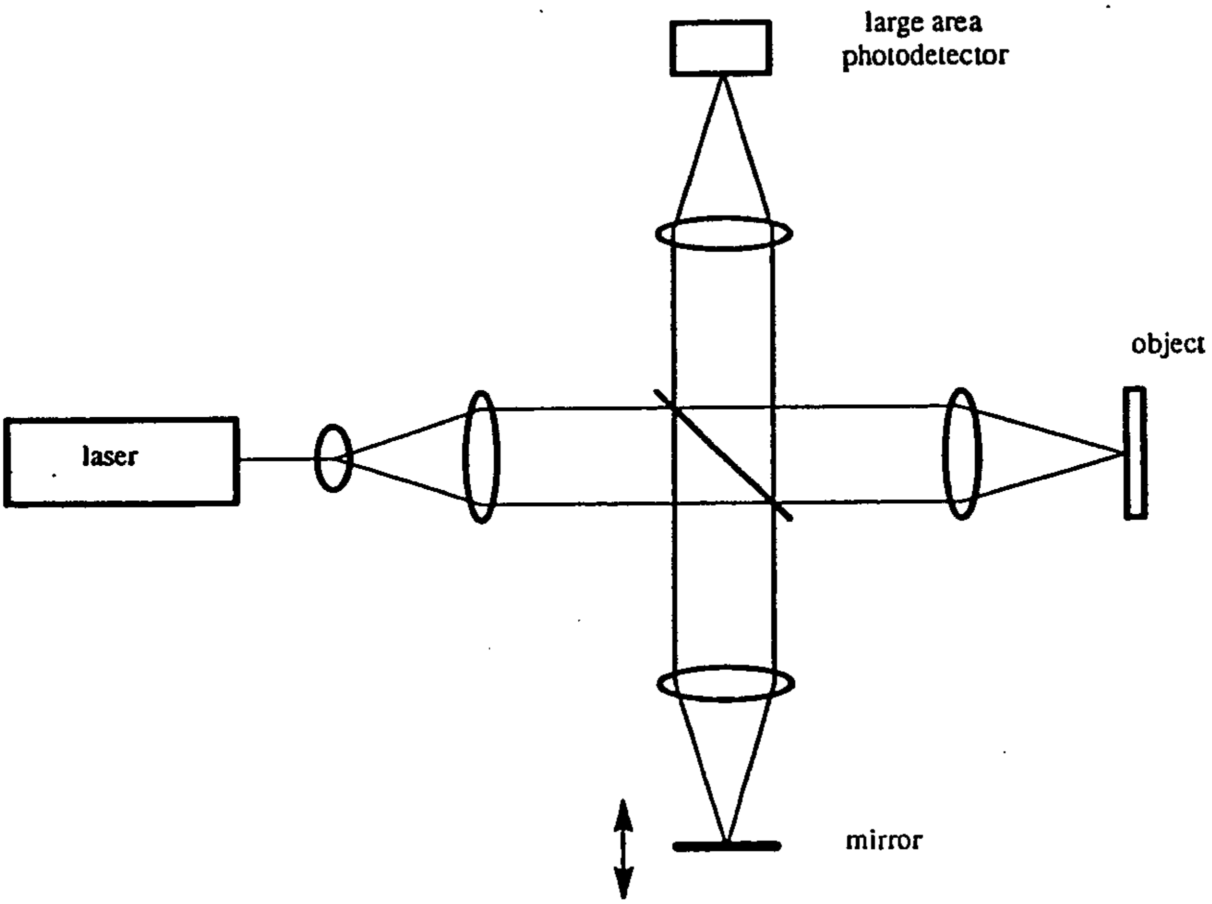
Interference Imaging



$$I = |U + r|^2$$

$$= \underbrace{|U|^2}_{\boxed{\text{Confocal Image}}} + \underbrace{|r|^2}_{\boxed{\text{Constant Reference}}} + \underbrace{2\mathrm{Re}\{r^* U\}}_{\boxed{\text{Interference term image}}}$$

≡ conv & confocal


large area
photodetector
object
laser
mirror

Modulation Schemes

Phase Modulation

$$\underline{\phi = \phi_o \cos\omega t}$$

$$I = |r + U \exp j\,\phi(t)|^2$$

$$= r^2 + |U|^2 + 2r\,[\mathrm{Re}\{U\}\,\underline{\cos(\phi_0 \cos\omega t)} - \mathrm{Im}\,\{U\}\,\underline{\sin(\phi_0 \cos\omega t)}]$$

Recall

$$\underline{\cos(\phi_0 \cos\omega t)} = J_0(\phi_0) - \underline{2J_2(\phi_0)\cos 2\omega t} + 2J_4(\phi_0)\cos 4\omega t + \ldots.$$

$$\underline{\sin(\phi_0 \cos\omega t)} = \underline{2J_1(\phi_0)\cos\omega t} - 2J_3(\phi_0)\cos 3\omega t + \ldots.$$

Synchronously demodulate (lock-in)

(i) $\underline{\cos\omega t}$

$$\underline{I_1 \sim \mathrm{Re}\{U\}\,J_1(\phi_0)}$$

(ii) $\underline{\cos 2\omega t}$

$$\underline{I_2 \sim \mathrm{Im}\,\{U\}\,J_2(\phi_0)}$$

Quadrature Interference term images

$$\underline{\text{Confocal} \sim \alpha I_1^2 + \beta I_2^2}$$

DEMODULATED SIGNALS

$I_1 \sim \mathrm{Re}\{U\} \; J_1(\varnothing_0)$

$I_2 \sim \mathrm{Im}\{U\} \; J_2(\varnothing_0)$

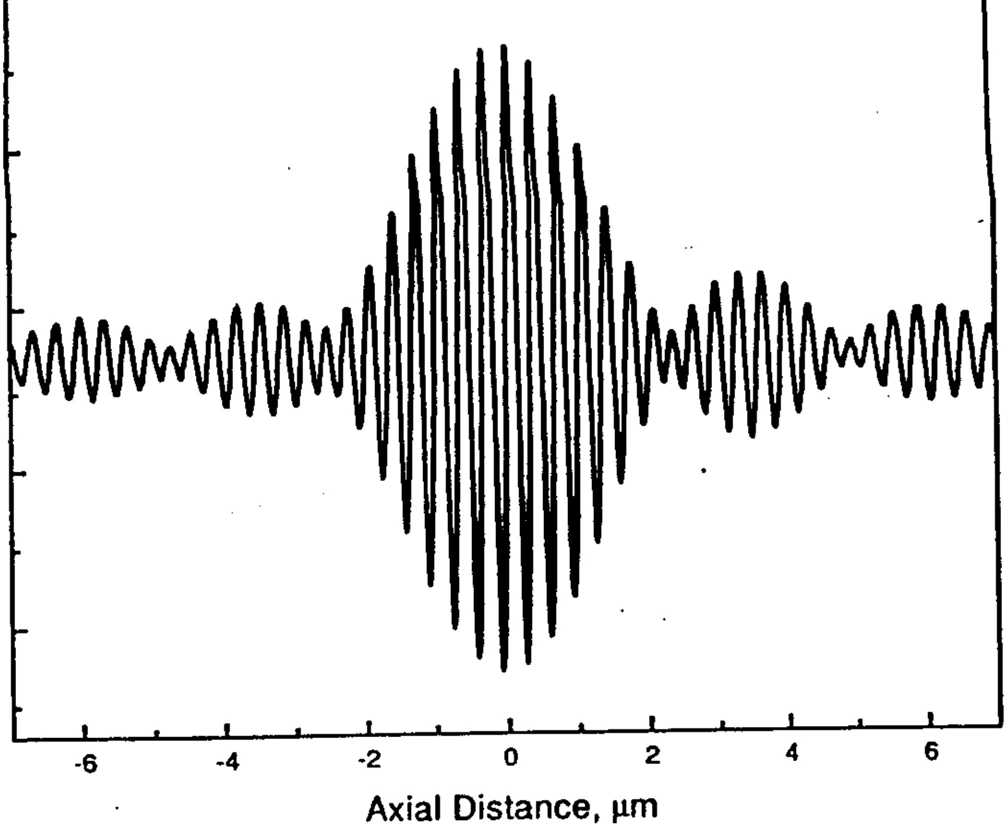
Dither

frequency	180 kHz
amplitude	$J_1(\varnothing_0) \approx J_2(\varnothing_0)$

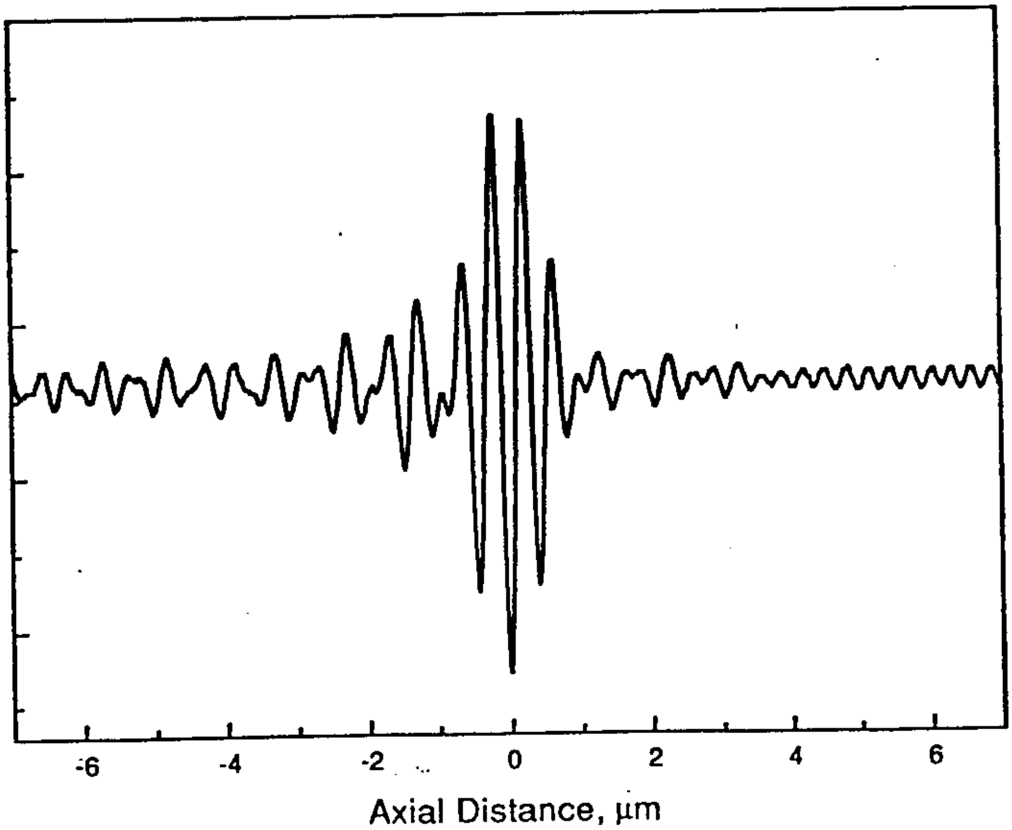
Axial Response

$I_1 = \mathrm{sinc}\,(u/2) \; \sin\,(2kz \cos^2 \alpha/2)$

$I_2 \approx \mathrm{sinc}\,(u/2) \; \cos\,(2kz \cos^2 \alpha/2)$

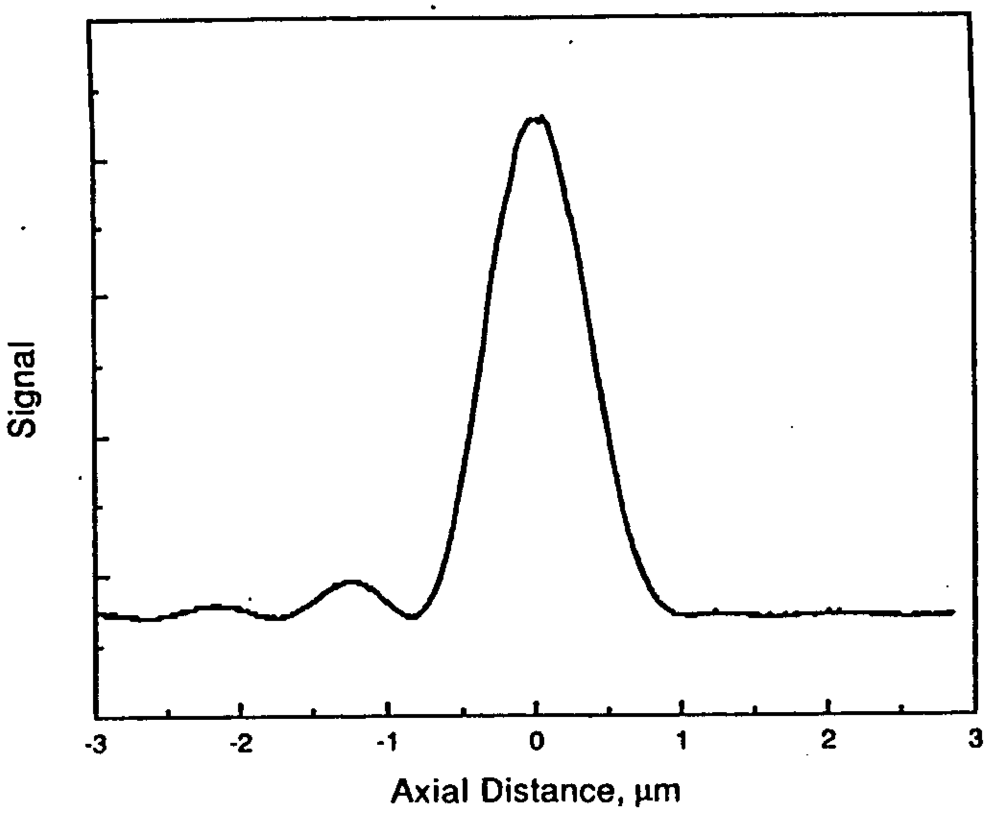


NA = 0·5

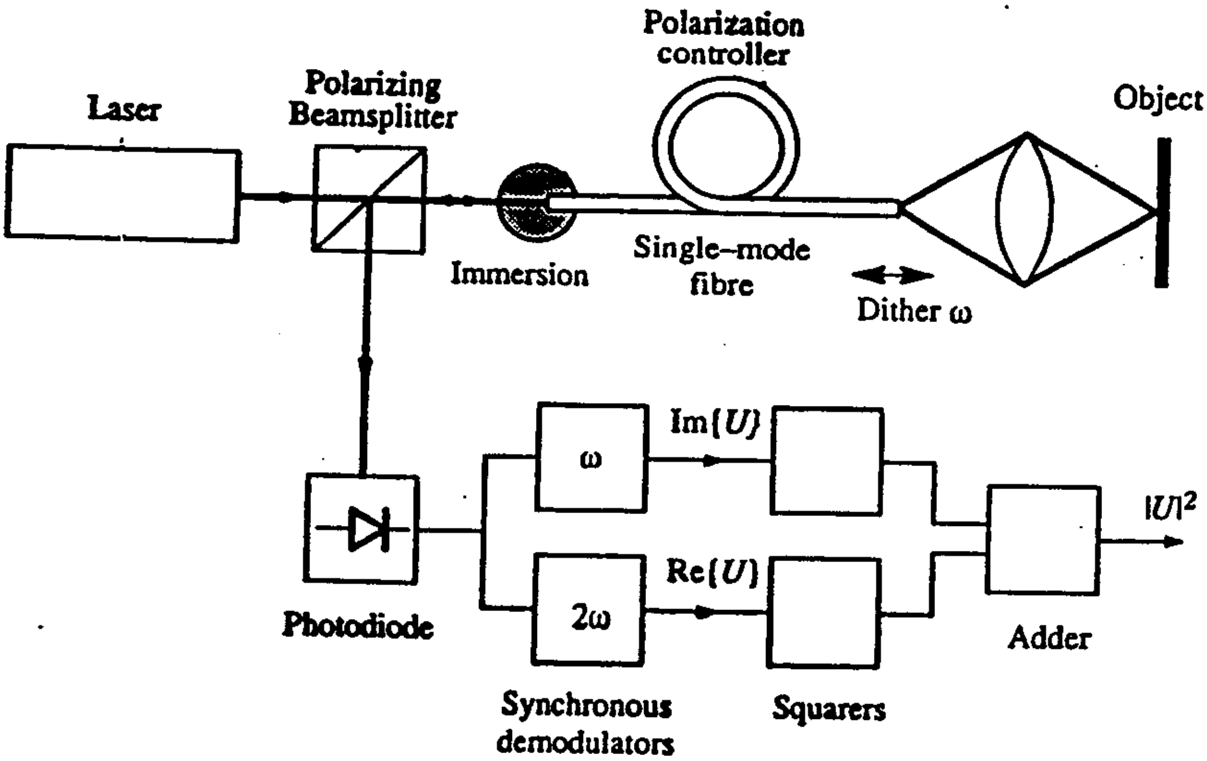


NA = 0·8

0·8 NA



Interference System

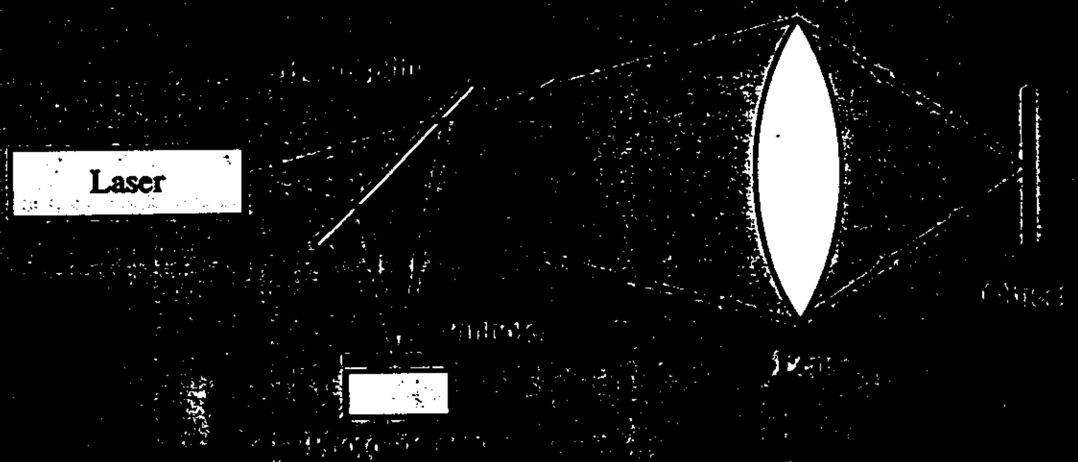


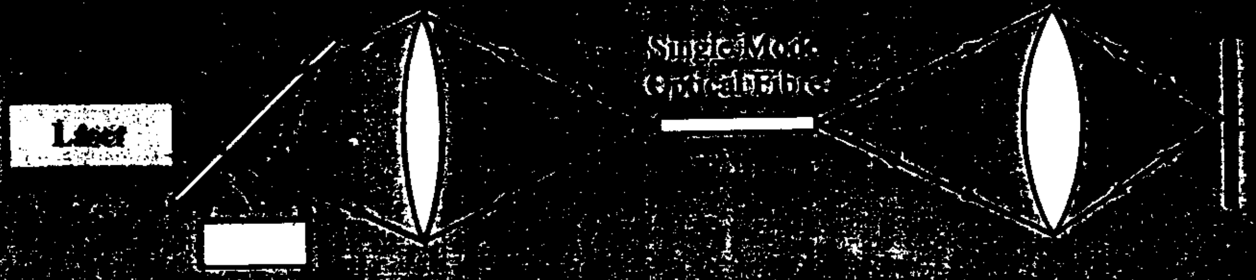
Axial Response

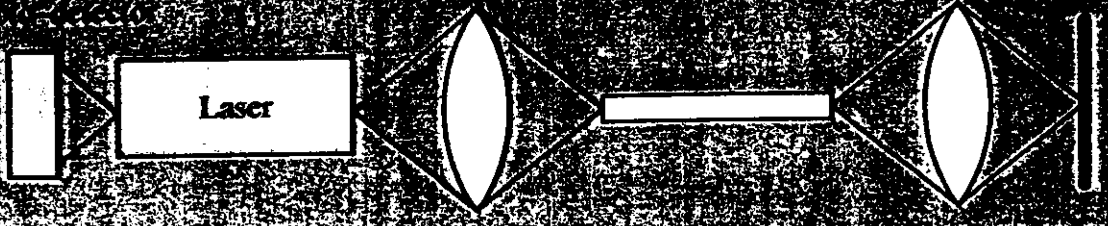
$$\mathrm{Im}\ \{U\} \approx \mathrm{sinc}\left(\frac{u}{2}\right) \quad \sin\left(2kz\cos^2\frac{\alpha}{2}\right)$$

$$\mathrm{Re}\{U\} \approx \mathrm{sinc}\left(\frac{u}{2}\right) \quad \cos\left(kz\cos^2\frac{\alpha}{2}\right)$$

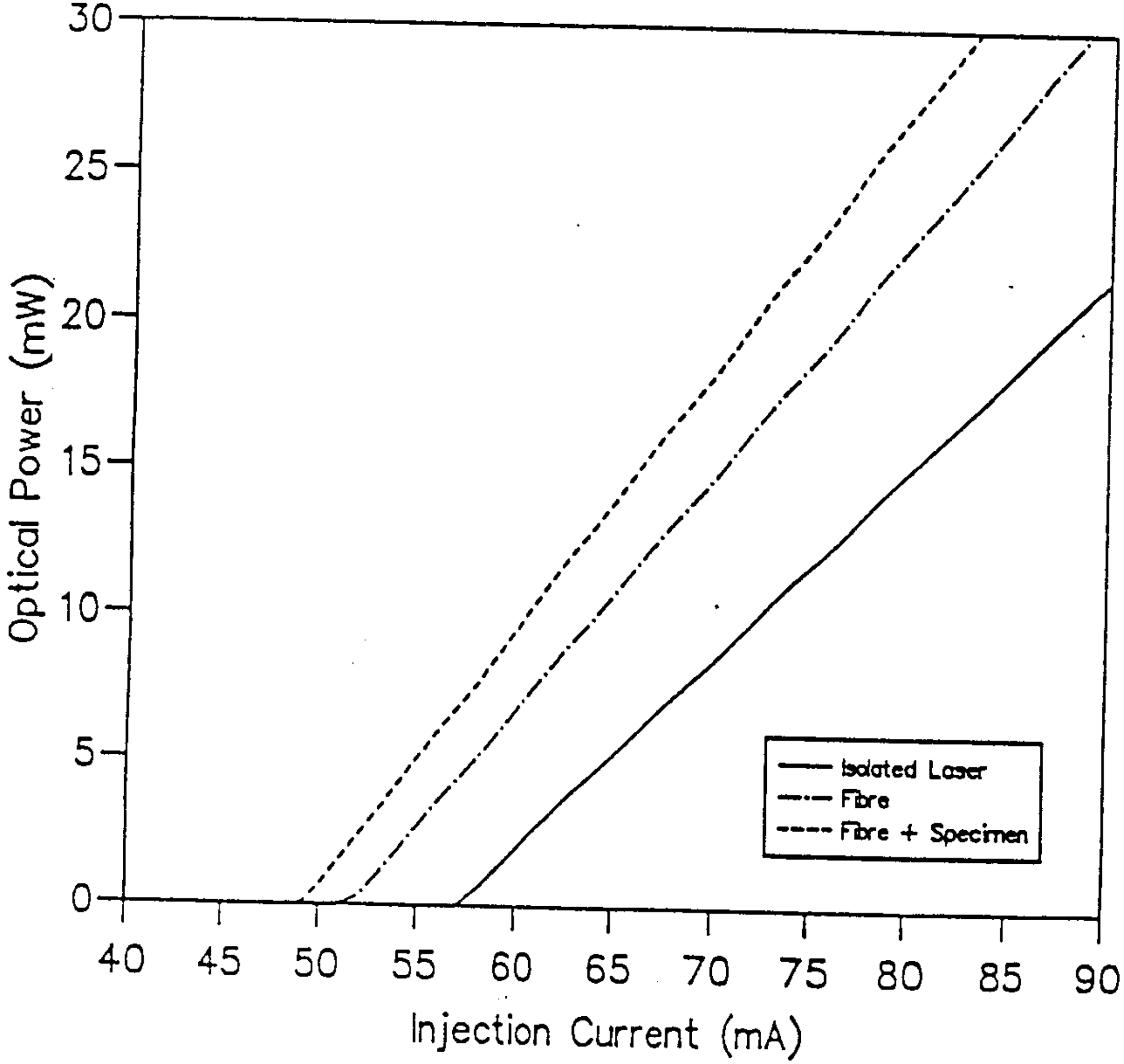
OPTICAL FEEDBACK CONFIGURATION



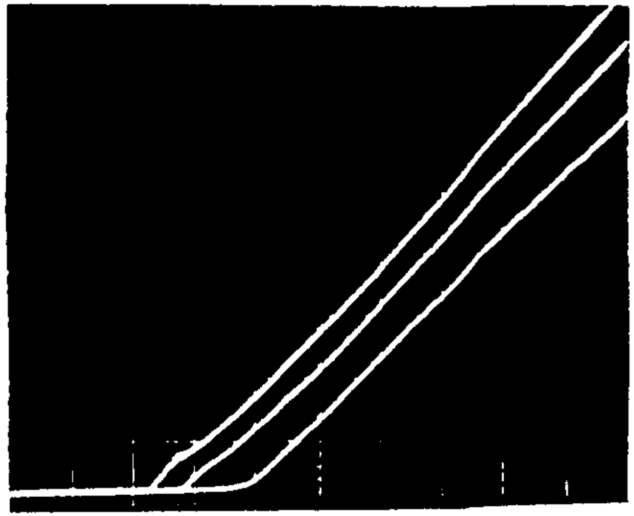




Theoretical Power vs Current



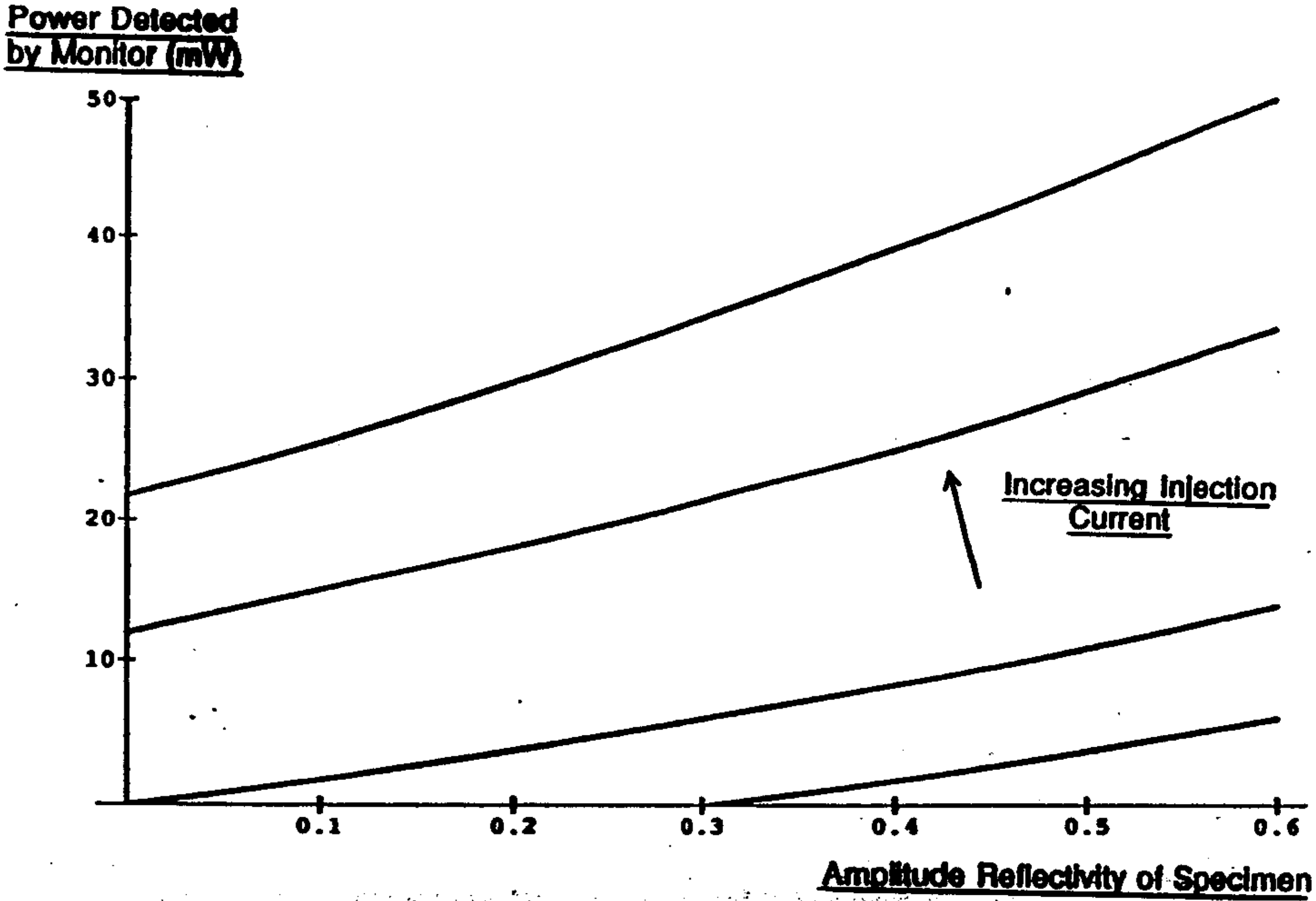
Experimental Results

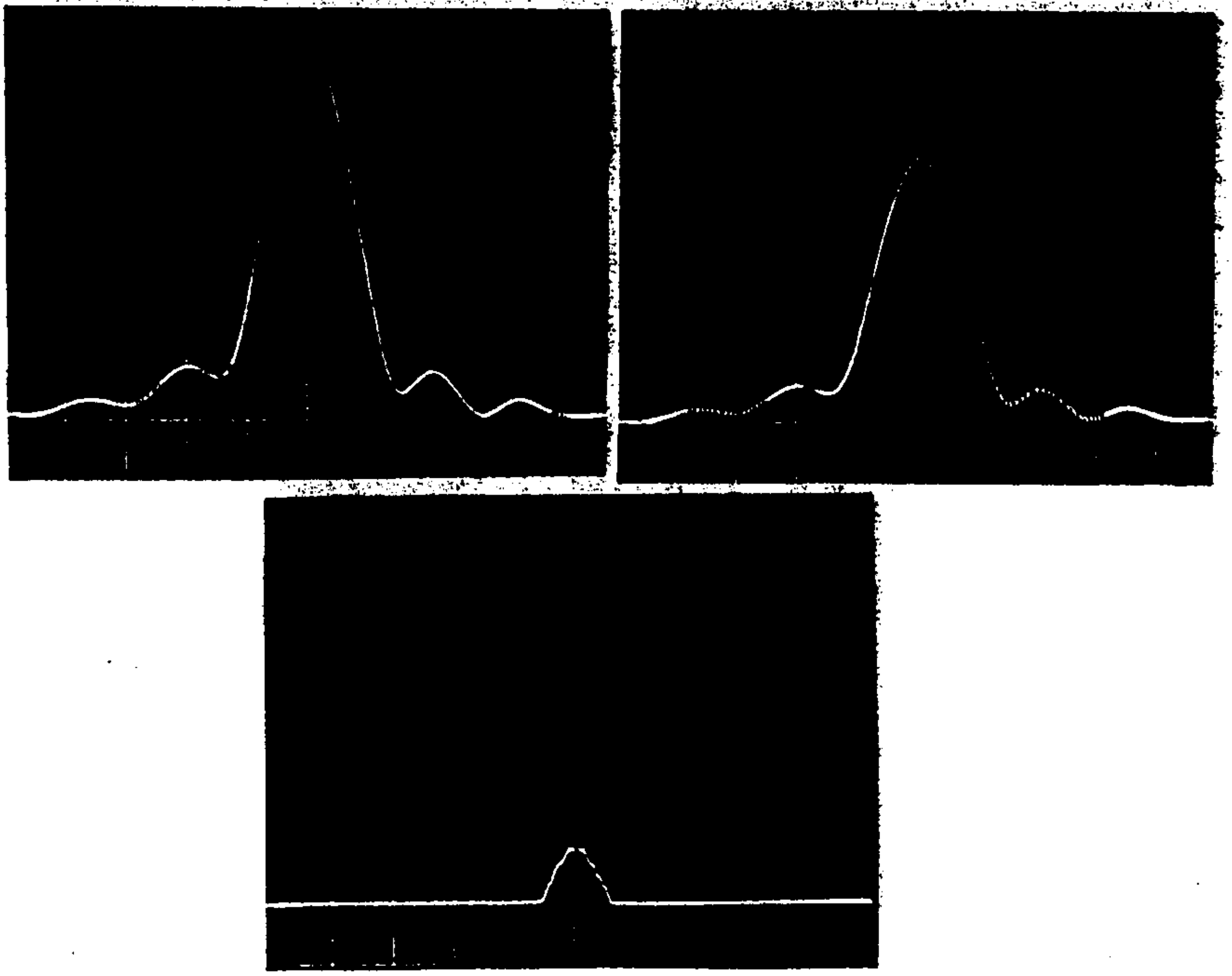


$$\text{Optical Power [mW]} = S_1(1-r^2) \times \frac{S_2\, L\, \text{Current[mA]} + \ln\{r(r + ct(1-r^2))\} - 1}{\{1 + r/(r+ct(1-r^2))\}\ \{1-r(r+ct(1-r^2))\}}$$

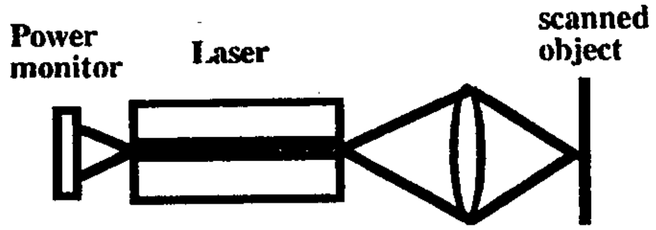
S_1, S_2 : Scaling Factors.
$S_1 = 33.95$, $S_2 = 128.89$

L : Active Length of Laser. L = 0.3mm
c : Coupling Coefficient of laser to fibre.
c = 0.80
r : Amplitude Reflectivity of "Isolated Laser" Mirror. r = 0.5477
t : Amplitude Reflectivity of Specimen.
t = 0.317, 0.200, 0.00 (from left to right)


Power Detected by Monitor (mW)
50
40
30
20
10
0.1
0.2
0.3
0.4
0.5
0.6
Amplitude Reflectivity of Specimen
Increasing Injection Current



Laser detection



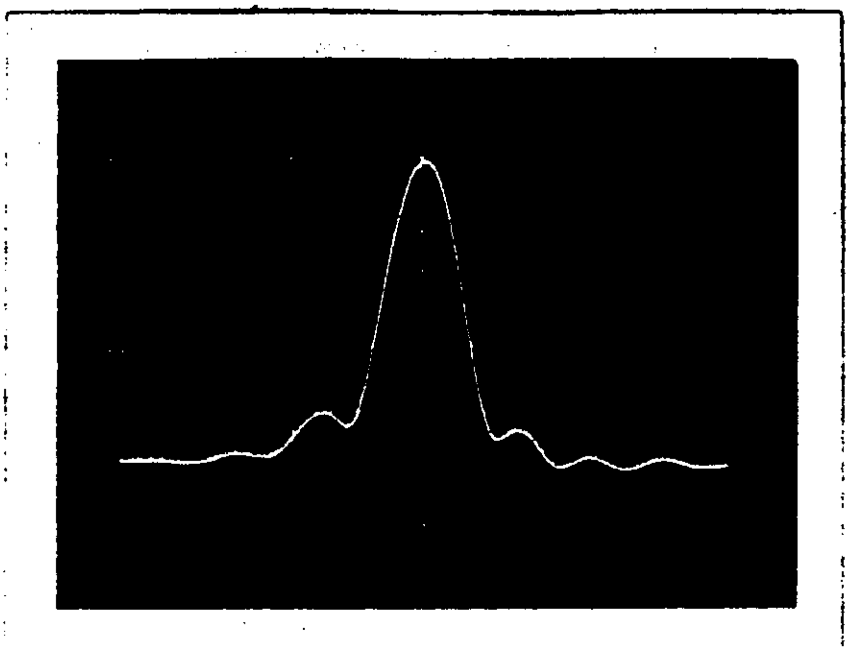
Laser mode acts as spatial filter

He-Ne laser gives $\Delta I \sim \mathrm{Re}\{s\}$

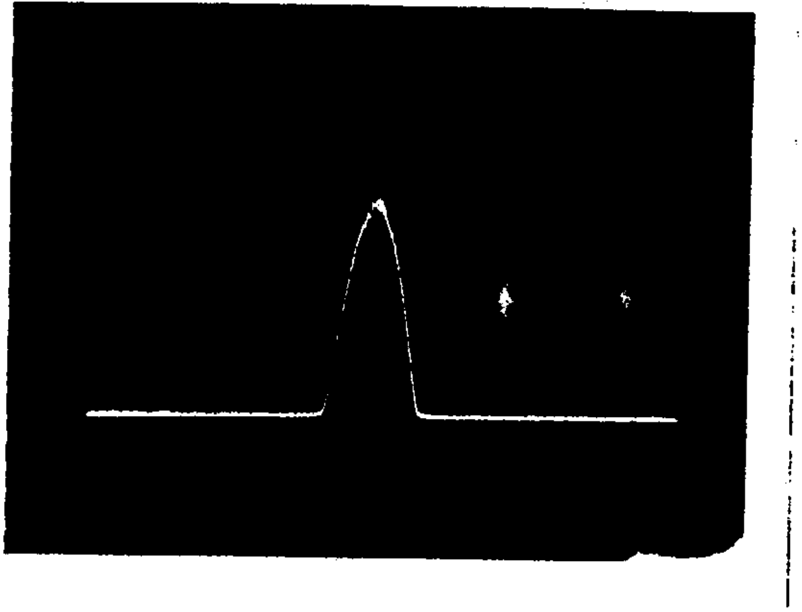
Semiconductor laser gives $\Delta I \sim |s|$

Semiconductor Laser [illegible]

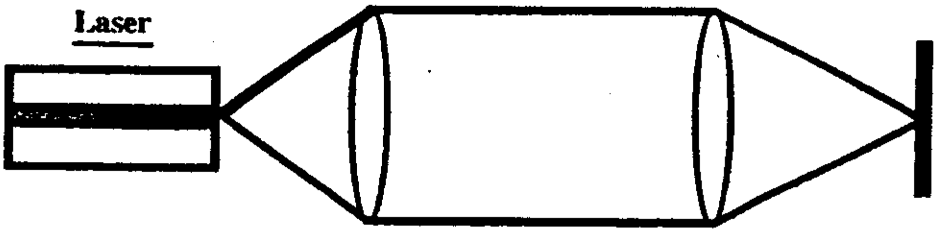
$|\delta|$

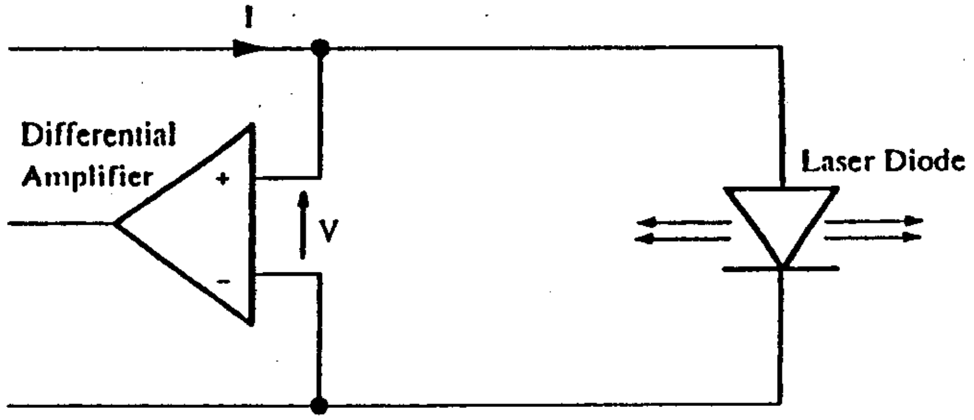


below threshold



Semiconductor laser confocal microscope

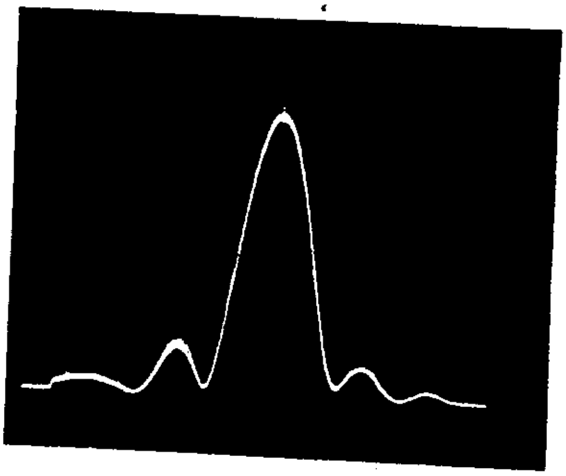




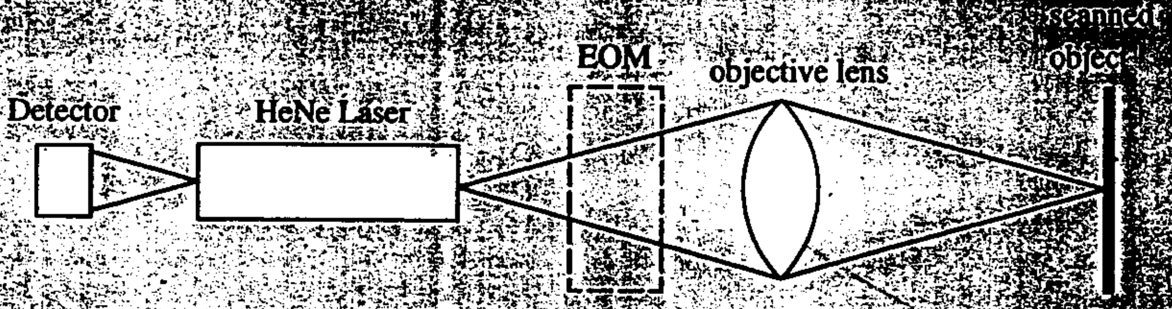
Perturbation on junction voltage

constant injection current

Axial Response



PROFILOMETRY



$$I = \cos\left[\phi + 2kz\cos^2\frac{\alpha}{2}\right]\operatorname{sinc}\left(\frac{u}{2}\right)$$

Feedback control of $\phi \Rightarrow$ Surface profiles (z)

(i) dc feedback

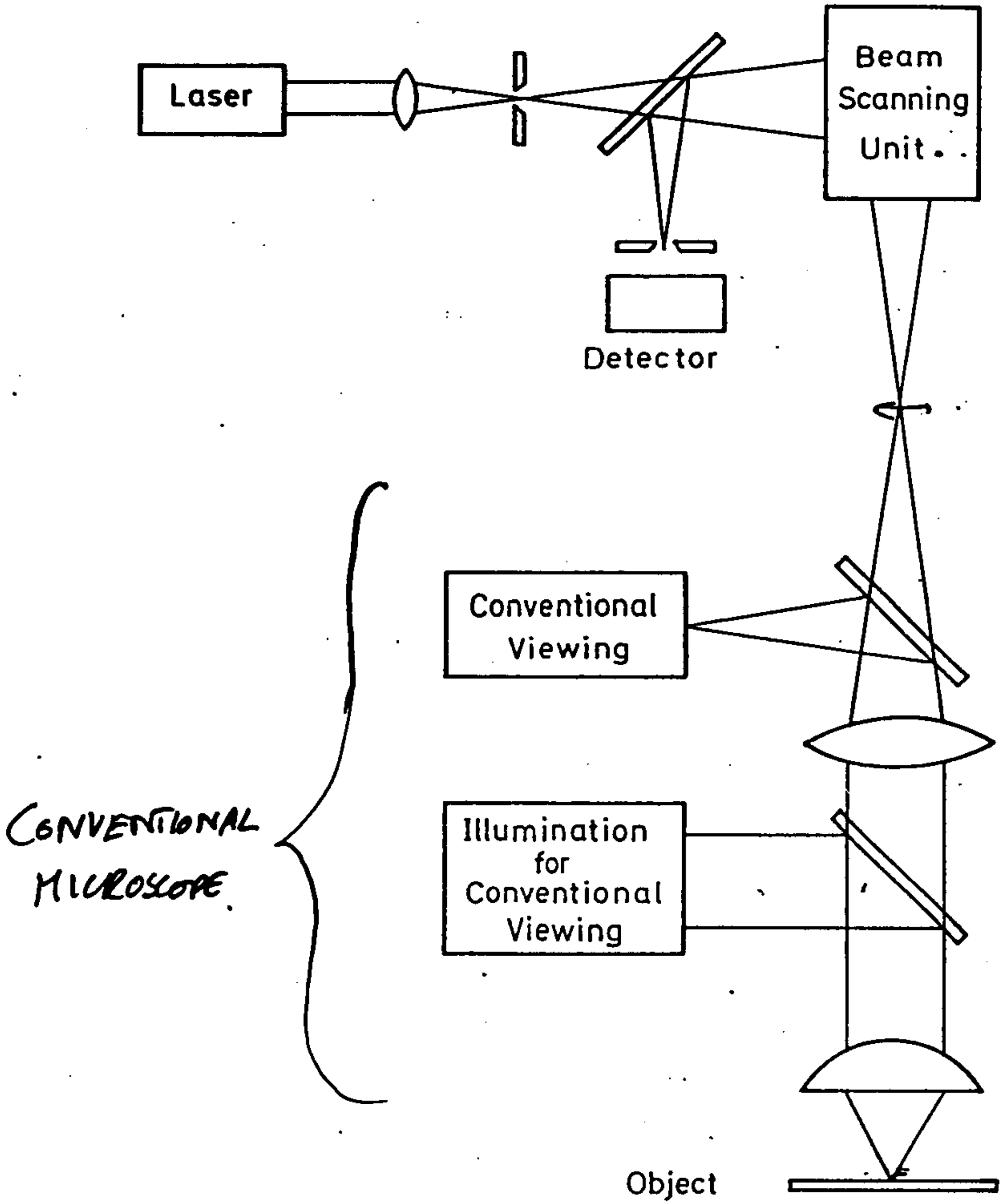
(ii) Modulation feedback $\phi = \phi_0 + \phi_1 \cos\omega t + \phi_2$

Lock-In: $\phi_2 \propto$ surface height

$\Rightarrow$ Confocal Image

Confocal Microscopy with White light.

- Tandem Scanning
- Aperture Correlation (SCAM)
- Grid Illumination
  (Structured Light)


Laser
Beam Scanning Unit
Detector
Conventional Viewing
Illumination for Conventional Viewing
Object
CONVENTIONAL MICROSCOPE

Some drawbacks

Lasers

- **brightness**
- **limited wavelength choice**

***usually* not real-time**

pinhole alignment problems

need to scan

Some goals

Easy to align

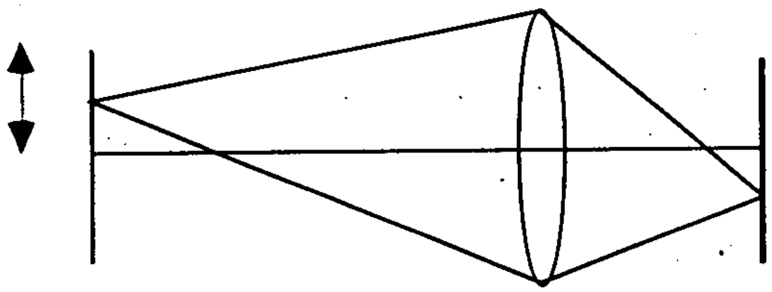
No scanning

real-time

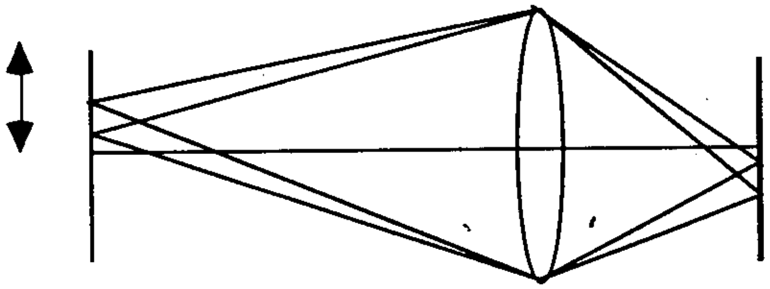
no laser

The Principle

- **Single point systems**

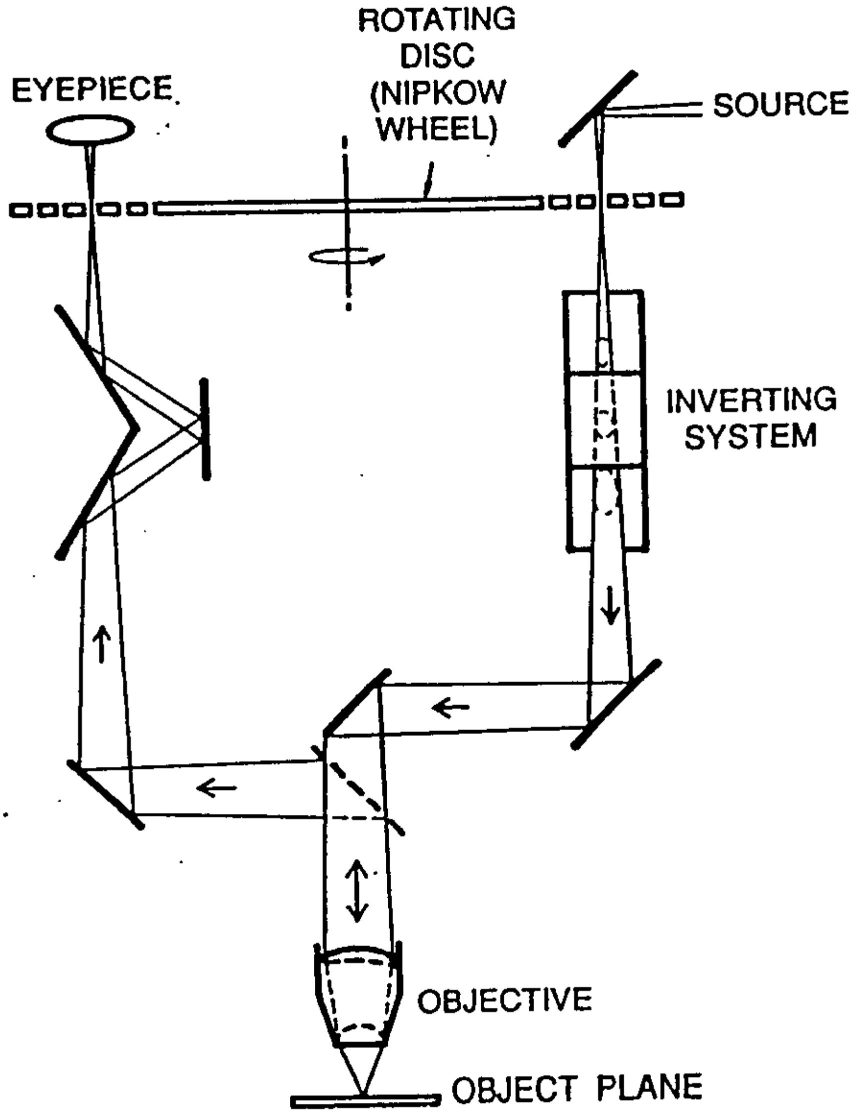


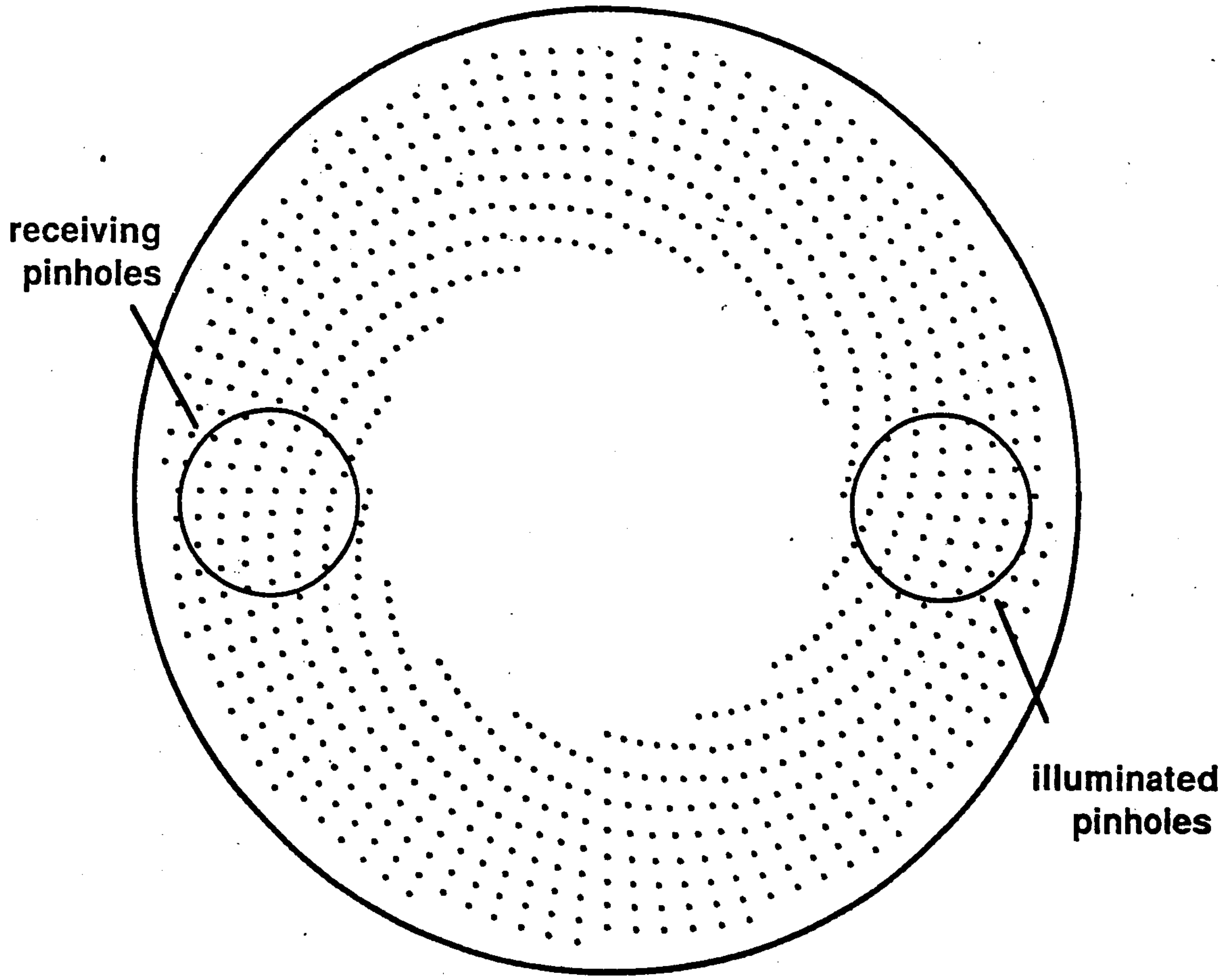
- **Two point systems**



- **Multiple point systems -- TSM**

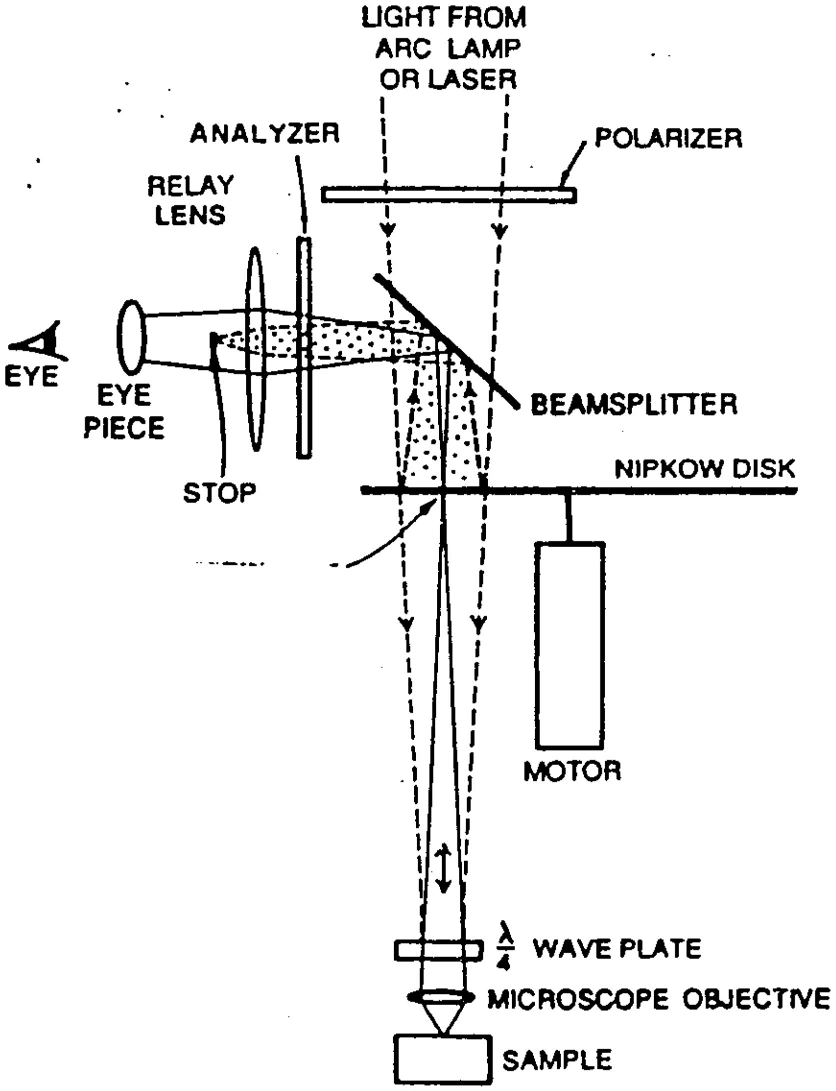
  space pinholes far apart -- cross talk


ROTATING DISC (NIPKOW WHEEL)
EYEPIECE
SOURCE
INVERTING SYSTEM
OBJECTIVE
OBJECT PLANE


receiving
pinholes
illuminated
pinholes

polarised light
black disc
stop
Tilt disc — not shown

GORDON KINO



tilt (maybe)

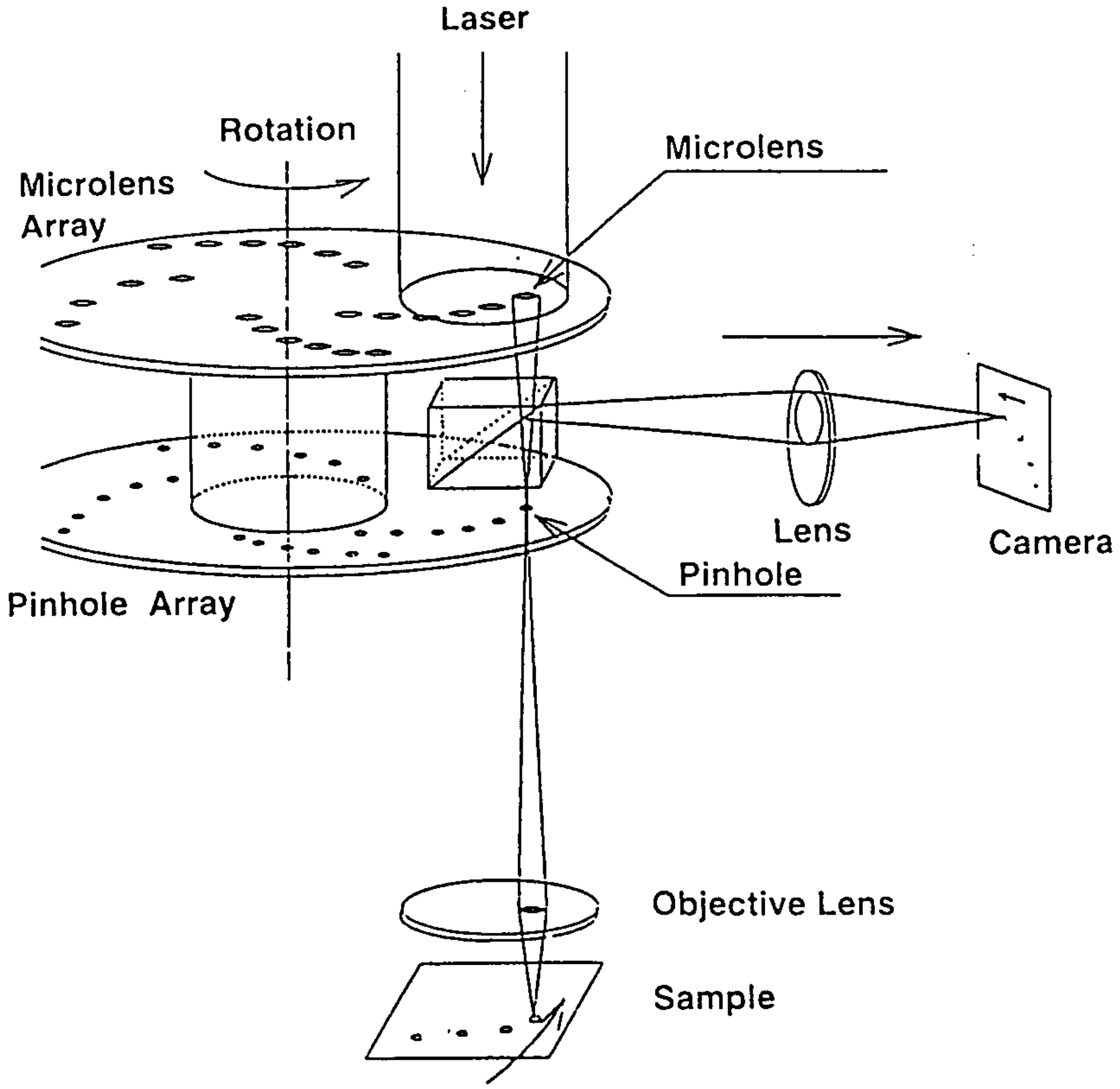


FIG.1 . Overview of CSU10

Confocal Microscopes

- **Single point systems**

  Lasers

    brightness
    limited wavelength choice

  ***usually* not real-time**

- **Multiple point systems**

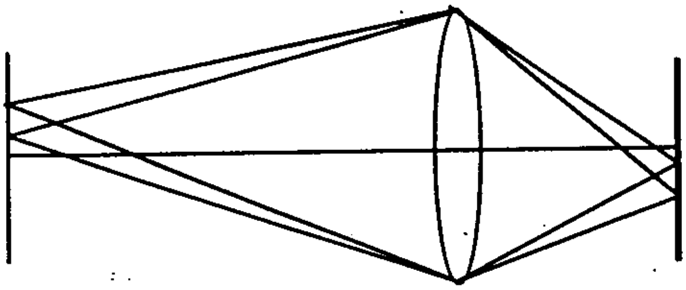
  Arc lamps can be used

    wide wavelength choice

  real-time

  low light budget

Aperture Correlation



aperture
mask

- **Place pixels close together**

- **Programmable SLM as aperture mask**

- **Use time sequential pixel transmission, $b_j(t)$.**

- **Choose $b_j(t)$ such that**

$$\langle b_i(t) b_j(t) \rangle = \begin{cases} 1 & i = j \\ 0 & i \neq j \end{cases}$$

i.e. no cross talk between pixels

- **Also need** $\langle b_i(t) \rangle = 0$

Correlation Sequences

- Choose $b_i(t)$ such that

$$\langle b_i(t) b_j(t) \rangle = \delta_{ij}$$

i.e. zero cross correlation between pixels

(i) Independent random binary sequences +1, -1

(ii) Complementary time shifted Golay sequences

<u>Both require negative intensities!</u>

Solution

Add constant dc offset to $b_i(t)$.

Pixel transmission $(b_i(t)+1)/2$ i.e. 0 or 1

Result

<u>Composite confocal and conventional image</u>

System implementation

- **Code apertures -- obtain composite image**
- **Obtain conventional image**
- **Subtract to obtain confocal image**

Implementation

- **Spatial light modulators**

  intensity or polarisation modulation of pixels

- **Spinning aperture discs**

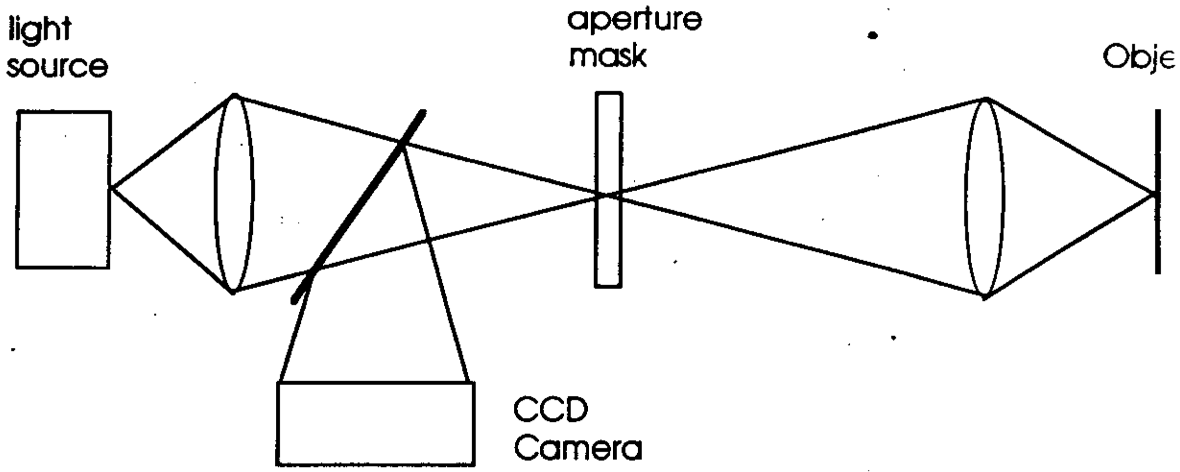
  impress correlation codes lithographically

  spin disc to achieve correlation

  use blank sector to obtain conventional image

  real time subtraction

The System

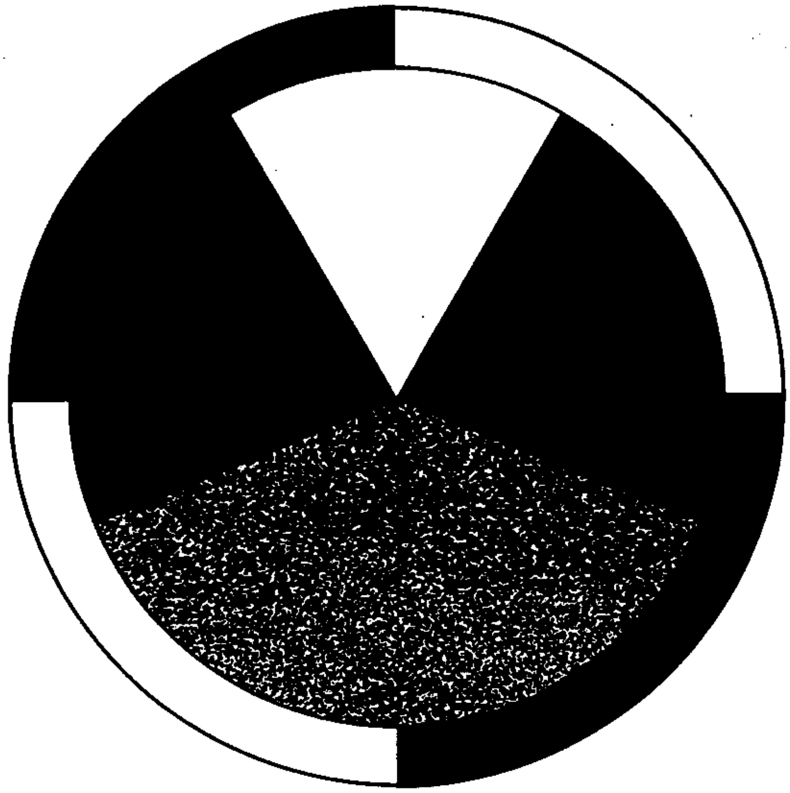


Aperture Disc -- **80 μm pixels on 80 μm pitch**
random sequence
equal black and white

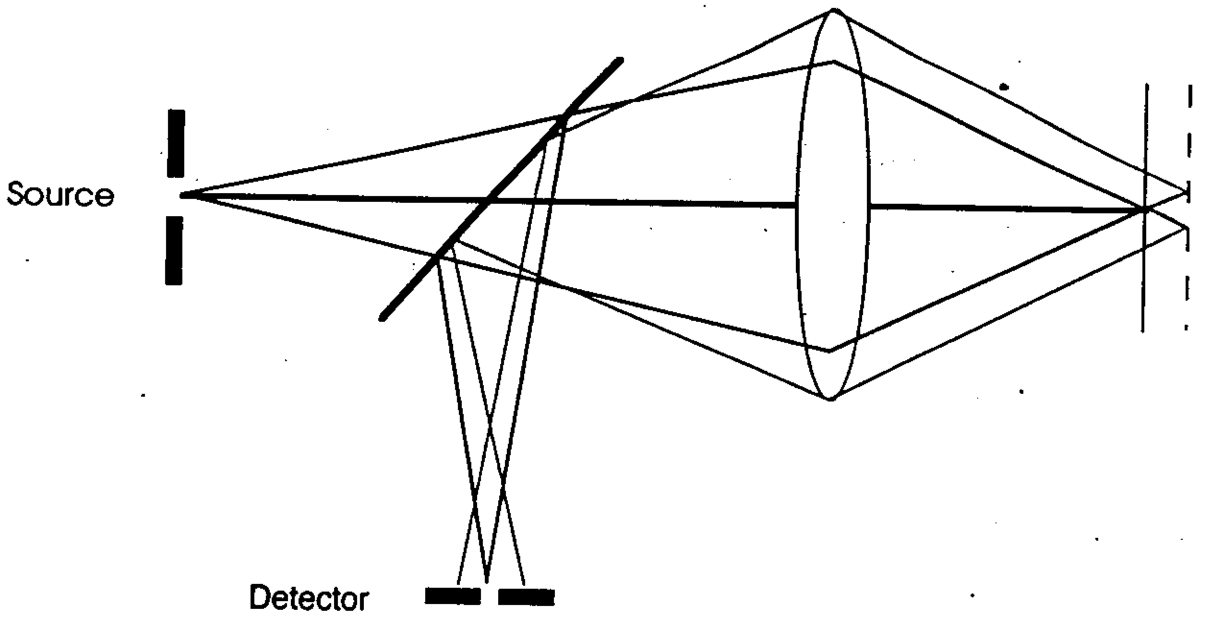
Camera -- **simple CCTV CCD camera**

Light source -- **incandescent projector bulb**

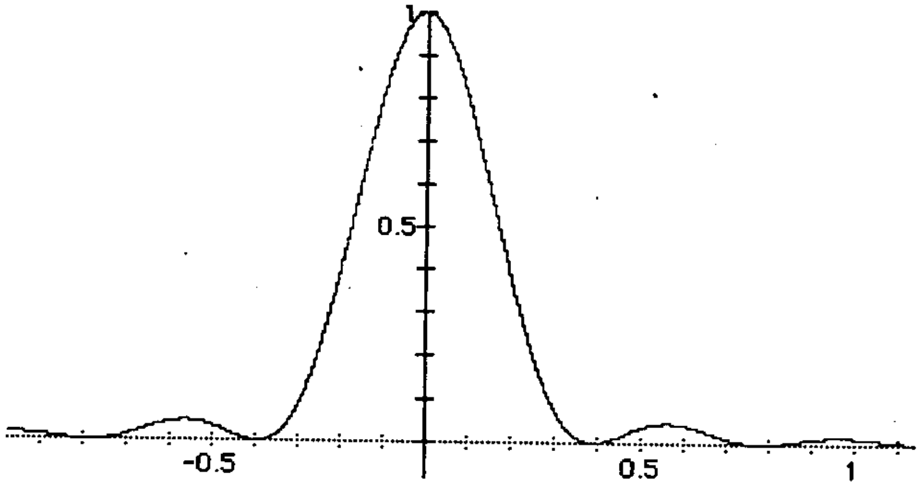
Image speed -- **17 confocal frames/second**

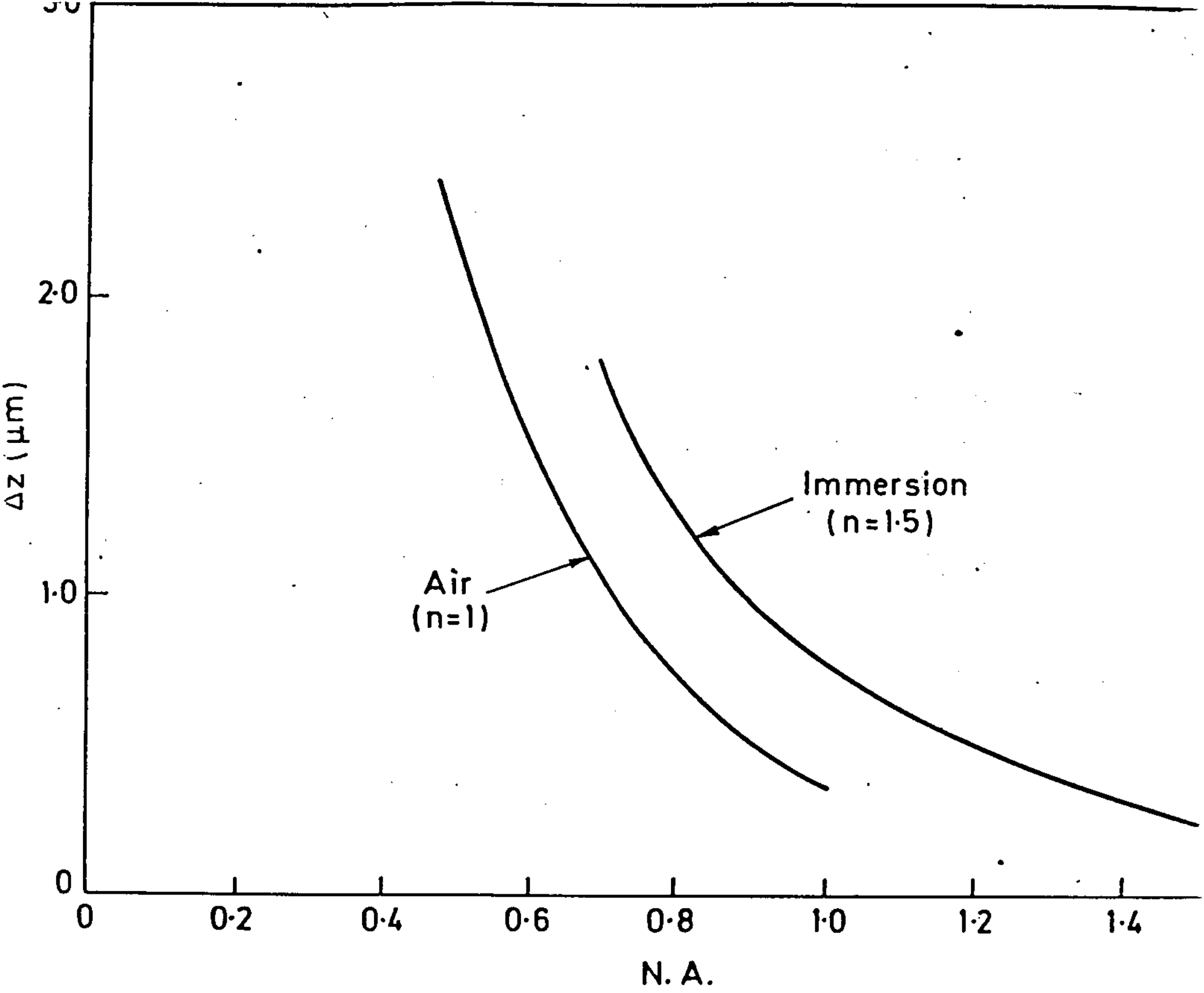


Optical sectioning



- **Scan plane reflector through focus**



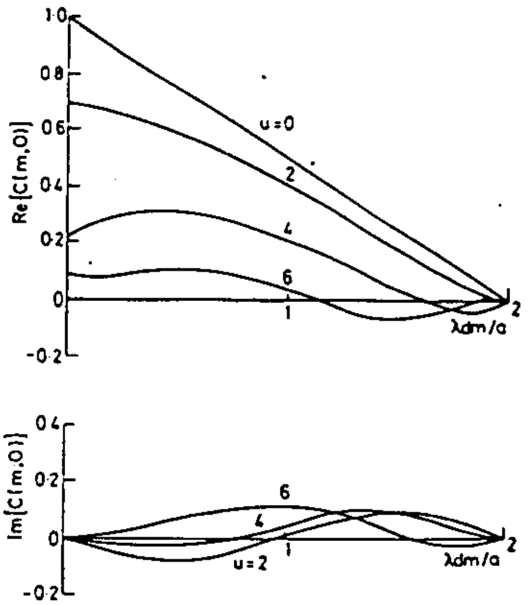


1.11

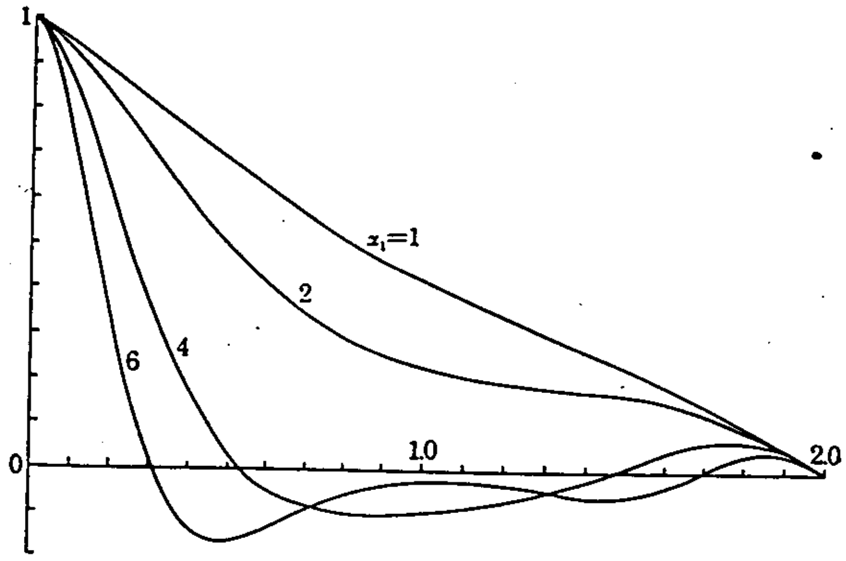
Optical Sectioning

- Require all spatial freqencies to attenuate with defocus.
- Confocal -- yes
- Conventional -- no
- Weak object transfer function reflection

Confocal



Conventional



- Non-zero spatial frequencies do attenuate with defocus.

<u>so</u>

- illuminate object with a single spatial frequency -- fringe pattern.
- obtain optically sectioned image but with fringe pattern super-imposed.
- remove fringe pattern
- optically sectioned image

Example

Actual image signal

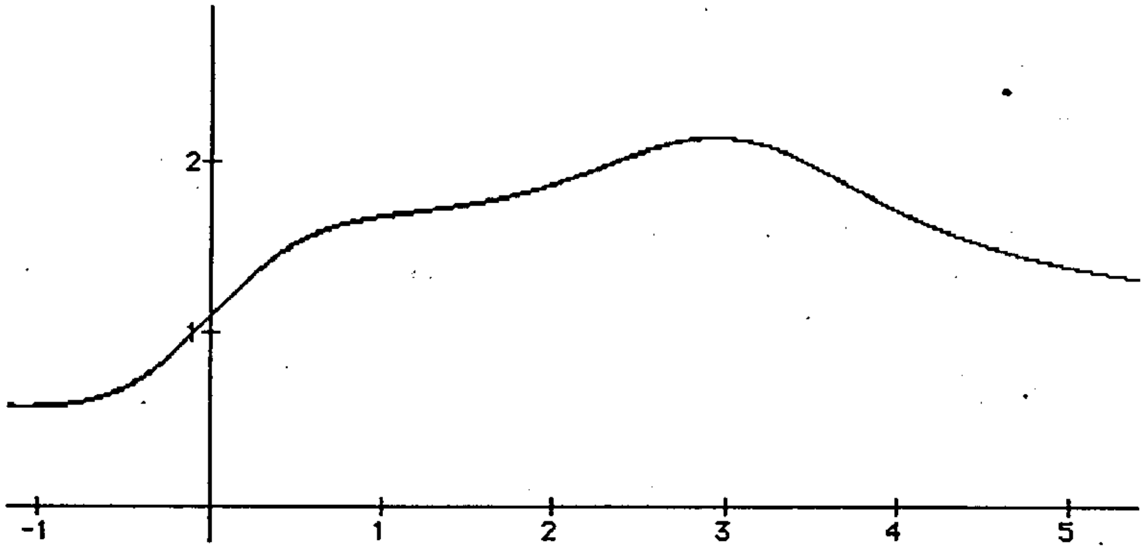
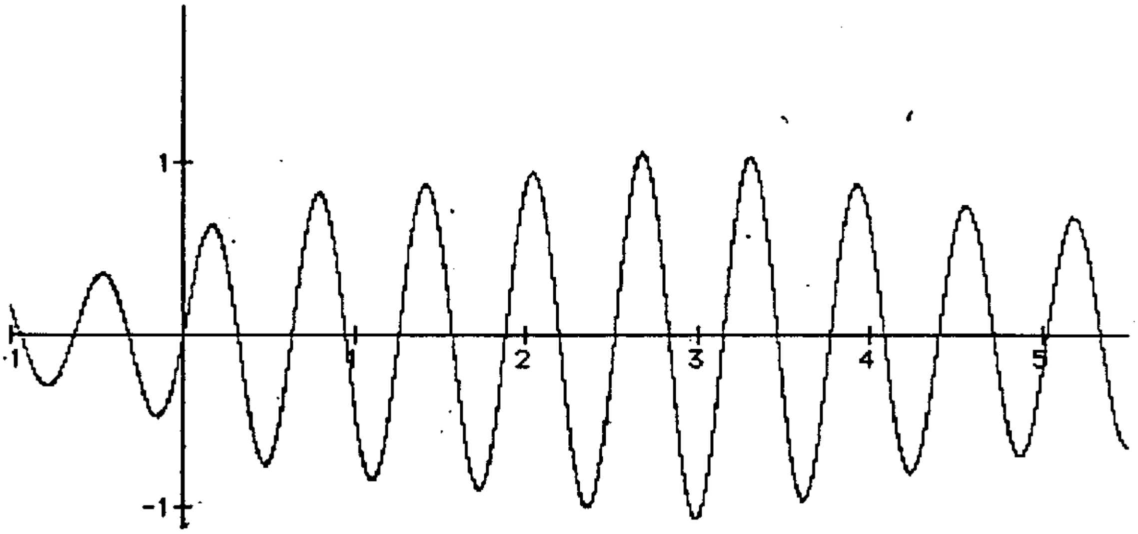


Image with fringes



Fringe Removal?

Implementation

- Introduce 1 D grating into illumination path

$$S = 1 + \cos(2\pi \upsilon x)$$

- Image $I = I_0 + I_{sf}$

$I0$ is a conventional image.

I_{sf} is the sectioning image with fringes.

Fringe removal

- Design of suitably matched detector array to sample at fringe maxima (followed by ± 1 multiplication).
- Very stringent alignment -- impractical.
- Mathematical technique using three images.
- Fast, simple, no alignment problems

Fringe removal

- 1 D grating in illumination path

$$S = 1 + \cos\{2\pi\upsilon(x+\phi)\}$$

ϕ represents a shift in the grating position

- Image crudely

$$I = I_0 + I_s \cos\{2\pi\upsilon(x+\phi)\}$$

I_S is the desired sectioning image.

Take three images.

I_1 corresponding to ϕ=0

I_2 corresponding to ϕ=120

I_3 corresponding to ϕ=240

Calculate

$$I_s = \sqrt{(I_1 - I_2)^2 + (I_1 - I_3)^2 + (I_2 - I_3)^2}$$

$$I_0 = I_1 + I_2 + I_3$$

Fringe Removal

$$I_s = \sqrt{(I_1 - I_2)^2 + (I_1 - I_3)^2 + (I_2 - I_3)^2}$$

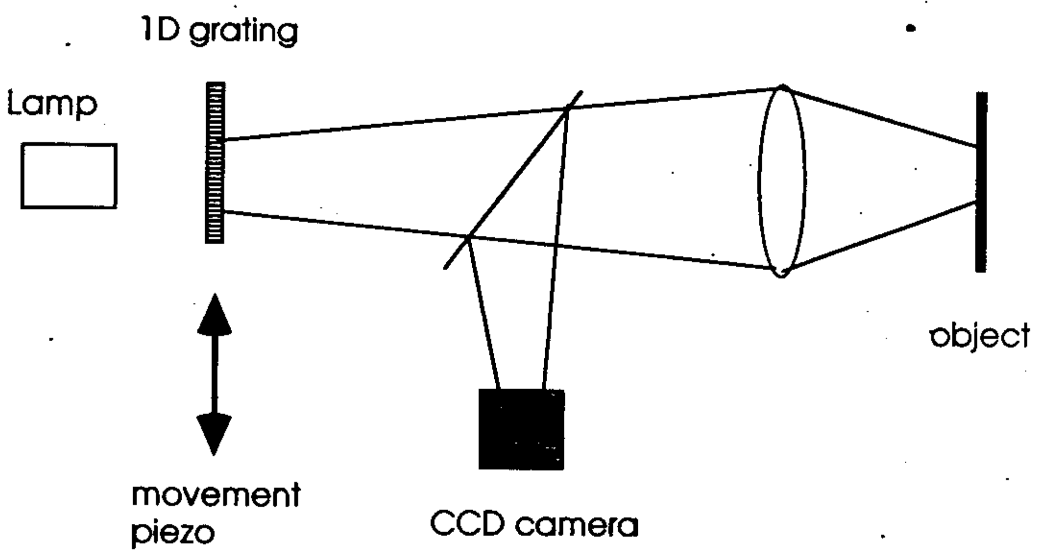
- Computationally intense
- I_1, I_2, and I_3 all from 8 bit, say, camera
- Fixed number of values
- Look-up table -- real time imaging.

Full theory gives

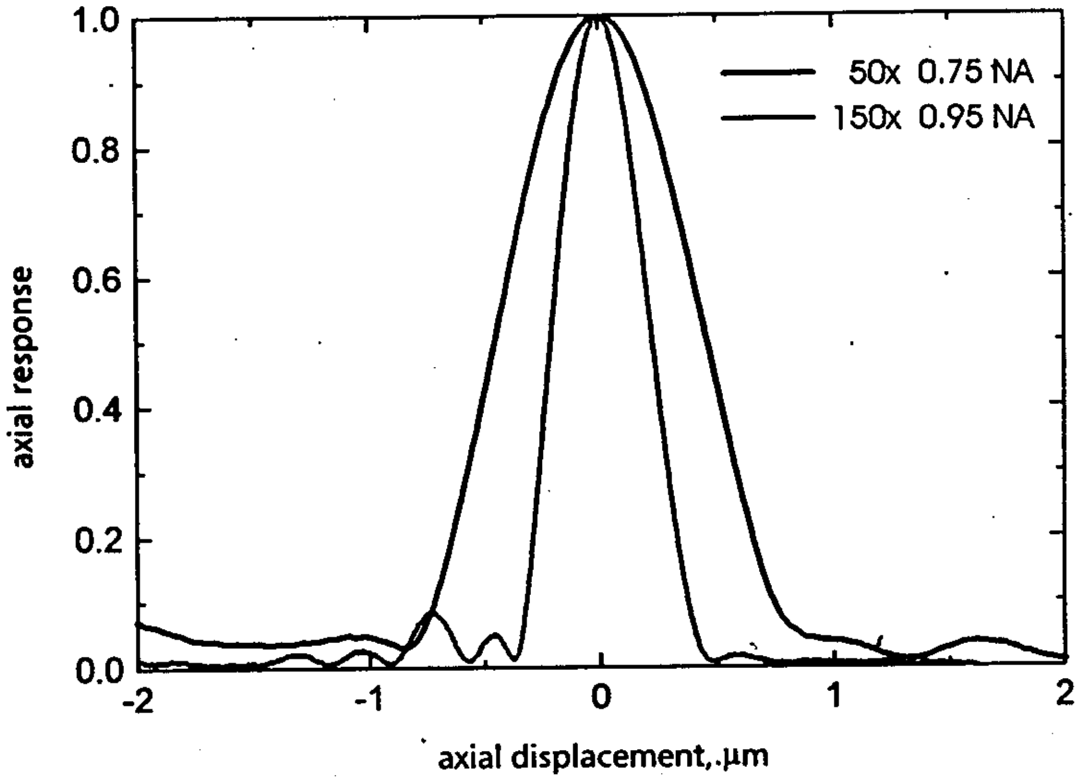
$$I = \left| \iint C(m,p)T(m)T^*(p)\exp - j(m-p)xdmdp \right|$$

- partially coherent
- axial response to plane mirror -- $|C(0,0)|$

Implementation



- **standard microscope illumination**
- **piezo-driven 25 μm pitch grating**
- **light efficient**
- **real-time imaging**
- **easily and cheaply implemented in conventional microscope**


1.0
0.8
0.6
0.4
0.2
0.0
axial response
-2
-1
0
1
2
axial displacement, µm
50x 0.75 NA
150x 0.95 NA