

Biomedical Optics

Introduction to Microscopy

Lecture 4 Advanced Techniques

Mike Hughes
Physics & Astronomy, University of Kent

Contents

1 Qualitative and Quantitative Phase Imaging [Not Examinable]	1
1.1 Phase Contrast	2
1.2 Quantitative Phase Imaging/Digital Holographic Microscopy	2
2 Other Microscopy Techniques [Not Examinable]	3

The content of this lecture is not examinable, but by looking at advanced techniques we will have a chance to deepen our understanding of the topics covered in Lectures 1-3.

1 Qualitative and Quantitative Phase Imaging [Not Examinable]

In a transmission microscope, we obtain image contrast because parts of the sample absorb different amounts of light. However, as discussed in Lecture 1, many objects (such as cells) are not very absorbing, and so contrast can be quite low.

As light travels a distance z through a material of refractive index n (i.e. of optical thickness nz), the phase progresses by knz where k is the wavenumber.

$$k = \frac{2\pi}{\lambda} \quad (1)$$

and where λ is the wavelength. So, if we initially have some light with phase ϕ , then after passing through the material it will have a phase of,

$$\phi' = \phi + nz k, \quad (2)$$

and so the electric field, E , becomes E' ,

$$E' = |E|e^{i[\phi+nzk]}. \quad (3)$$

Remember that the phase wraps around every time we get to 2π , since $\cos \phi = \cos(\phi + 2\pi)$.

Now, if we consider a sample such as a cell on a microscope slide, the refractive index of the cell is different to the surrounding medium. So the light which has travelled through the cell will have a different phase to the light which hasn't. So, if we could see the phase this would provide some extra contrast in the image. If we could measure the phase, we might also be able to measure the optical thickness of the cell (although this is complicated if the phase difference has gone past 2π and wrapped around). If we know the physical thickness this can tell us n , or if we know n then it tells us the thickness.

A second benefit of phase imaging is that, once we have determined the 'complex field' (combination of amplitude and phase), we can use the numerical refocusing techniques to determine the field at other axial positions. This means we can 'focus' at different depths in the sample without moving anything (or even go back and refocus our images later on). It also means that, in some cases, we can dispense with a lens altogether, and perform all the focusing numerically.

1.1 Phase Contrast

Phase contrast imaging is a very old idea, it was first developed in the 1930s and won Fritz Zernike the 1953 Nobel Prize in Physics. The idea is to somehow convert phase information into intensity information so that we can see it (either by eye or on a camera). This is possible using interference, because the intensity pattern produced by the interference between different beams depends on their relative phase.

There are two common methods: Zernike phase contrast and Differential Interference Contrast (DIC) microscopy. Both improve contrast for specimens which add an optical delay but do not significantly absorb (known as **phase objects**). You can read more about them at:

<https://www.olympus-lifescience.com/en/microscope-resource/primer/techniques/dic/dichome/>

<https://www.olympus-lifescience.com/en/microscope-resource/primer/techniques/phasecontrast/phaseindex/>

Note that, in both cases, the relationship between the actual phase and the intensity pattern we see is quite complicated and generally not reversible, i.e. we can't figure out the actual value of the phase. So we sometimes refer to this as **qualitative phase imaging**.

1.2 Quantitative Phase Imaging/Digital Holographic Microscopy

The goal of Quantitative Phase Imaging (QPI) is to reconstruct the complex field (amplitude and phase) by encoding phase information in the intensity recorded by the camera in such a way that we can recover the actual value of the phase. There are various ways of achieving this, mostly involving using interference between light from the sample and a reference beam. Because the interference pattern depends on phase, we can then work backwards to figure out the actual values of the phase.

This has several important applications:

1. **Contrast:** As for conventional phase contrast imaging, QPI allows samples which do not absorb or scatter strongly enough to be seen in intensity images to be viewed.

2. **Thickness measurements:** Since the phase delay depends on the thickness of the sample (assuming constant refractive index) then the phase measurements can be used to infer the thickness of the sample at each (x, y) point and hence recover a 3D representation of the sample.
3. **Numerical Refocusing:** Once the complex field is known at some distance from the sample, we can adjust the focus position numerically using the Fresnel propagator to compute the field at any other distance. This allows post-acquisition adjustments in the focus position, or acquisition of extended depth of field images, with no physical turning of a focusing knob.
4. **Refractive Index Measurements:** The phase changes induced by a sample depend on the optical path length, D , through the sample. $D = nL$ where L is the physical thickness and n is the refractive index. Hence if the physical thickness of an object is known then phase measurements can be used to recover the refractive index (or rather the line integral of $n(z)$ through the sample).

2 Other Microscopy Techniques [Not Examinable]

There is a huge range of other microscopy techniques which we do not have time to study in detail and which will not be examined. However, you now have the basic tools to understand them, and can read further yourself if interested. For example:

- **Super-Resolution Microscopy:** It is possible to go beyond the fundamental resolution limits discussed in Lecture 2. Structured Illumination Microscopy (SIM) can achieve a factor of 2 improvement, and can be applied to any sample. STED and related techniques exploit saturable fluorophores, while STORM and related techniques use fluorophores that blink on and off. These fluorescence techniques can achieve resolution far below the diffraction limit, ultimately limited by factors including noise.
- **Fluorescence Lifetime Imaging Microscopy (FLIM):** In a normal fluorescence microscope, we are merely detecting the presence of a fluorophore, and distinguishing between different fluorophores by their different excitation wavelengths (e.g. we successively image using different wavelength lasers). However, we can also illuminate using a pulse from a laser (or a cyclically amplitude-modulated laser), and look how the fluorescence decays as a function of time. This provides additional information, particularly from intrinsic fluorophores (i.e. naturally occurring tissue fluorescence).
- **In Vivo and Endoscopic Microscopy:** By adding microscopic imaging to an endoscope we can perform microscopy directly on living tissue (as opposed to the normal technique of first removing the tissue using a biopsy). You can look up *Mauna Kea Technologies* who produce the *Cellvizio* endoscopic microscope, and this is also a [topic of research at Kent](#).