Practical Transmission Light Microscopy

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Kohler Illumination

- The aim is to have the incident light focussed on the specimen
- All extraneous light is cut out
- The specimen is illuminated by a small cone of light
- Scattered light is also cut out
Bright Field Setup

- Clean up the light path.
- Remove all filters, phase rings, prisms etc.
- Check objectives are clean
- Set condenser to bright field
Bright Field Setup 2

- Focus on the specimen
- If you can’t find it try a low power first
- Identify the field diaphragm
- (It is usually in the path from the light bulb to the condenser)
- Stop it down until you can see its edges.
- If you can, you have found the field diaphragm!
Bright Field Setup 3

• Focus the edges of the field diaphragm by raising and lowering the condenser.
• Open the diaphragm until it just disappears from view.
• Centre it if necessary
• Remove an eyepiece
Bright Field Setup 4

- Find Iris diaphragm (usually in the condenser itself).
- Looking down empty eye tube, close iris diaphragm until it covers 10% or the field of view.
- Replace eyepiece and check contrast levels.
- Adjust the iris diaphragm as required.
Bright Field Setup 5

- Check focus using the fixed eyepiece
- Without adjusting focus rotate the other eyepiece until it too is in focus when both your eyes are relaxed.
- Interpupil distance can then be adjusted to give binocular fusion of the two images
The Virtual Microscope

• If you want to practice this there is a virtual microscope with instructions at:

  • http://microscopy.fsu.edu/primer/

• Here you can see clear examples of the effect of each step.
Polarizing Microscopy

Polaroid filters only allow one plane of light to pass through. This technique depends on having one polarising filter below the condenser and one above the objective. When these are “crossed” no light passes. Anything in the object that polarises light will cause light to pass through the second filter.
Differential Interference Contrast Microscopy (DIC)
(also known as Nomarski IC.)

- In this technique of polarised microscopy two extra prisms are added to the light path.
- These Woolaston prisms of two calcium fluorite wedges cemented together are placed above and below the polaroid filters, usually in the condenser and objective holder.
Nomarski (DIC)

The three dimensional appearance is actually an artifact of the technique indicating only differences in optical density.

The contrast can be altered by fine adjustments of the polarising filter or the Woolaston prism above the objective.

The addition of a Lambda plate to the light path can add colour.
Phase Contrast Microscopy

- The eye and film perceive variations in colour and intensity. Neither is sensitive to phase.
- However transparent objects may alter the phase of light passing through them.
- F. Zernike won the Nobel prize in 1953 for showing how to convert phase differences to amplitude differences we can observe.
Unstained vs Phase vs DIC
Phase Contrast 2

- Transparent materials will have little effect on transmission (brightness)
- However they may well alter the phase of the light due to the different refractive index of the object and its surroundings.
Phase Contrast 3

The phase plate has a neutral density filter in a ring surrounded by material which shifts the wavelength of the light by a quarter (a 1/4 Lambda plate).

- To convert phase changes to density changes a special condenser with a ring shaped mask is used.
- The rings are matched to objectives.
- Special 'phase' objectives are used with a "Phase ring" fixed to the back focal plane of the...
A few manufacturers offer different objectives with thicker or thinner neutral density plates that can affect contrast.
Phase Contrast 3

- Phase is a sort of darkfield microscopy, the background is darkened due to the neutral density filter,
- Only diffracted light passes through the 1/4 lambda part of the phase ring
- The interference created lead to the phase difference being changed into intensity differences.
Phase Contrast 4

- Light in phase will brighten
- Light out of phase will darken
- In phase cells have an artificial halo of bright light, this is NOT good for quantification but IS good for finding unstained cells
Phase Contrast 5

- In order for phase contrast to work the phase ring must be carefully aligned.
- A Bertram lens or an eyepiece telescope is used to bring the condenser ring in to focus.
- Two screws are adjusted to centre the phase ring.
- If the wrong phase ring is in place it will be difficult to achieve clarity.

Phase Plate and Light Annulus Alignment

(a)  (b)  (c)

Figure 4
Hoffman Modulation Contrast

- This technique is a sort of “poor mans Nomarski”
- However it is really good at low magnifications and will work through plastic
- It is very good for general tissue culture work
- It is a directional effect like Nomarski so a rotating stage can be an advantage although on
Principles of Hoffman Modulation Contrast

Central Modulator and Slit Plate

Modulator

Objective Lens

Specimen Slide

Condenser Lens

Slit Plate

Polarizer

Offset Modulator and Slit Plate

(a) Figure 3 (b)
Hoffman 3

- Rather like phase a slit (rather than a ring) is aligned with a plate in the objective plane that darkens light out of phase.
- The separate polarizing filter allows better control of contrast than with Nomarski.
- Hoffman is a patented technique so the cost is often a condition of sale (i.e., a license fee is paid).
Hoffman 4

- As with phase it is crucial that the plates are aligned correctly.
- The Bertram lens is used to focus on the modulator plate.
- It is rotated to line up with the condenser slit.
- Screws are then used to move it laterally until there is only a small slit admitting light.
Darkfield Microscopy

In this technique the specimen is illuminated by a cone of light which bypasses the objective. The NA of the condenser must exceed that of the lens. Resolution is poor. Objects in the light path scatter light into the objective. Largely replaced by DIC or Phase.
Darkfield 2

- In darkfield microscopy an opaque disc is inserted in the condenser. In more expensive systems a spherical mirror is employed to keep light levels up.
- With careful alignment and after opening the diaphragms the specimen is illuminated with a cone of light.
- This is particularly good for autoradiography.
Rheinberg 2

(a) Brightfield  
(b) Darkfield  
Figure 5  
(c) Rheinberg

Good for winning macrophotography competitions!
Rheinberg Illumination

Figure 1

Figure 3

Figure 5
Other Techniques

• Oblique Illumination
  – The condenser is set far to one side resulting in a dramatic shadowing effect.
  – Confusing for anyone that follows you!
  – New systems with fibre optic oblique lighting appearing.