

Scinapse

A high-contrast, false-color microscopy image of a neural network. The neurons are depicted with bright yellow and white outlines against a dark background. Some cell bodies and processes are highlighted in cyan and magenta. The overall appearance is that of a complex, interconnected web of biological structures.

Improve the
contrast of your
microscopy
images

 Scientifica

A note from Scientifica

Scientifica specialises in providing cutting-edge equipment optimised for electrophysiology, multiphoton imaging and optogenetics research.

We have created this magazine to help you improve the contrast of your electrophysiology images. We have explained how each technique works, the equipment needed as well as the advantages and disadvantages of each.

The guides included in this magazine can be found online at www.scientifica.uk.com. We have collated them in this magazine so they can be easily referenced in the lab.

Please get in touch if you any feedback and suggestions, we'd love to hear your thoughts via info@scientifica.uk.com.



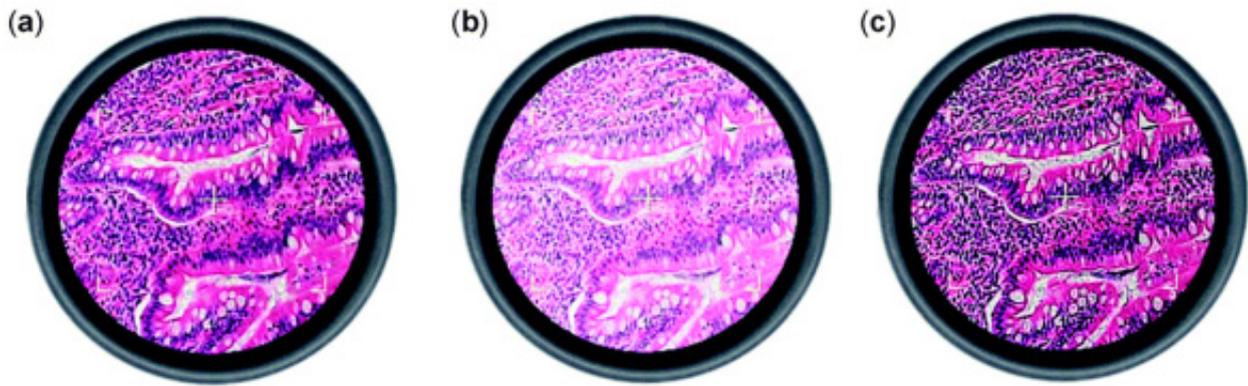
Thank you

The team at Scientifica



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Koehler Illumination

Koehler Illumination provides optimum contrast and resolution by focusing and centring the light path and spreading it evenly over the field of view.

To allow a microscope to be set up for Koehler, it must have two adjustable iris diaphragms: the aperture diaphragm at the substage condenser and the field diaphragm nearer to the lamp.

The aperture iris diaphragm controls the angular aperture of the cone of light from the condenser, while the field iris diaphragm controls the area of the circle of light illuminating the specimen.

Technical requirements

The substage condenser must be capable of being focused up and down, and must be fitted with an aperture iris diaphragm that can be opened and closed by a lever or knob.

The light path must be fitted with a condensing lens, a collector lens and a field iris diaphragm that can be opened and closed.



Focusing the condenser

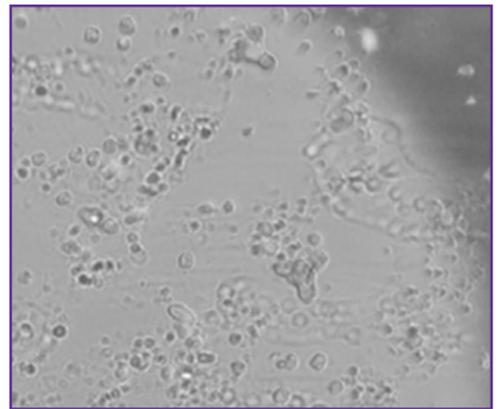
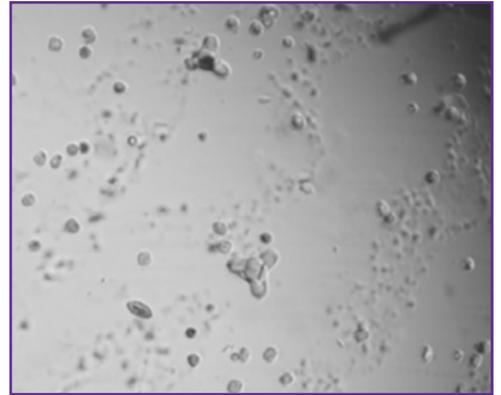
1) Place a thin sample on the stage and focus on it using a 4x or 10x objective.

For an appropriate starting position, make sure the condenser front lens is approximately 0.5cm from the bottom of the coverslip.

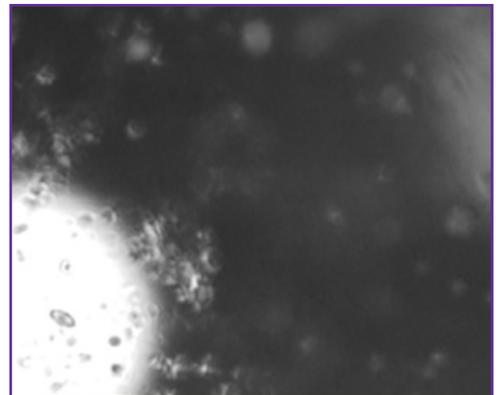
2) Using the field iris diaphragm control, located on the front surface of the substage optics, close the diaphragm right down while looking at your monitor.

You will see a dark circle encroaching on the screen.

Note: If this dark circle does not fall within your field of view, you may need to use the two silver adjusting screws on the condenser arm to centre your condenser



3) Move the condenser up or down until the edge of the dark circle (the blades of the iris) appears in sharp focus on the monitor. Once you have positioned the condenser correctly switch the user interface back to position (I) to avoid accidental re-positioning of the condenser.



Note: Depending on the working distance of the condenser you may be in a close proximity to the sample.

Centering the condenser

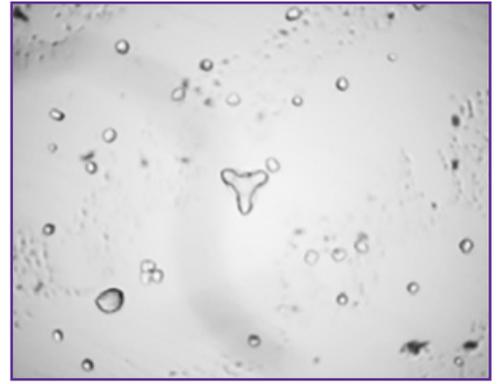
4) Located on the condenser arm are two silver adjusting screws used for centering the condenser. Turn the screws to centre the now multi-edged shape. It should now appear in the centre of the field of view.

This process is made easier in the final stages by opening the diaphragm almost to the edge of the monitor's field of view.



5) Once the condenser has been focused and centred in this way, the diaphragm can be opened so that it is just outside the field of view.

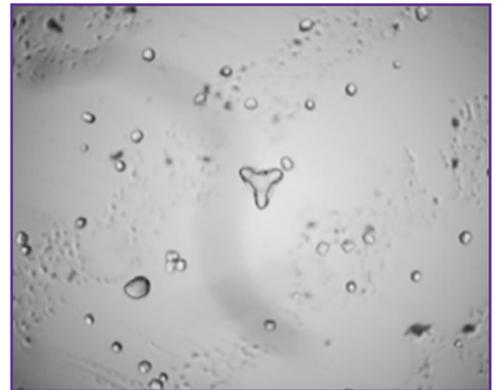
The condenser will remain centred when different objectives are selected, but the field iris diaphragm will have to be adjusted to just outside the field of view at different magnifications.



Adjusting the aperture iris

This important step is often neglected, leading to sub-optimal resolution and/or poor contrast.

6) Locate the control for the aperture iris, which will often be a thin silver lever protruding from the condenser. With the condenser in place, focused and centred, the iris should be closed down so that it occupies the outer 20% or so of the field. This increases the contrast, making observation easier.



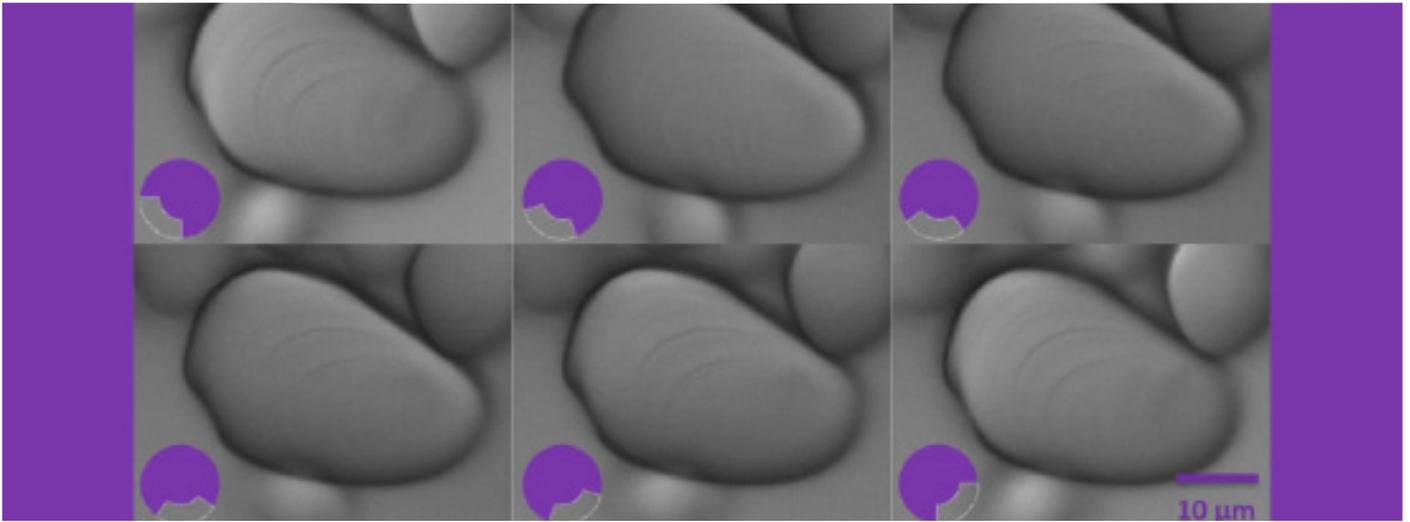
Some specimens may need variation on the 20%, beware of closing the iris too much as resolution will be drastically reduced.

Although the human eye will accept sub-optimal images, once these images are captured by a camera, the results will speak for themselves (take a look at some of the photomicrographs in scientific journals and judge for yourself!).

Note: A more accurate way of adjusting the aperture iris is to note the numerical aperture (or NA) on the objective, then set the NA on the condenser to 20% less.

For example, with a 40x objective with a numerical aperture value of 0.65, set the graduation on the condenser to 20% less, approximately 0.5

Taking a little time to check your microscope before each use will be well worth the effort.



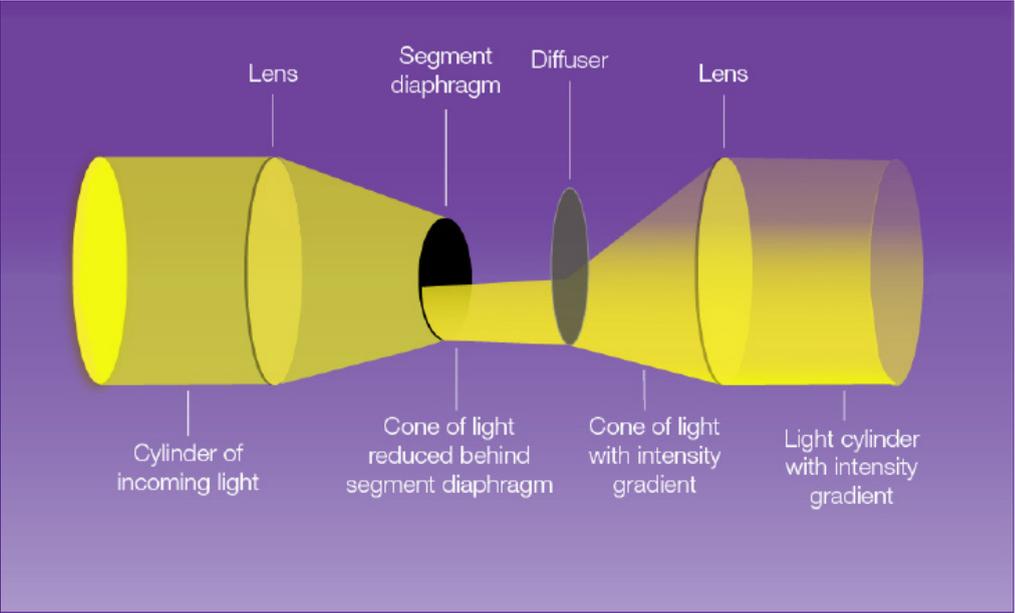
Dodt Gradient Contrast

Dodt Gradient Contrast (DGC) was developed by Professor Hans-Ulrich Dodt during the 1990s while working at the Max Planck Institute of Psychiatry in Munich.

By using a similar principle to oblique contrast, DGC produces images with contrast comparable to those made by Differential Interference Contrast (DIC) (see page 13), and without the imaging artefacts associated with other techniques like phase contrast (see page 18).

Like IR-DIC (infrared DIC), DGC is a form of infrared videomicroscopy (although visible wavelengths can also be used) that improves the contrast of images of unstained tissue

In DGC, a gradient contrast tube is placed between the light source and the condenser. Within the tube, there is a lens that focuses the light towards a diffuser positioned a short distance after a quarter annulus. The annulus blocks some of the light, and the diffuser creates a gradient of illumination. A second lens then collimates this light before it enters the condenser. The difference in intensity across edges in the sample enhances interference in the light path turning the phase shift through the sample into a change in amplitude.



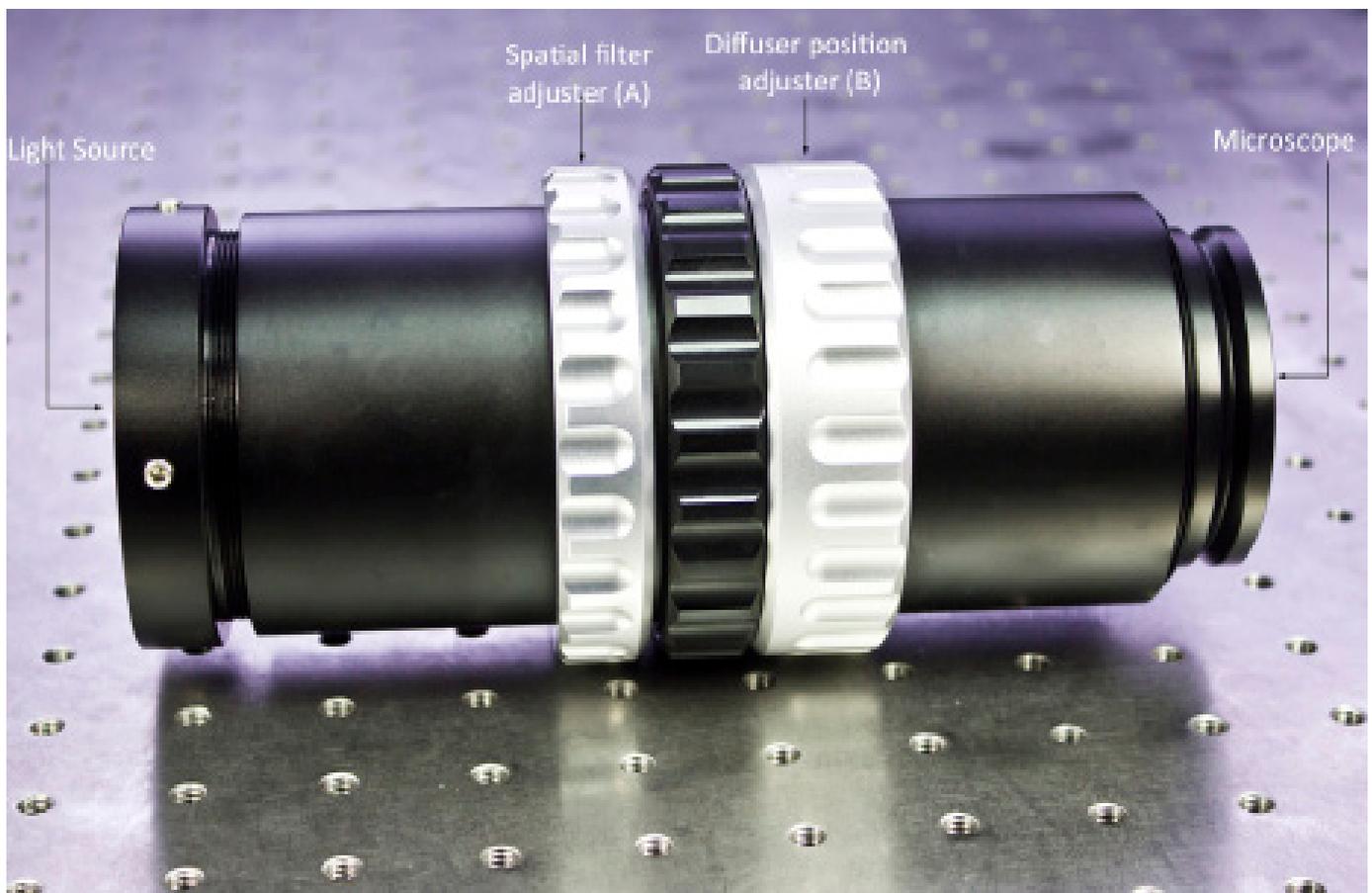
The light path through a gradient contrast tube

DGC allows some light to illuminate the entire area initially lit by the light cylinder, making the image comparable to the actual object and reducing optical artefacts.

As much of the light is blocked, DGC generates less stray light at the sample, enabling the use of a 1.4 numerical aperture (N.A.) oil condenser. Additionally, the curved slit of the quarter annulus means that the gradient of illumination occurs in two perpendicular directions. This feature becomes advantageous when attempting to visualise the dendritic branches of neurons travelling in various directions.

Setting up gradient contrast

The only additional components required to make a brightfield microscope into a scope capable of DGC are the gradient contrast tube itself and potentially a high-intensity infrared light source. The tube is placed between the transmitted light source and the transmitted illumination port of the microscope.



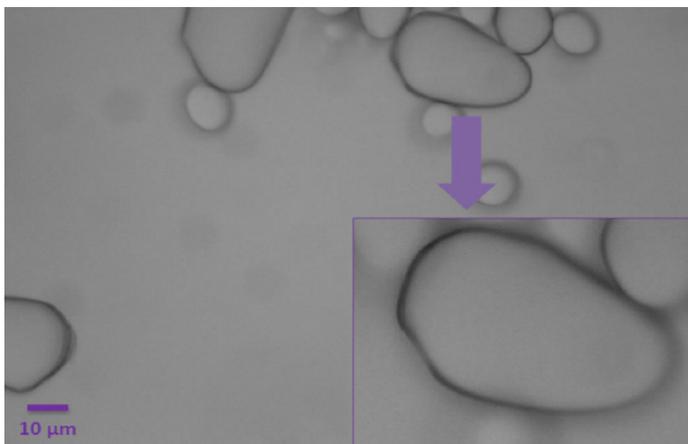
The Scientifica gradient contrast tube

After installing the contrast tube, the first thing to do is to set up Koehler illumination (see page 1) on the microscope. Additionally, if your microscope has an additional diffuser between the illumination port and the condenser this must be removed, as it will dilute and disperse the contrast output provided by the spatial filter.

Once the Gradient Contrast Tube is installed and the microscope is properly set up for Koehler illumination, the microscope can be optimised for Dodt Contrast. The spatial filter adjustment ring (A) can be rotated to adjust the position of the quarter annulus by approximately 90° enabling you to change the direction of the gradient across the sample. Finding the best placement for ring A will depend on your sample and is best optimised by trial and error. The direction of the gradient will highlight structures running anti-parallel to the spatial filter.

The diffuser position adjuster (B) can be rotated to move the diffuser axially along the light path (closer or further from the microscope) to alter the amount of contrast on the image by increasing or decreasing the sharpness of the gradient.

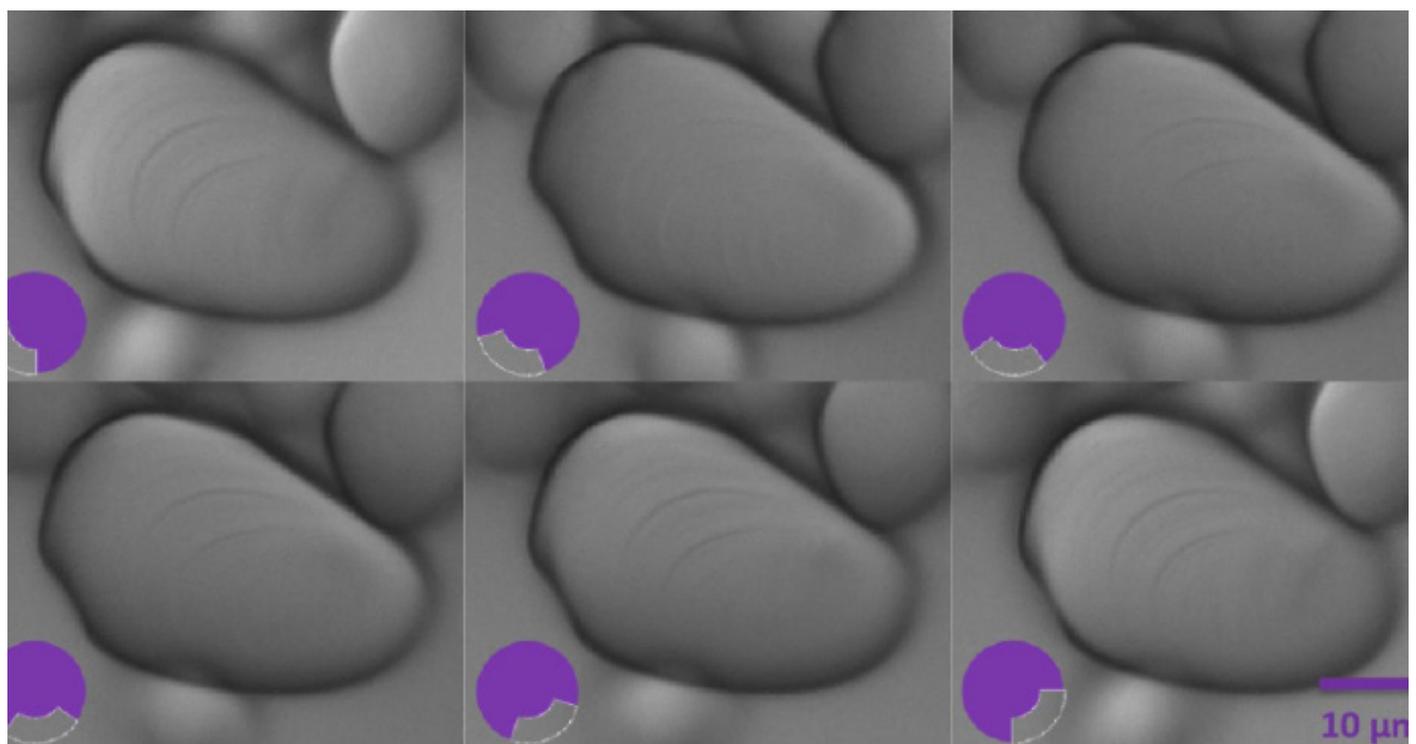
As an example, this image shows starch granules obtained from potato starch (*Solanum tuberosum*) observed without a Dodt contrast tube. The starch granules appear to be smooth objects without much definition beyond the outline.



However, with the insertion of the gradient contrast tube, the growth rings (or lamellae) can be observed thanks to the increased contrast.

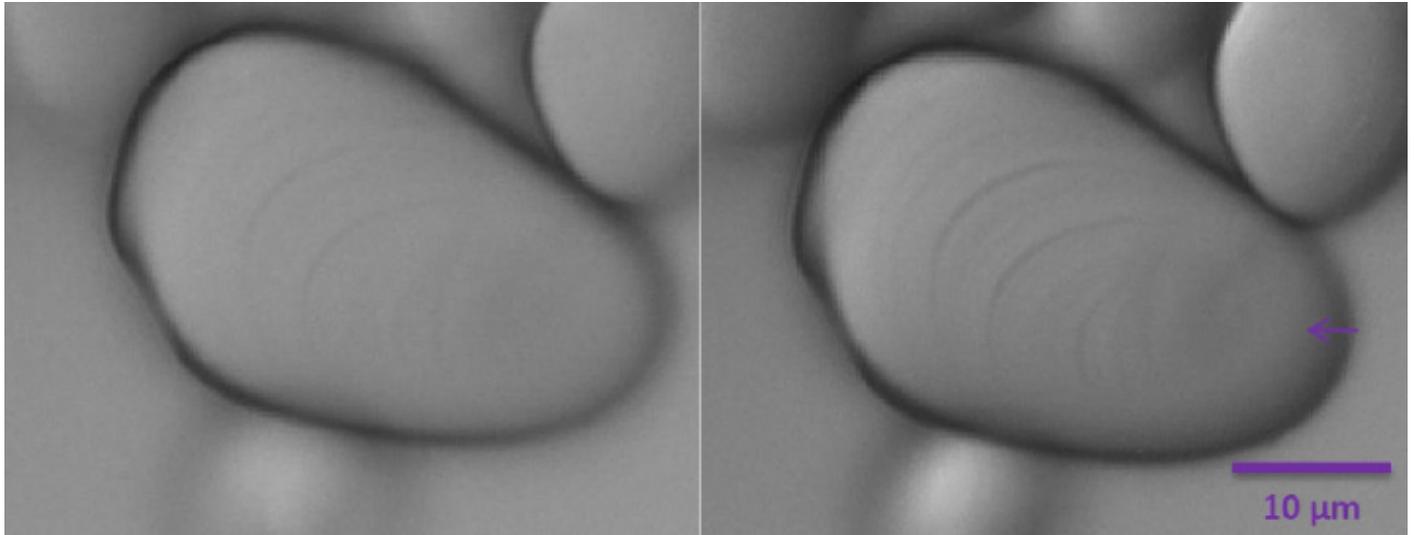
Starch granules visualised without employing the Dodt contrast tube.

Inset: zoomed version of the starch granule.



Starch granules visualised with the Dodt contrast tube enabling the clear observation of the growth rings. The spatial filter is rotated 18 degrees between each image to outline the different features of the sample.

By rotating the quarter annulus using ring A, it is possible to see the outline of the growth rings in their different positions as they are radially oriented. These layers accumulate around the hilum, which is the granule's point of botanical origin.



Starch granules visualised with Dodt contrast with diffuser closer to the microscope (left panel) and closer to the light source (right panel). The arrow indicates the hilum.

Adjusting the position of the diffuser with ring B alters the amount of contrast on the image (see above). In the right panel, the position of the diffuser closer to the light source enables the observation of the hilum as a slightly depressed zone (purple arrow).

If after adjusting the spatial filter and diffuser you are still unable to obtain a clear image, there is a further method that can be employed to enhance the contrast. Laterally offset the condenser and bring the shadowed edge of the iris into the field of view to create additional oblique illumination within the light path (do not move the condenser axially). This will cast a gradient of light across your field of view (from darker to lighter), which you can adjust by altering the light source intensity. This process should enable you to obtain an enhanced contrast image in the centre of your field of view.

Please note that the Scientifica gradient contrast tube can be installed without any particular radial orientation. Therefore the absolute position of the spatial filter will change depending on the alignment of the tube when affixed to the microscope. Additionally, the optimum position of Rings A and B will depend on the experimental sample.

Advantages

DGC makes unstained living tissue visible without harming it.

It is cost-effective as it does not require the purchase of expensive optical elements. Additionally, no further elements are added into the light path between the sample and camera or detector, making it an ideal contrast technique for patching in combination with two photon excitation fluorescence microscopy.

With a good camera, the fast frame rates available with this approach make it an excellent tool for positioning micropipettes during patch clamp experiments to enable patch clamp electrophysiology in thick brain slices (<300 μm).

Limitations

The amount of light lost through the quarter annulus is significant. This loss can lead to dark images which will, in turn, cause a lack of contrast. This loss can be exacerbated when using an LED which has a lower maximum power output than a traditional arch discharge illumination source.

DGC is not as simple as other contrast techniques to align and set up correctly. The requirement to readjust the settings regularly can be an annoyance to some users.

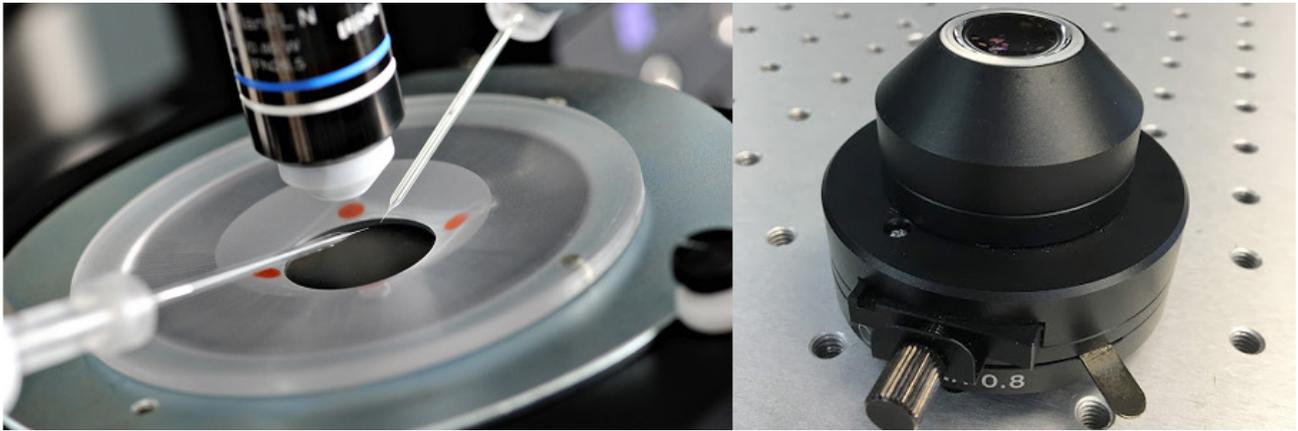
Applications

The primary use of DGC is to visualise unstained neurons, which are mainly phase objects i.e. they alter the phase of the light waves that pass through them but not their amplitude. DGC is particularly useful when attempting to observe neurons in thick brain slices.

DGC is also ideal for performing patch clamp experiments when used in combination with two photon illumination as there is no requirement for optics between the collection module placed after the objective lens.

Take a look at www.scientifica.uk.com for more useful guides!





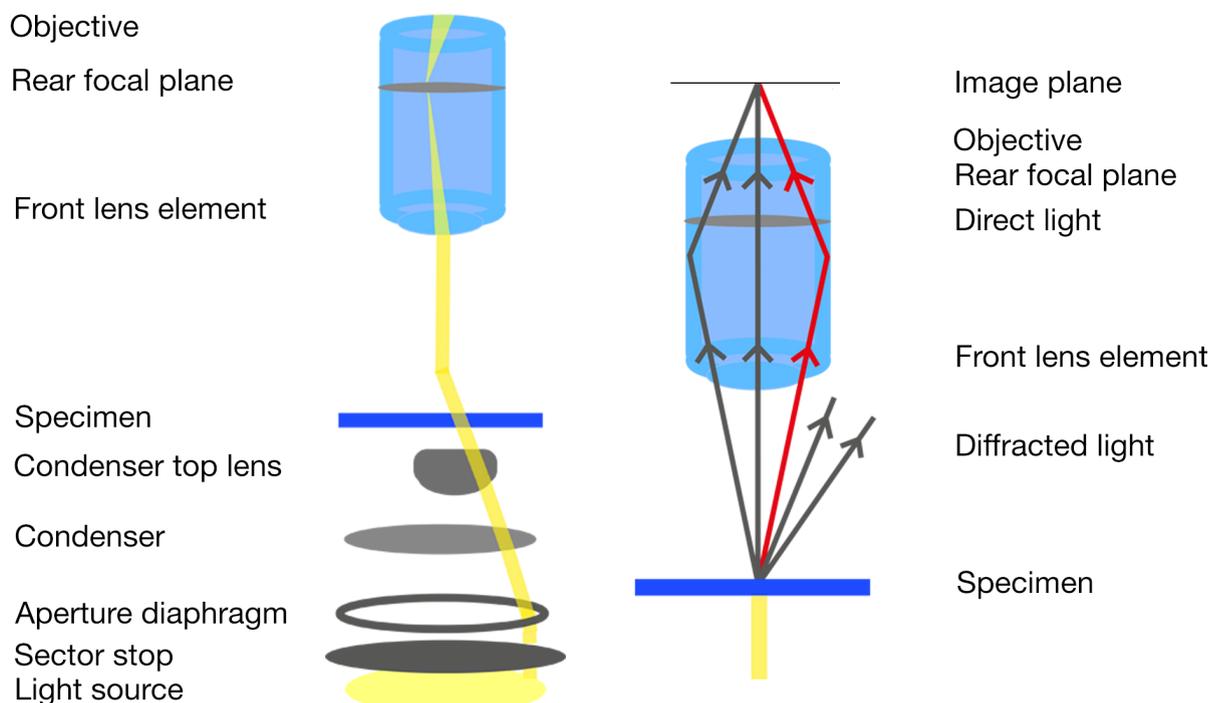
Oblique Contrast

Oblique contrast is used to increase the contrast of images of thin, transparent, unstained samples, the details of which can be difficult to visualise using brightfield microscopy.

Similarly to differential interference contrast (DIC) (see page 13), images produced using oblique contrast have shadowed regions, giving a pseudo three-dimensional effect. The light strikes the specimen at an oblique angle, which introduces phase changes to reveal features with a much greater contrast.

How it works

With a standard condenser used in brightfield microscopy, light passes through the centre of the specimen, diffracting at different angles as it hits the specimen before recombining at the back of the image plane. As the light diffracts at different angles and therefore travels a longer distance before reaching the image plane, the phase of the light is affected. However, when light of equal amplitude and wavelength and inverse phase combines it cancels out so there is no overall change in phase. This is called destructive interference. Therefore, there is no overall change in the image.



Oblique contrast utilises an oblique condenser containing a sector stop with a small off-centre slit which makes the light go through the sample at an angle, so the specimen is illuminated from one direction only. The sector stop is located beneath the lens and aperture diaphragm of the condenser. Light from the source passes through the slit which causes it to pass through the condenser lens at an oblique, off-axis angle. The specimen is then illuminated an angle.

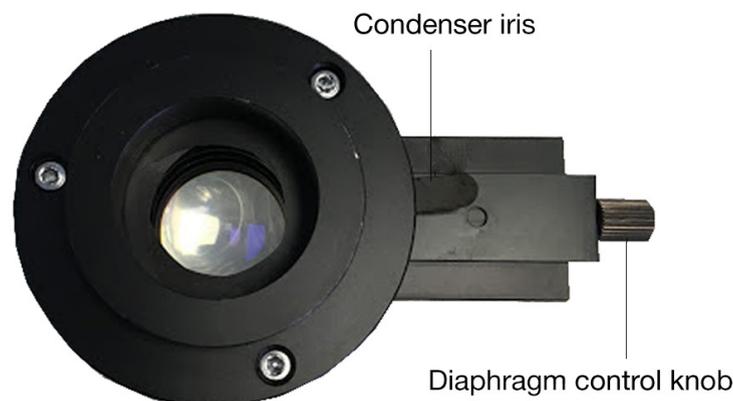
After passing through the specimen, the light rays enter the microscope objective and converge at the rear focal plane before they are observed through eye pieces or on a camera. As the light in oblique contrast hits the sample at an angle, only the diffracted sidebands of light on one half of the direct beam will be collected by the objective. This means that there is constructive and destructive interference between the direct light beams and collected sidebands.

Setup

To make a brightfield microscope capable of oblique contrast, the only additional components needed are an oblique condenser and an infrared or visible light source. The condenser is placed in the condenser housing above the light source, below the condenser lens and aperture diaphragm.

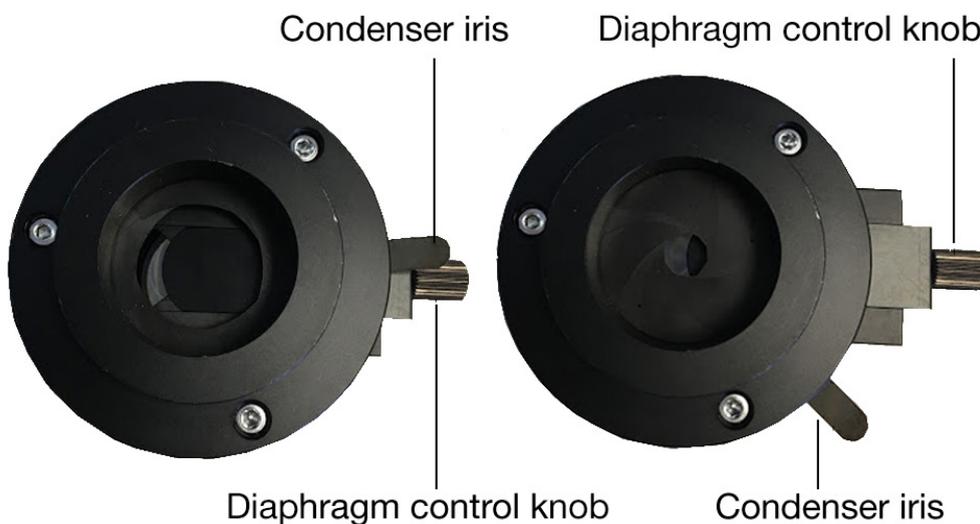


Firstly, pull out the oblique slider and open the oblique iris so the condenser is adjusted for brightfield illumination.

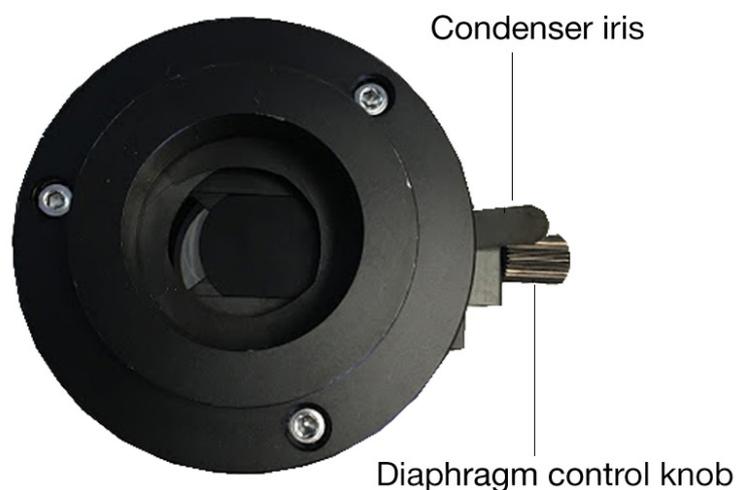


Secondly, set up Koehler illumination (see page 1) on the microscope. If using a manual microscope, remove the condenser stop before setting up Koehler illumination and replace it once Koehler illumination has been set up.

Close down the condenser iris to match the numerical aperture of the objective and then adjust the direction of the sector stop using the diaphragm control knob. Close down or open the stop until the desired image is achieved. Adjust the amount of oblique illumination in this way, depending on the specimen and the amount of detail required. The oblique condenser enables the angles of illumination to be altered through 360° without moving the specimen.



Then, adjust the direction of the sector stop by turning the diaphragm control knob. Close down or open the stop to adjust the amount of oblique illumination until the desired image is achieved. The amount of oblique illumination needed depends on the specimen and the amount of detail required. The oblique condenser enables the angles of illumination to be altered through 360° without moving the specimen.



When making adjustments, check that the sector stop is still positioned correctly and that the slider is not too far in or out.

Advantages

- As oblique contrast does not use polarised light, it is not affected by birefringent samples (birefringent samples have a different reflective index depending on how the light is polarised)
- It is simple to use – a small disc is moved around to adjust the image
- It is cheaper than other methods, such as DIC (Differential Interference Contrast)
- It produces pseudo three-dimensional images to aid techniques such as patch-clamping.

Limitations

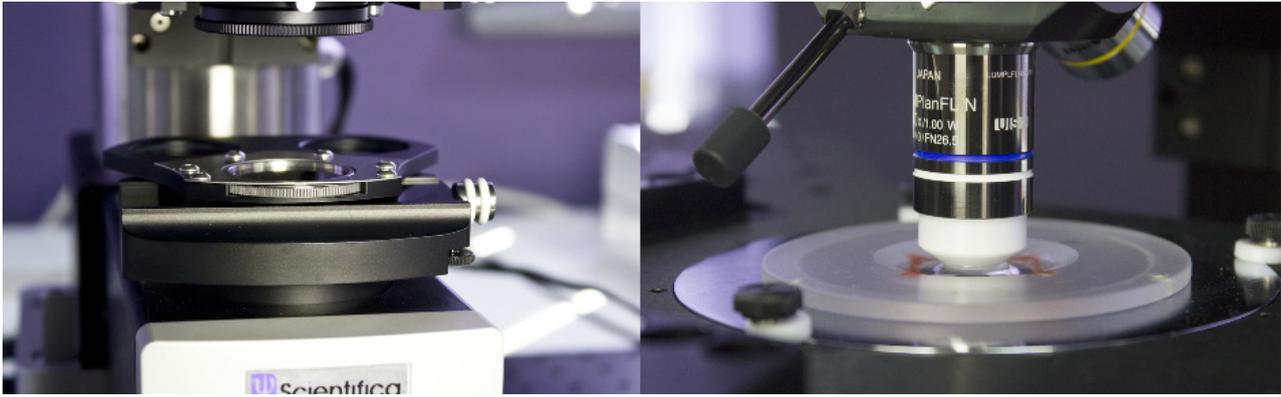
- Oblique contrast can generate false images in some samples, depending on the angle that the light goes through the sample.
- The same specimen can appear very different if the orientation of it is changed which can be misleading.
- The 3D effect is not representative of the actual geometry of the specimen, so you can see 3D depths that aren't real, similar to optical illusions.
- Oblique contrast may affect image resolution as the full numerical aperture of the objective and condenser is not being utilised.

Applications

Oblique contrast is suitable for thin, transparent or nearly transparent samples such as cell cultures or thin brain slices. It can be used for visualising specimens that can't be stained, such as living cells, tissues or organisms.

As oblique contrast doesn't use polarised light, it is suitable for imaging vertebrate axons. Vertebrate axons are myelinated by a birefringent material and, unlike polarised light, the unpolarised light doesn't affect the refractive index of the myelin. As petri dishes are also birefringent, oblique contrast is ideal for imaging samples within these.

There are no additional optics in the above sample collection path, making oblique contrast ideal for two-photon and confocal imaging, as no intensity is lost. It is also used to introduce contrast in patch-clamp experiments.



Differential Interference Contrast

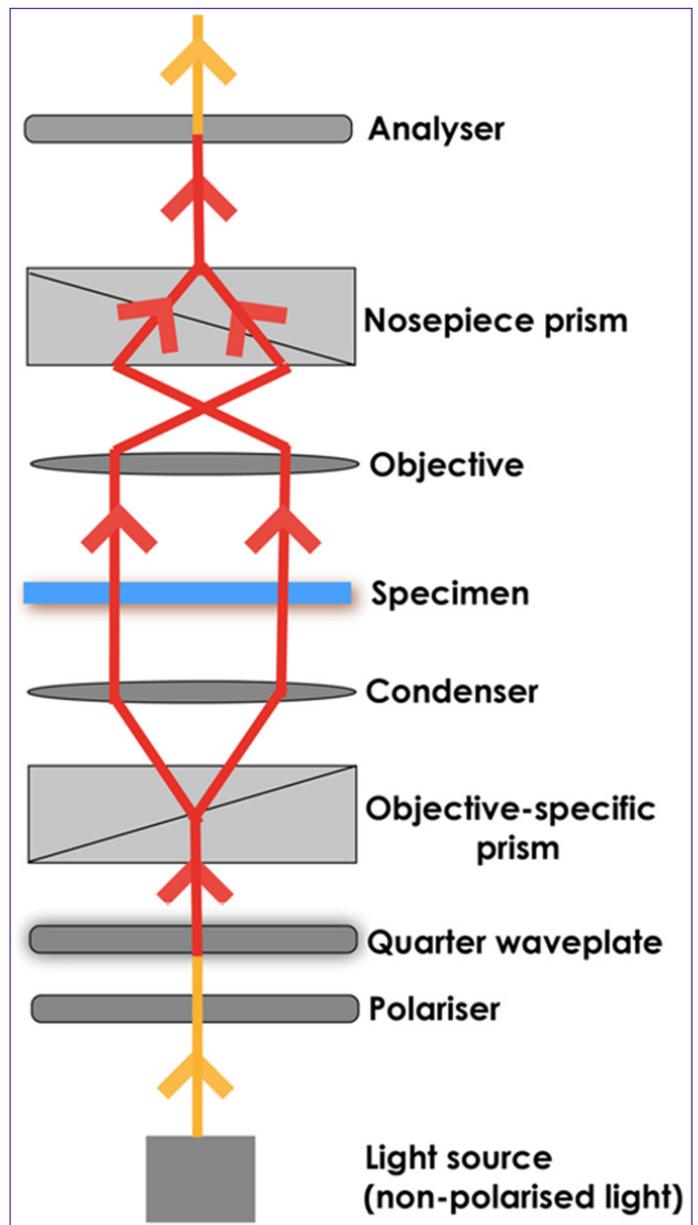
Differential Interference Contrast (DIC) introduces contrast to images of specimens which have little or no contrast when viewed using brightfield microscopy. The images produced using DIC have a pseudo 3D-effect, making the technique ideal for electrophysiology experiments.

How DIC works

In DIC, light emitted from the source is linearly polarised by a polariser. The linearly polarised beam of light enters an objective-specific prism, which splits it into two rays that vibrate perpendicular to each other. The rays are parallel as they pass through a condenser, but as they are vibrating perpendicular to each other, they cannot cause interference.

The split beams pass through the specimen. The specimen's varying thickness and refractive indices alter the wave paths of the beams. They then enter the objective, which focuses them above the rear focal plane. The two beams enter a second prism, in the nosepiece, which combines them. The beams have different lengths, because they passed through different parts of the specimen.

The analyser, which is a second polariser, brings the vibrations of the beams into the same plane and axis, causing destructive and constructive interference to occur. The light then travels to the eyepiece or camera, where a DIC image with differences in intensity and colour can be seen.



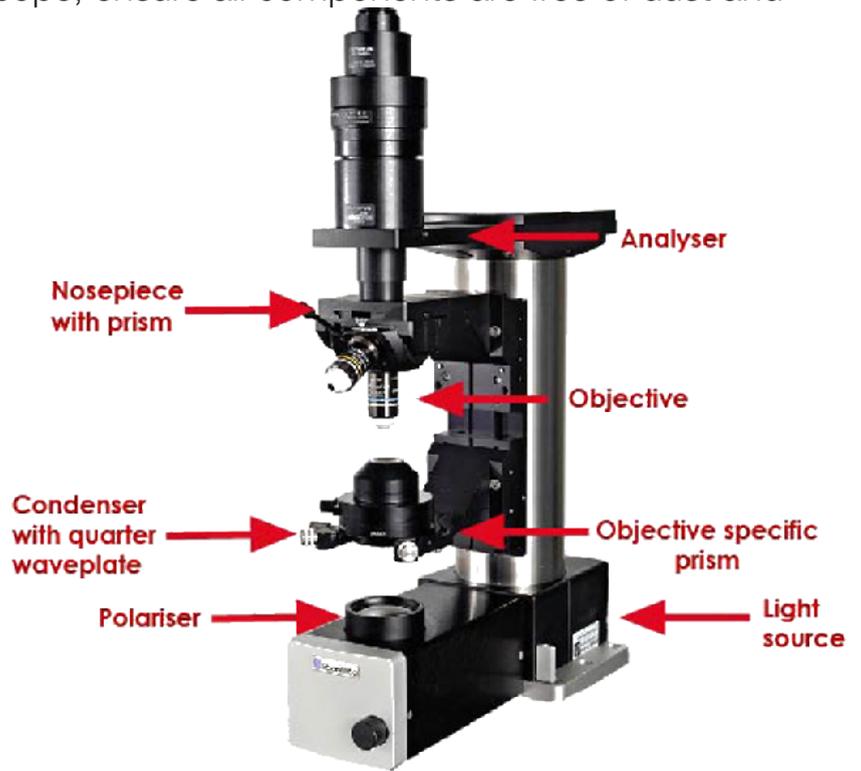
Setting up DIC

DIC can be utilised on virtually any upright or inverted microscope, as long as all components can be installed. In the image below of the Scientifica SliceScope, although not all DIC components are present, their locations are labelled.

Before setting up DIC on a microscope, ensure all components are free of dust and debris, as dirty objective, condenser and lens elements can reduce image quality.

1. If using a white light source, insert an infrared bandpass filter into the slider on the SliceScope filter wheel and secure in place with a locking screw. This ensures only infrared light passes through to the polariser.

2. Insert the polariser into the silver-plated section of the filter wheel, underneath the condenser, shown in the image below. The polariser is on a rotatable mount, enabling it to be rotated when aligning DIC.

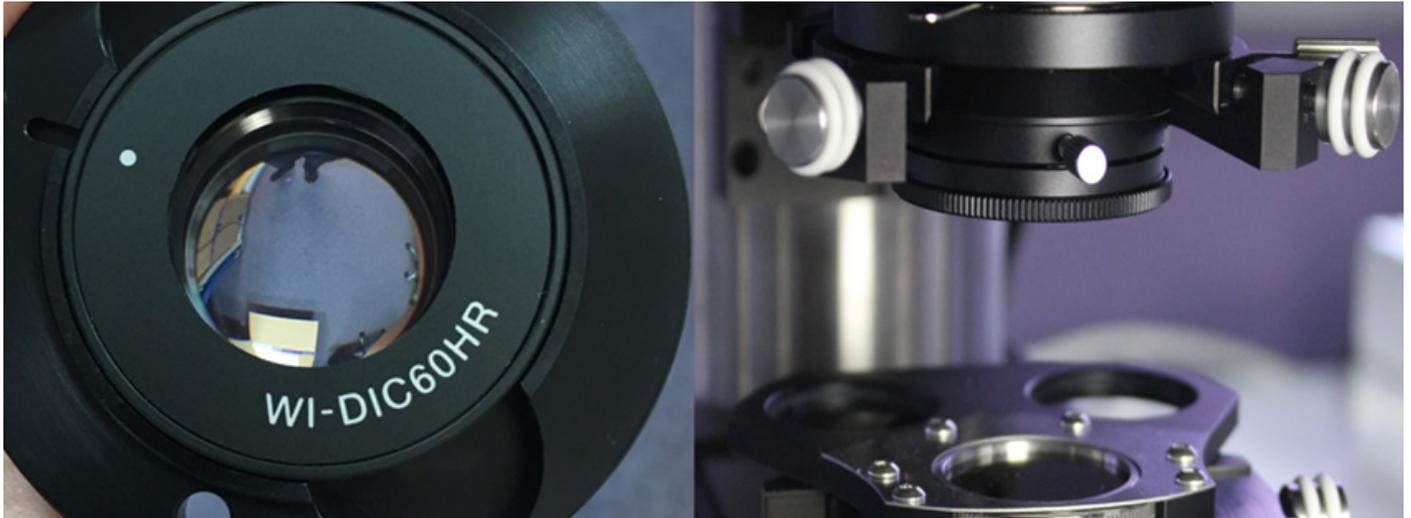


Filter wheel with rotatable mount, showing the installed polariser

3. Ensure your condenser does not have a protective covering on it, as this will obscure images produced using DIC.

4. Keeping the condenser facing upwards, use a hex key to unscrew the two screws either side of the condenser to open it. Turn the top section only upside down.

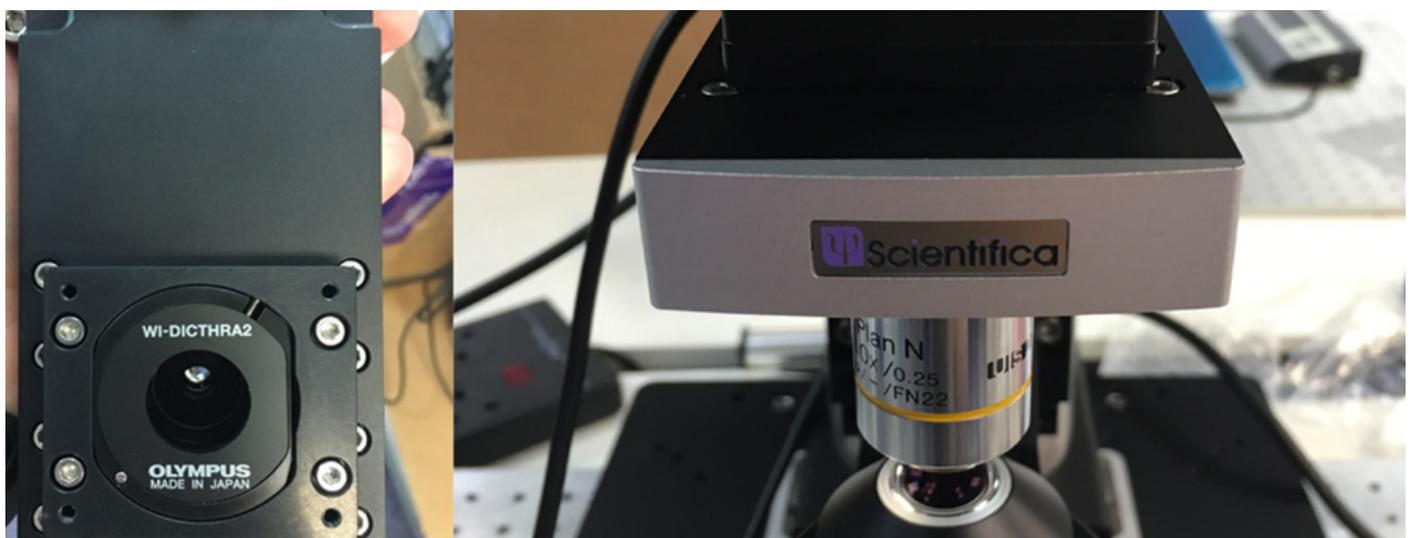
5. Loosen the grub screw on the side of the condenser and remove the spacer ring from the top half, as this is not needed. Insert the objective-specific prism, lining up the metal pin on the prism with the slot in the condenser. Tighten the grub screw and put the condenser back together. Each objective requires a different objective-specific prism, based on the magnification of the objective, for example a 40x objective requires a WI-DIC40HR objective specific prism. Usually, a prism is only required for the high magnification objective you are using.



The left image shows the WI-DIC60HR prism in the condenser. On the right is the condenser setup on the SliceScope with the filter wheel below

6. The second DIC prism (WI-DICT or WI-DICTHRA) is in a fixed mount which is inserted into the nosepiece above the objective. Loosen the grub screw on the right-hand side of the nosepiece arm and slide the nosepiece (WI-SRE) forward. Insert the second DIC prism into the nosepiece and secure it using the small screw at the rear-right corner of the nosepiece.

If using a Manual Objective Changer (MOC), loosen the four screws to open it and then follow the instructions above.



The image on the left shows the objective prism in the MOC. The image on the right is the nosepiece, where the objective prism is located

7. If fluorescence is being used, insert the analyser (U-AN) into the fluorescence turret, as shown on the next page. If fluorescence isn't being used, insert the analyser (U-ANT) into the U-KPA.

8. The U-KPA can then be secured on the SliceScope under the imaging lens or trinocular head.

9. The polariser and analyser need to be aligned so that their transmission pathways cross at a 90 degree angle to one another. This can be achieved by rotating the polariser in the filter wheel. As this can be rotated 360 degrees, the analyser and U-KPA can be mounted in any orientation.



The image on the left is a U-AN analyser, and the image on the right shows the U-AN analyser inserted into a fluorescent turret on the SliceScope.

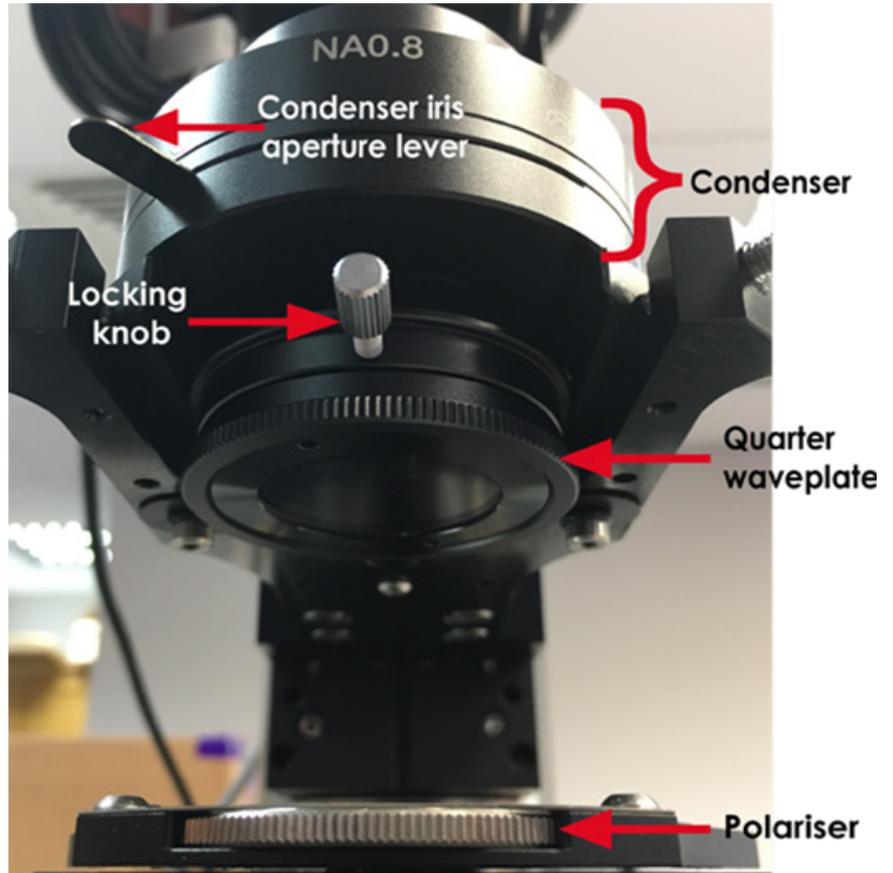
10. For DIC microscopy to be performed, Koehler illumination must be first set-up. Once this has been set-up, DIC will need to be aligned.

Aligning DIC

1. Focus on a blank sample plate using either a 4x or 10x objective.
2. Move the DIC slider with the U-AN analyser into the light path. The U-ANT is not on a slider and is in a fixed position which is always in the light path.
3. If using a trinocular head, remove one eyepiece and view the sample directly down the trinocular head.
- 4.a. When using eyepieces: rotate the polariser until there is a dark stripe through the centre of the field of view. This indicates that the polariser and analyser are aligned at 90 degrees to each other.
- 4.b. When using a camera: rotate the polariser until the image is at its darkest.

5. If the condenser and objectives were removed, put them back at this point. Double-check that your condenser is still set-up for Koehler illumination.
6. Use your DIC objective to bring your sample into focus.
7. Add a specimen so that contrast can be optimised for the sample you are viewing.
8. As the SliceScope uses de Sénarmont DIC, a quarter waveplate is required.

Adjusting the quarter wave plate changes whether structures appear concave or convex. Unscrew the locking knob at the front of the image below and adjust the quarter wave plate as desired.



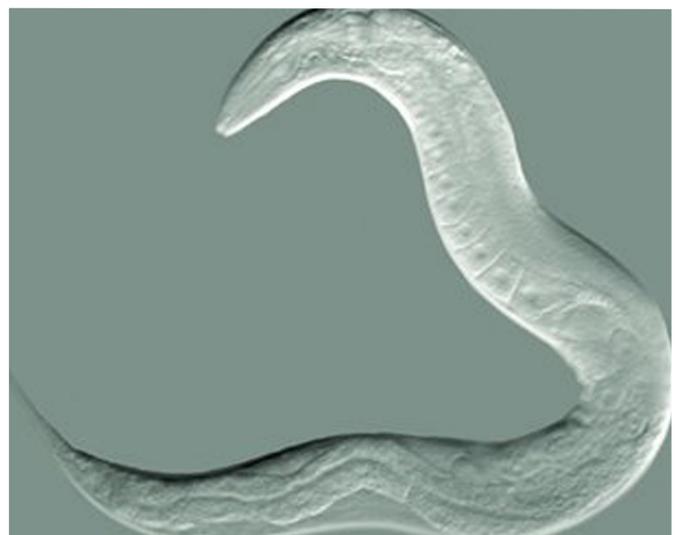
9. Lock the quarter waveplate in position using the locking knob.

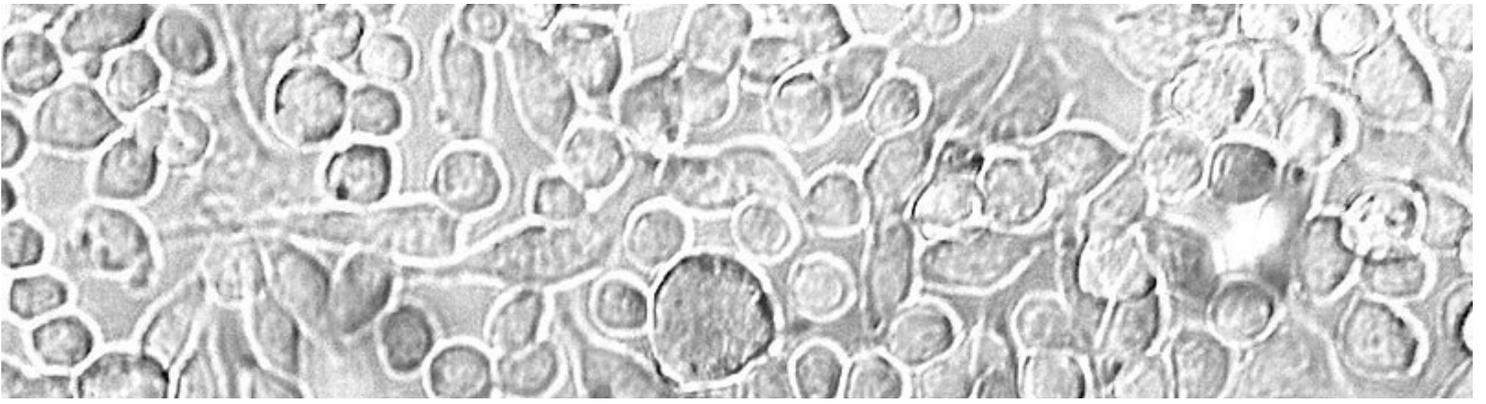
10. The lever in the image above controls the condenser iris aperture. If using eyepieces, remove one eyepiece and look down so you can see the internal condenser iris. Adjust the lever until the condenser iris just disappears from the field of view. When using a camera, adjust the lever until the image is at its sharpest.

11. As DIC is a function of specimen orientation, rotating the sample can improve or change the image contrast.

You should now have a clear DIC image! Here is an example of a DIC image; the image has good contrast and the *C.elegans* has a 3D effect.

An adult Caenorhabditis elegans nematode imaged using DIC by Bob Goldstein





Phase Contrast

Phase contrast is used to enhance the contrast of light microscopy images of transparent and colourless specimens. It enables visualisation of cells and cell components that would be difficult to see using an ordinary light microscope.

Phase contrast does not require cells to be killed, fixed or stained. The technique enables living cells, usually in culture, to be visualised in their natural state. This means biological processes can be seen and recorded at high contrast and specimen detail can be observed. Fluorescence staining can be used in combination with phase contrast to further improve the visualisation of samples.

Phase contrast is ideal for thinner samples, therefore an inverted microscope system can be used. This provides the additional advantage of having more working space. Phase contrast can also be installed on upright microscopes.

If thicker samples need to be visualised in high-resolution, differential interference contrast (DIC) is a more suitable technique to use.

Applications

Phase contrast is used to visualise transparent specimens, when high-resolution is not required, including:

- Living cells (usually in culture)
- Microorganisms
- Thin tissue slices
- Fibres
- Subcellular particles, including organelles

Components needed for phase contrast

It is relatively simple and inexpensive to adapt a light microscope for phase contrast. A phase contrast condenser with a condenser annulus (also known as a phase ring or phase annulus) needs to be installed, along with a set of phase contrast objectives, each of which contains a phase plate. The phase plate is located in the objective rear focal plane.

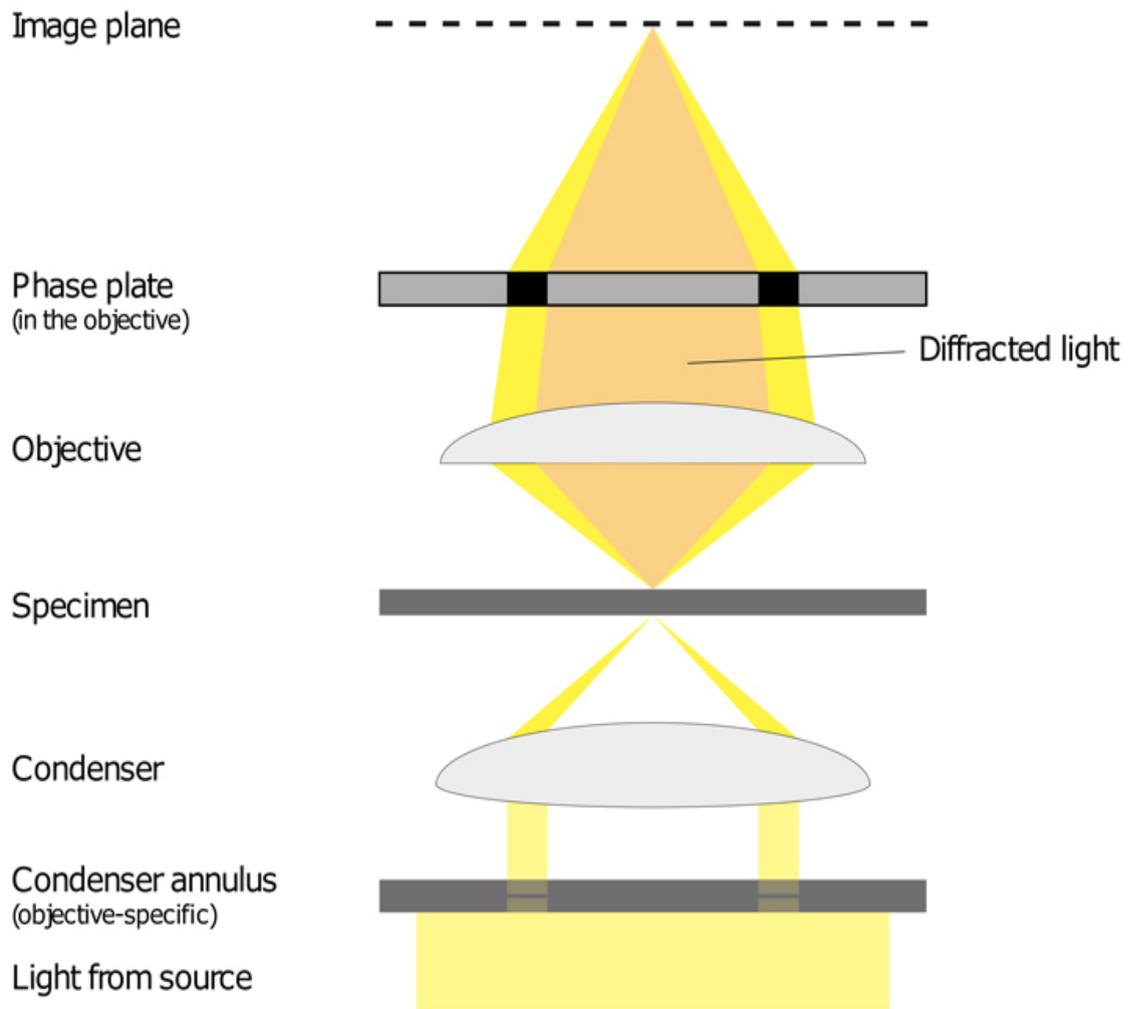
How the technique works

Phase contrast microscopy translates small changes in the phase into changes in amplitude (brightness), which are then seen as differences in image contrast.

Unstained specimens that do not absorb light are known as phase objects. This is because they slightly change the phase of light that is diffracted by them; the light is usually phase-shifted by about $\frac{1}{4}$ wavelength compared to the background light. Our eyes are unable to detect these slight phase differences as they can only detect variations in the frequency and intensity of light.

Phase contrast enables high contrast images to be produced by further increasing the difference of the light phase. It is this characteristic that enables background light to be separated from specimen diffracted light. The difference of the light phase is increased by slowing down (or advancing) the background light by a $\frac{1}{4}$ wavelength, with a phase plate just before the image plane. When the light is focussed on the image plane, the diffracted and background light cause destructive (or constructive) interference which decreases (or increases) the brightness of the areas that contain the sample, in comparison to the background light.

Some of the light that passes through the specimen will not be diffracted (bright yellow in figure 1). These light waves form a bright image on the rear aperture of the objective. The light waves that are diffracted by the specimen pass the diffracted plane and focus on the image plane only. This allows the background light and the diffracted light to be separated.



The path of light in an upright microscope set up for phase contrast

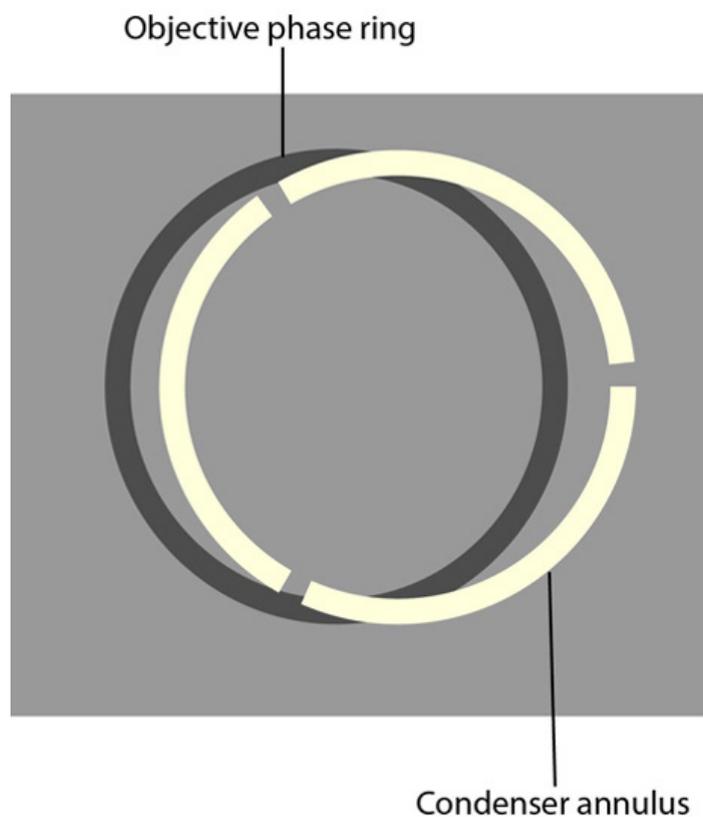
The phase plate then changes the background light's speed by $\frac{1}{4}$ wavelength. When the light is focused on the image plane, the diffracted and background light will cause destructive or constructive interference, which changes the brightness of the areas that contain the sample in comparison to the background light. Often the background is also dimmed by 60 - 90% by a grey filter ring.

Aligning your microscope for phase contrast

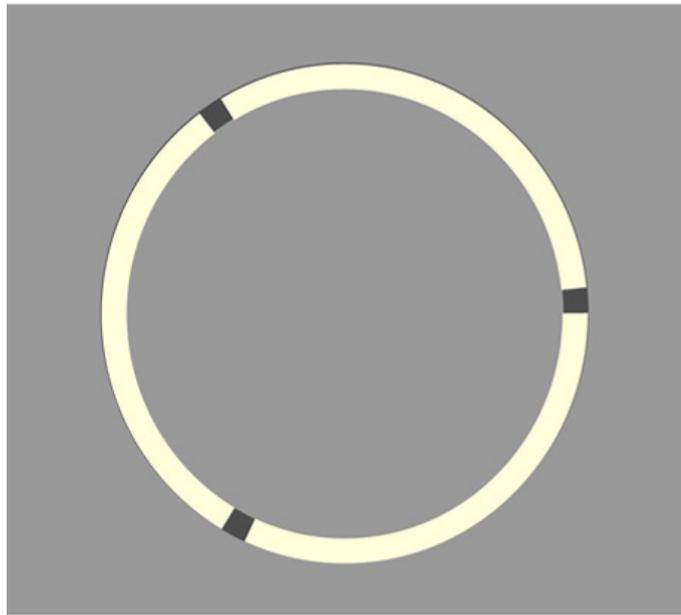
All of the components required for phase contrast need to be aligned and centred. Some phase sliders are pre-centred, we therefore recommend that you check beforehand. Details of how to centre and align phase components are below:

1. If possible, set up Koehler illumination on your microscope. Read Scientifica's 6 step guide to Koehler illumination to help you set this up: www.scientifica.uk.com/neurowire/koehler-illumination-what-why-and-how

2. Install the phase rings in the condenser.
3. Remove the eyepieces and replace these with the phase contrast centring telescope.
4. Put the phase contrast telescope into focus, so that the phase plate and phase ring are in focus.
5. Put the lowest magnification phase objective and corresponding phase annulus in place. For example, a 10x Ph1 objective with a Ph1 phase annulus.
6. Look at the phase plate and phase ring through the phase telescope.
7. Using the adjustment screws on the condenser, centre the phase plate and phase ring so the segmented circle of light sits on the black ring.



Here, the objective phase ring and condenser annulus are mis-aligned. See page 22 for a diagram of the phase ring and condenser annulus correctly aligned.



Here, the components are correctly aligned, as the segmented circle of light created by the condenser annulus sits on the black ring.

8. Repeat steps 4-6 for the rest of the objectives.
9. Once all objectives have been aligned and centred, remove the phase contrast centring telescope and replace this with the eyepieces.

You shouldn't need to re-centre the phase contrast microscope. It is, however, recommended to regularly check that the set-up is centred using the phase contrast telescope.

Limitations

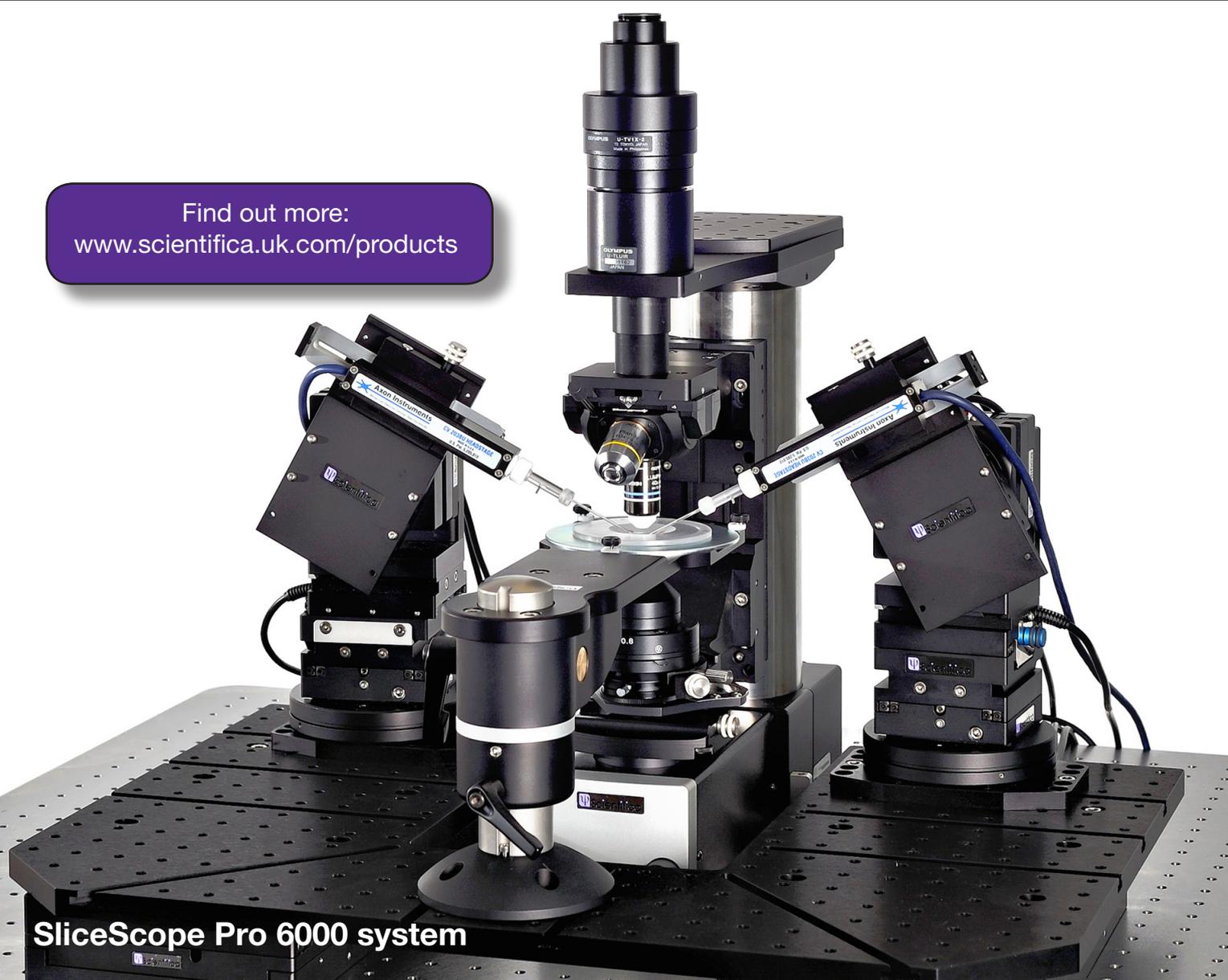
- Images generated using phase contrast often have halos surrounding the outline of details that have a high phase shift. These halos are optical artefacts and can make it hard to see the boundaries of details.
- The resolution of phase images can be reduced due to the phase annuli limiting the numerical aperture of the system.
- Phase contrast doesn't work well with thick specimens as these can appear distorted.

For more information about any of the contrast techniques described in this magazine, please contact our team of experts at Scientifica at info@scientifica.uk.com or visit www.scientifica.uk.com

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