Aim

To provide an overview of light and fluorescence microscope techniques for localising DNA, RNA and their protein products in cells and tissues.

Introduction to microscopy: an outline of light and fluorescence microscopy with selected methods for staining gene products and other cell and tissue components.

Hands-on work: use of the microscope, general stains for plant and animal tissues, fluorescent localisation of the actin cytoskeleton, DNA and ER in fixed and living cells and tissues

Introduction

Why would a molecular biologist ever need to look down a microscope? If the experimental work involves only sequence analysis, protein identification, or quantification of nucleic acids or protein, microscope work may not be needed. However, in many cases, once the gene, RNA message or protein of interest has been isolated and characterised, you will want to know its location and activity in the source tissue. This requires some type of microscopy to observe the DNA, RNA or proteins after they have been tagged to make them visible to the human eye either directly (staining) or after further processing (e.g. isotopic labels).

There are three main types of questions that can addressed by this technique:

1. Where is the gene of interest active or protein of interest produced?
   DNA can be localised within chromosomes or nuclei at various stages of the cell cycle; the mRNA produced by transcription of specific genes can be detected; as can the protein from translated message. Both nucleic acid and protein can be labelled in the same tissue or in sequential sections of tissue using different markers for each.
2. When is the gene/mRNA active?
   Quantitative analysis of tissue extracts (Southerns, Northerns, Westerns) can be combined with localisation to specific cells and/or organelles.
3. What internal or external stimuli induce this activity?
   Observe production of message or protein in response to hormones, other ligands and agonists, temperature stress, drought, salt stress, sugars, etc.

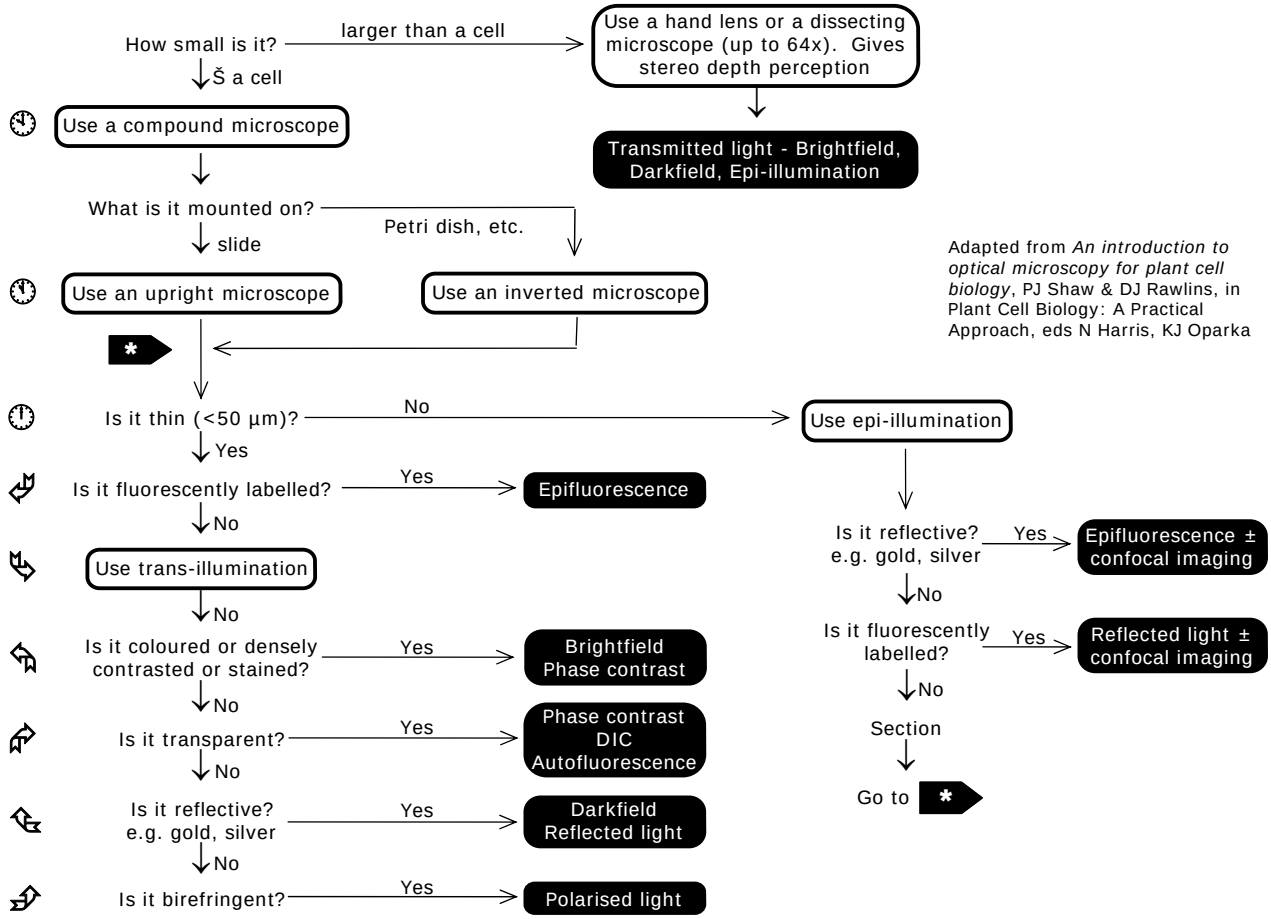
In each case, there are two main ways to localise the DNA, RNA or proteins:

1. Stain the tissue with a compound(s) that binds only to the molecule(s) of interest.
   For DNA, use oligos, dsDNA (cDNA or genomic) tagged isotopically or with biotin, DIG or fluorochrome.
   For RNA, use sense or antisense ssRNA, cRNA, oligos or riboprobes tagged as for DNA.
   For protein, mainly use antibodies to specific proteins, and the signal is usually amplified with a secondary antibody tagged with fluorochrome, biotin, enzyme, gold, etc.. Can also use histochemical stains for specific classes of proteins, or specific ligands, e.g. lectins.
2. Tag the nucleic acid or protein of interest with a genetic marker, such as GFP, GUS, etc. which can be observed either directly (GFP) or after further staining (GUS) in transgenic organisms or tissues.

The stain or tag is observed in transmitted or fluorescent light, depending on the type of probe used. Isotopic tagging requires exposure to film and comparison with source tissue. Most of these stains or tags can be observed in fixed and sectioned tissue, but there is now a wide range of fluorescent probes that enable observation of many nucleic acids, proteins and other cell components in living tissue.

Today, we will go through the basics of transmitted light and epifluorescence microscopy, discuss relevant applications for molecular biology and examine different staining techniques for light and fluorescence microscopy. We will also compare conventional fluorescence and confocal microscopy to illustrate their different applications.

Microscope methods and when to use them



Adapted from *An introduction to optical microscopy for plant cell biology*, PJ Shaw & DJ Rawlins, in Plant Cell Biology: A Practical Approach, eds N Harris, KJ Oparka

1. In most cases, localisation will be in cells or tissues observed under the compound microscope.
2. If the preparation is a living cell culture or other tissue that must be maintained in liquid, observation is best on an inverted microscope, and may need a temperature-controlled chamber.
3. For very thick tissue, or highly pigmented tissues (most animal tissues, pigmented plant tissues) which must be observed with minimal manipulation or pigment extraction, the images must be collected from the surface layers - epi-illumination. Confocal microscopy greatly enhances the depth from which high resolution images can be collected.
4. Fluorescent stains are detected by epifluorescence, which allows the emitted fluorescent light to be separated from the incident excitation light. Conventional fluorescence microscopy uses dichroic mirrors, which allow transmission of shorter wavelengths from the light source to the specimen, but longer wavelengths emitted from the specimen are reflected by the mirror to the observer (see below).
5. Cell cultures, thin slices of living tissue, or thin sections of fixed, embedded and sectioned tissue can be observed with transmitted light.
6. Thin sections of highly pigmented tissue, or tissue with high-contrast staining, can be examined in brightfield. Staining is a chemical method of enhancing contrast of thin, transparent specimens.
7. Optical contrast methods - either phase contrast or differential interference contrast (DIC), are used for very transparent, lightly stained specimens in which you need to see the tissue around the stained areas of interest. Many plant and animal tissues are autofluorescent (which may interfere with fluorescent probes).
8. Stains that produce reflective crystals (peroxidase reactions) or that use colloidal gold or silver-enhanced gold can be seen in either darkfield or reflected light. The stains are very bright against a dark background.
9. For a few applications in plant and animal biology, the ability of the material to rotate polarised light is used to assess particular properties, e.g. orientation or thickness of layers in bone or plant cell walls.

Brightfield microscopy and Köhler illumination

For all light microscopy, it is essential to adjust the microscope for optimal imaging. Microscopes use a type of illumination named after its inventor, which is called Köhler illumination. The following protocol is used to set up the microscope:

1. Focus on the specimen with the field diaphragm (on the light source) and condenser diaphragm fully open.
2. Close the field diaphragm until the edges can just be seen.
3. Adjust the height of the condenser until the edge of the field diaphragm is sharply focussed.
4. If necessary, centre the image of the field diaphragm with the centring controls on the condenser.
5. Open the field diaphragm until it just disappears from the field of view. The condenser centration may need fine adjustment at this stage.
6. Remove one eyepiece and look down the eyepiece tube; the condenser diaphragm should be fully open. Close this diaphragm so that about two-thirds of the original field is visible. This is the standard setting for optimal contrast and resolution, but may be adjusted according to personal preference or for difficult specimens. Opening the condenser diaphragm increases resolution but decreases contrast and vice versa.
7. For low power objectives on older microscopes, you will need to swing in an accessory lens in the condenser and readjust for Köhler illumination. You will need to make minor adjustments to the field diaphragm, condenser height and condenser diaphragm when switching between objectives.

Phase contrast

This technique was invented by Zernicke (and earned him a Nobel Prize). Here, diffracted light from the edges and interfaces in the specimen is made to interfere with the direct light beam such that the two beams are exactly half a wavelength out of phase with each other. This will give rise to constructive or destructive interference, producing bright outlines at the edges of refractile objects.

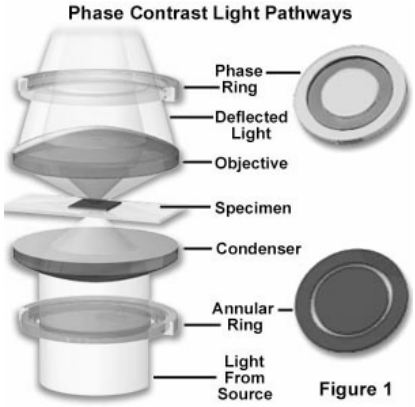
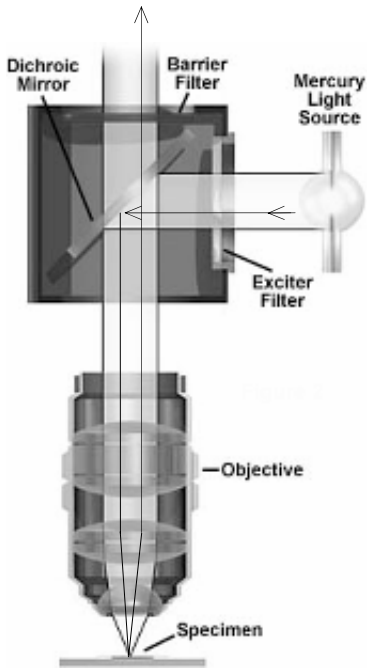


Figure 1

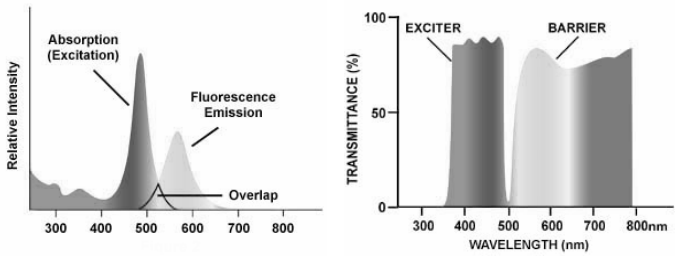
Fluorescence

Certain compounds will absorb light, usually in a narrow wavelength range (waveband), then re-emit some energy in the form of light of specific, longer waveband. If the **fluorochrome** only emits light during the absorption and excitation from a primary light source, this is **fluorescence** (as opposed to phosphorescence). Since we need emission of visible light, the excitation wavelengths used are long-wave UV, violet, blue and green light (approx. 360-570 nm). The resulting fluorescence is violet, blue, green, yellow, orange or red.

Epifluorescence microscopy uses dichroic mirrors that reflect short wavelengths of the exciting light onto the specimen, then allow transmission of the longer-wavelength emitted light back through the mirror to the eyepiece or camera. The objective also functions as the condenser in epifluorescence.



The **exciter filter** transmits short wavelengths of the exciting light, which matches the absorption peak of the fluorochrome, onto the specimen, and the **barrier filter** transmits only the longer-wavelength light emitted from the fluorochrome.



Objectives used for specific applications

Microscope objectives are designed specifically for phase contrast, DIC, fluorescence, etc., although they can often be used for more than one application, e.g. DIC and fluorescence. They are corrected to varying degrees for flatness of field and colour aberrations. They may be used either dry, with oil immersion or water immersion. Some highly corrected objectives give best results when used with coverslips of correct thickness, or may have a correction collar for use with different types of coverslip. These features are indicated on the objective, as seen at right.

References

There are some very good tutorials and reference sites on the web. Some of these are listed below.

micro.magnet.fsu.edu/primer/index.html
Excellent - highly recommended for those who have not done much microscopy and who wish to find out how it works and what information is obtained using the different options. This is Molecular Expressions' Microscopy Primer, and covers all of the basic types of microscopy discussed above and listed in the flow diagram, plus has very detailed tutorials illustrating how the images form, with Java applets that let you manipulate the "microscope" and observe how the image is affected.

micro.magnet.fsu.edu/primer/webresources.html
A comprehensive listing of links about many aspects of microscopy, including other microscopy sites on the web, history of microscopy, virtual microscopy, various facilities and microscope labs, educational sites, societies, booksellers, journals, newsgroups, glossaries, and last but not least, equipment and other suppliers. Maintained by Molecular Expressions - supported by Olympus Microscopes and Florida State University.

swehsc.pharmacy.arizona.edu/exppath/micro/index.html
This is another extensive list of microscopy and imaging resources on the web, maintained by the Experimental Pathology Service Core, Southwest Environmental Health Sciences Center (an NIEHS Center), Center for Toxicology, University of Arizona College of Pharmacy. Good starting point for finding out about any aspect of microscopy.

www.probes.com
A commercial site that has a vast amount of information on its fluorescent probes, including much useful detail about absorption and emission spectra and how these are affected in different chemical environments.

www.cellbio.com/protocols.html
An on-line data base of molecular biology protocols with links to other relevant pages.

There are many general books and review articles that deal with specific techniques. The series Methods in Molecular Biology, Methods in Cell Biology (American Society of Cell Biology) and the Practical Approach series (Oxford U Press) are particularly good, with some relevant volumes are listed below. They should be in your library!

Antisense Technologies (1997)	eds Conrad Lichtenstein and Wolfgang Nellen
Electron Microscopy in Molecular Biology (1991)	ed. Robin Harris
Essential Molecular Biology 1 & 2 (1991)	ed. T.A. Brown
Gene Probes 1 & 2 (1995)	eds B.D. Hames and S.J. Higgins
Immunocytochemistry 1 & 2 (1997)	eds Alan P. Johnstone and Malcolm W. Turner
In Situ Hybridisation 2nd edn (1998)	ed. David G. Wilkinson
Light Microscopy in Biology 2nd edn (1999)	ed. Alan J. Lacey
Monoclonal Antibodies (2000)	eds Phil S. Shepherd and Chris Dean
Oligonucleotides and Analogues (1991)	ed. Fritz Eckstein
Plant Cell Biology (1994)	eds N. Harris and K.J. Oparka
Plant Cell Biology (2001)	eds C. Hawes and B. Satiat-Jeunemaitre
Plant Molecular Biology (1989)	ed. C.H. Shaw
Protein Localisation by Fluorescence (1999)	ed. Viki Allan

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Stevens JK, Mills LR, Trogadis JE (eds) (1994) Three-Dimensional Confocal Microscopy: Volume Investigation of Biological Specimens. Academic Press, London.

Suppliers (this list is not exhaustive)

Australian Instrument Services Pty Ltd, P.O. Box 2 Croydon, Victoria, 3136 www.ausinst.com.au Email: sales@ausinst.com.au	ph 03 9729 9399 1 800 625 008 fax 03 9720 5776
Carl Zeiss Pty Ltd, 114 Pyrmont Bridge Road, Camperdown, 2050 http://www.zeiss.de/C12567BE0045ACF1?Open info@zeiss.com.au	ph 9516 1333 fax 9519 5642
OED Pty. Ltd., Suite 8, 6 Pound Road, Hornsby, 2077 members.ozemail.com.au/~sbwisbey/index.html jbwisbey@ozemail.com.au	ph 9482 1149 fax 9482 1196
Olympus Australia, 152 Forster Rd, Mt Waverly	ph 132 992
Nikon, 47 Overseas Drive, Noble Park www.nikon.com/	ph 131 404 fax 790 1900
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Selby Scientific Instruments, 352 Ferntree Gully Rd, Notting Hill	ph 132 990
York Optical and Scientific, 256 Flinders St, Melbourne	ph 654 3938 fax 654 4223