Applied Precision DeltaVision v.2.0
Deconvolution System

The Applied Precision DeltaVision Deconvolution microscope uses a standard Olympus IX-70 inverted microscope with a mercury arc bulb as the illumination source and is capable of exciting fluorophores from the UV to the far Red ((DAPI, Hoechst, FITC, Rhodamine, Texas Red, Cy5) and will also collect transmitted/DIC light images. The microscope can separate up to 4 dyes and can visualize triple labels in a single image window. After acquisition, the raw data is deconvolved using the Agard & Sedat inverse matrix algorithm. The software package automates the computations and will perform 3D reconstruction of deconvolved sections.

The DeltaVision software runs on a Silicon Graphics Indy computer running IRIX 6.2 (UNIX) operating system. This manual will mention only necessary UNIX commands, as all of the controls for running the microscope are done using the DV software. This manual is designed to highlight the functions used by most CSIF users, but cannot cover any of the subjects in depth. For a more complete description of functions, consult the DeltaVision manual.

On March 26, 1997, the software was upgraded to DeltaVision version 2.0. The username is still dvuser with the same password. This manual contains all of the changes new to using version 2.

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Conventions used in this manual:

SOFTWARE "BUTTONS" ARE SHOWN IN SMALL CAPS

File menu options are given in bold

Italics are used to notify of common problems

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Powering on the system:

In order to use the DeltaVision, you must be trained by one of the CSIF staff. If you are the first session of the day, you will need to power on the entire system as follows:

1. Camera cooler - flip *two* toggle switches on cooler unit, located under the desk to the left of the vibration table. Check in a few minutes that the two tubes filled with glycerol attached to camera are, in fact, cool. This is *MEGA-important* - if the camera is not cooled, it will be destroyed.
2. Mercury arc lamp - turn on toggle switch on lamp power supply on the floor to the left of vibration table.
3. Turn on camera controller (immediately under PC monitor - says Photometrics, PXL) - toggle switch is in back, top right corner. Wait until green light comes on
4. Turn on power strip - located to the right of the computer/control boxes on the desk. The Applied Precision box should turn on (small orange light comes on).
5. Turn on photodiode - small black box attached to the left side of the black box just behind the microscope. The switch is just above the three red buttons.
6. Turn on computer (button on front panel of CPU)

Let the equipment warm up for ~5 minutes

7. Double click "DeltaVision 4.0" icon (on PC monitor - use mouse on desk to the right of the equipment stack). Make sure that the DeltaVision PC 4.04c display comes up on monitor - the usual one with the square around the red cross with yellow text describing the current filter settings.

*** **SGI computer is always left on** - the screen will lock when the screen savers are activated. The password to unlock the screen is the one for the dvuser account.

To turn off equipment

Follow above instructions backwards (step 6 to step 1).

Make sure that equipment is turned off at the end of your session if no-one is around or it is after hours. The mercury arc lamp lifetime is only 200 hours and can break the mirrors in the housing if it is left on too long

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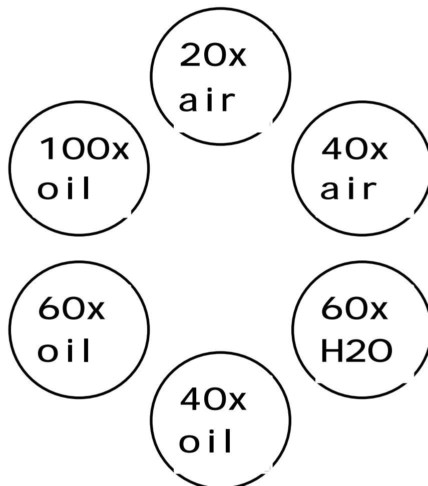
Viewing specimen through eyepieces:

Brightfield illumination

1. Make sure dial on the right side of the microscope base shows the eyeball, not SP (Side Port).
2. Turn on transmitted light source by pushing the green button next to the words "Light Off" on the left side of the microscope. When the green light goes *off*, the transmitted light is *on*. Adjust the brightness with the sliding lever.
3. Open shutter in front of light source using the button with the gray tape on the control pad (labelled "TRANS/DIC shutter").
4. To focus the specimen
    a. Use the large black knob at the lower right base of the microscope for gross focus adjustment.
    b. Fine focus adjustments are made with the yellow Z buttons on the control pad or by twisting the joystick knob to the right or left.
    c. The dial with the red tape should never be touched. The fine focus is motorized, so turning the knob by hand will strip the motor.
    d. Don't worry about getting the image perfectly focused because the eyepieces are not parfocal with the camera.

Lenses on Olympus microscope



air objectives:
20x, 0.75 NA, green ring
*40x, 0.85 NA, lt blue ring

water objective:
*60X, 1.20 NA, royal blue/white rings

oil objectives:
**40x, 1.35 NA, lt blue ring
60x, 1.4 NA royal blue ring
100x, 1.4 NA, white ring

* objective has coverglass correction collar
** objective has NA Iris (0.65 - 1.35 NA)

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DIC lenses

We have only two DIC lenses within the system: the 60x oil and the 100x oil. To obtain a DIC image, you must use the prism in the condenser that matches the objective. Choose the DP 100 for the 100x oil objective lens, or the DAPO 60 for the 60x oil objective lens. If you don't use the correct condenser position, then you will collect a brightfield image instead of the DIC image.

Important - Each time you want to collect a DIC image, you must align the optics according to the Aligning DIC on Olympus IX-70 instructions at the end of this manual.

Fluorescent illumination

1. Close the transmitted light shutter (grey button on keypad labelled "TRANS/DIC shutter")
2. The excitation filter wheel is controlled by the computer, so you must check on the PC computer monitor (on the stack of components on the desk to the left of the microscope) to see which filter is in the light path.

   To change the filter, use the red EX and EX buttons on the keypad. To open and close the shutter, push the green button in the lower left corner of the control pad (labelled "EX shutter").
3. The eyepieces have separate emission filters from those used by the camera and must be selected manually. These are found on the filter wheel just behind the eyepieces and are coded by dots along the rim of the wheel:
   a. no dots = brightfield image
   b. 1 dot = filter for DAPI (UV)
   c. 2 dots = filter for FITC
   d. 3 dots = filter for TR, rhodamine, PE
   e. 4 dots = filter for Cy5

Beginning an acquisition session:

1. Log in as dvuser.
2. Open DeltaVision by double clicking the DV icon on the desktop. The DV toolchest will appear at the top of the screen.

IMPORTANT NOTE:
The computer has a finite amount of disk space available. The DV Toolchest has a yellow status bar indicating what % of the hard drive is full. If the percentage climbs above ~90%, stop working and delete unwanted files and/or save data to your Jaz drive.

3. To start Resolve3D, choose
   File - **Acquire** from the DV menu.
   a. Note that the Resolve3D module uses standard SGI conventions
      - Gray boxes are for display only, do not fill in values.

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- Pink boxes are for entering parameters. After entering the value, *hit the return key to have the computer accept the change*.

4. The important parameters to monitor are as follows:
   a. Choose the acquisition window size for number of pixels collected along X axis and number of pixels along Y axis. You may use the SIZE button bar menu, or just type in the desired values in pink box to the right of Size menu. The default is 512 by 512. The program will calculate the pixel size.
   b. Adjust exposure (in sec). Longer time = brighter image. Ignore "FIND EXPOSURE"
   c. Specify the LENS you are currently using: 20x air, 40x air; 60x water, 40x oil, 60x oil, 100x oil.
   d. Check the Auxiliary magnification box if you are using the 1.5 magnifier (knob on the right base of the microscope is pulled out)
   e. Defaults are Speed = 1000, Bin = 1 and Gain not checked.
5. Adjusting illumination of the fluorophore
   a. Skip down to the Filter Control panel.
   b. The excitation and emission filter wheels are controlled by the computer, as well as the neutral density filters which decrease the amount of light hitting your sample.
   c. The excitation and emission filters are paired by default, so if you choose the FITC excitation filter, it will automatically choose the FITC emission filter. To override the default, choose one of the other emission settings using the "em filter" button (for example, for PI use the FITC excitation filter with the Rh-TR emission filter).
6. Turn the dial on the right of the microscope to side port (sp).
7. Click the ACQUIRE button in the top left corner of the window - this will open the excitation shutter for the specified exposure time and display image in acquisition window (#21-50)
   a. If the camera is saturated (computer will beep, "camera saturated" will display in dialog box at bottom of Resolve3D window and image window may be white), decrease the exposure time or add a neutral density filter.
   b. If nothing appears in the display window, the image may be out of focus, the exposure time may be too short, or you forgot to turn the beamsplitter dial to sp.
   c. The correct exposure/neutral density will be when the separation of the minimum and maximum intensity values captured on the camera is *at least* 200. However, recognize that 200 is an absolute minimum and the deconvolution will not be optimal. The separation between min (black bar) and max (red bar) should be at least 400 to get a decent deconvolution; 1000 is better.
   d. It is important to note that the image window will automatically scale the intensity values for maximal separation (the lowest value will be assigned to 0 and the maximum value will be assigned 255); therefore, your images will look the same even though the amount

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of fluorophore in two samples may be different. *You must keep track of the real intensity values* (recorded in the grey "mmm" boxes - min, max and mean, respectively.

Acquiring DIC Image

To collect the DIC image in the Acquire window, you need to change the default shutter to the transmitted shutter (EX =fluorescent/mercury bulb, TRANS = transmitted/halogen bulb). To specify the shutter, click on the SETTINGS button in the Resolve3D window. Next choose the SELECT button which is immediately to the right of Illumination System. Select TRANS and deselect EX. Then click done to close the select window. To go back to acquiring fluorescent images, deselect TRANS and select EX.

DeltaVision image window:

1. The number of the display window depends on whether you are displaying images that have been saved, images generated during post-collection processing or raw images from live acquisition. Saved/processed images are loaded into windows are numbered from 1 to 20, while raw acquired images are in windows numbered from 21 to 50.
2. Each window has three banks (= wave or wavelength) each of which can display the output from a single fluorophore or transmitted acquisition.
3. Zoom in and out by using the wheel on the bottom left of the window (click the small square beneath the window to reset the size to 1).
4. Move the zoomed image right/left or up/down using the sliders at bottom and right of image window OR by clicking the hand icon at top left and using mouse to move the image.
5. A region of interest can be centered in the window by clicking on the centering tool (circle with + through it) and clicking on spot you want to appear in the center.

Collecting a stack of images for deconvolution:

1. Determine upper and lower limits of specimen
   a. First, you need to determine the top and bottom of the specimen that you wish to image. Just below the Filter Control panel of Resolve3D, there is a panel with up and down arrow buttons for moving the objective up and down in set increments. You can choose the amount of movement by entering a value in the dZ field (pink box, suggested value = 1.0 micron).
   b. Determine the top of the specimen by clicking the up arrow *and then acquiring an image*, and repeat until the top has been determined (write the value down). Do the same process for the bottom and determine the total specimen width you want to image.

Calculate the value for the middle of the specimen [(Top value - bottom value)/2 + bottom value]. Move the objective to this middle position.

c. An acquisition window (#21-50) only stores the 20 most recently collected images. The section number will be displayed in the bottom left of the image window.

d. To toggle between images, use the slider at the left of image. Alternatively, use the right mouse button to move forward in the stack and the middle mouse button to move backward.

2. Collect the stack of images

a. Click the EXPERIMENT button at the top of the Resolve3D module.

b. Click the DESIGN button to write a macro (sequence of commands)

i. Choose the optical section step size (.15 to .3 um for a lens with N.A. ~1.4) and the number of sections. Hit enter and the computer will compute the total width of the specimen that will be imaged. Adjust these two parameters to specify the range you determined in step 1b.

c. For each fluorophore you wish to measure,

i. Click box on left (check mark will appear),

ii. Enter the exposure time, and the ex, em and nd filter settings.

iii. Choose the window and wave you want the output to go to. We suggest specifying a specific window (21 to 50) in which to display all of fluorophores (max=3) and put each fluorophore into a different wave (1, 2, 3).

iv. Illum. should read "default"

v. Ignore the time lapse setup.

d. Save the settings by choosing **File** - **Save & Exit** (not Save As) from the DV menu. Click YES to overwrite the old macro settings file. The macro window will automatically close.

c. Click RUN and then DOIT to run the experiment.

i. Type in the name of the file (Keep it short!!). The program will add .r3d automatically.

Common problems encountered while collecting a stack

Q1- Why is first image collected different than what was seen when the bottom was determined in step 1b?

This is a common problem and is due to a number of factors. The motor has moved the objective back to the same physical place, but if your sample moved, then what you see is different. Common reasons for sample movement:

1 - The slide moved on the stage

Correction: Clamp edges of slide before focussing

2 - Too much or too little immersion oil used, causing tension or vacuum between objective and coverslip

Correction - clean both slide and objective and apply small drop of immersion oil on objective

3 - Sample is not fixed to coverslip and movement of objective results in movement of sample

Correction - Calculate top and bottom of specimen as outlined in step 1b, but leave objective at bottom (do not move objective to middle position). In the Design window (step 2b), Enter 0 (zero) in box labelled "Offset from current z" and hit enter. Make sure this is still zero before saving Experiment macro! This will cause less movement of the objective and, hopefully, sample won't move. If sample still moves, then you need to modify sample preparation to stabilize your sample.

Q2 - Why can't I focus on my specimen?

The high magnification objectives on the microscope all have fairly high numerical apertures (~1.4). This means that the depth of focus (how far into the sample you can focus) is very small, so your sample must be close to the objective. The most common problems are:

1 - The coverslip is incorrect for high NA lenses and you can't see "through" the coverslip to the specimen.

Correction - Use only #1 or #1.5 coverslips.

2 - The cells were grown on the glass slide, then mounting medium was applied and then the coverslip. If too much mounting medium is used, then the objective can't focus through to the specimen.

Correction - Apply mounting medium sparingly **or** grow cells on coverslip and mount with glass slide.

3 - Cells were grown in chamber slides with gasket and gasket was not removed during mounting. Specimen is too far away from objective.

Correction - remove gasket **or** grow in chamber slides with coverslip bottoms (instead of glass slide bottom)

Collecting DIC Stack

To run an experiment which includes both fluorescent and DIC images, click on the EXPERIMENT button in the Resolve3D window. Next choose DESIGN, and fill in most of the sections as before. There is one new column, labelled Illum., which says "default." For dual fluorescence/DIC stacks, you must change this to "custom." Choose EX for the fluorescent banks and TRAN for the DIC bank. In addition, for the DIC image, choose DAPI for the filter set (exposure time will be ~0.1 second) **or** put the UV filter in place in front of the lamp (otherwise the halogen bulb will saturate the camera). Run and analyze the experiment as before.

Post-collection processing:

A normal processing session consists of three separate steps -

1) Deconvolve (with correction for photosensor data),
2) VolumeViewer (formerly Projection), and
3) converting files to TIFF format.

The Drag & Drop processing of DeltaVision v 1.2 has been replaced with a menu interface. While the processes are running, it is important not to close the pink macro or grey log windows (this may cause the computer to hang). To get the windows out of the way, it is suggested that you "iconify" them. Click the SMALL square in the top right corner of the window and the window will collapse down to a small icon along the top of the monitor. To re-open the window, click once on the icon.

When the process is done, your data file will be displayed with an icon showing a green prism and a red check mark. The other newly created files may be log files (*.log), shell scripts (*.sh), and other accessory text files. Important - *.sh files are the executable files for the macro to run the process. DO NOT double click on any *.sh file or it will start running the process all over again and you'll have to wait for it to finish.

Step 1:Deconvolve and correct dataset

1. Open the raw data file you wish to deconvolve (double click dataset icon).
2. In the DV toolchest, choose **Process** - **Deconvolve**. Next, drag the number of the display window into the input box. Make sure the cursor is positioned in the pink box and hit enter - the output filename and OTF will automatically be chosen for you. Check the "apply correction" box so the data will be corrected prior to running the deconvolvution algorithm. Notice that the output filename does not contain "_cor" but the data will be corrected. While the process is running, a scratch file named "filename_cor_TBH" will appear containing the corrected data and will be erased when the process is finished.

2a. (Optional) To confine the deconvolution to a specific xy region, click SELECTREGION and then scroll a box around the region you want analyzed. To confine the deconvolution to a subset of z sections, click DETAILS... and fill in the desired starting and ending z sections.

2b. (Optional) For dual fluorescent and transmitted datasets, only deconvolve the fluorescent channel(s). The raw transmitted data can be merged with the deconvolved fluorescent image later.

3. Choose DOIT.
4. Don't close the Deconvolution window while it is working or it may hang the process.
5. The output file will now be called "filename.r3d_d3d".

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Optional - To run several deconvolutions consecutively:

1. Follow steps 1 and 2 above.
2. After you hit enter, click on RUN OPTIONS. The run options window will appear.
3. The log file and the command file will be filled in for you if you hit enter in the above step. Click the RUN OPTIONS button bar menu, and select "add to queue."
4. Check the box to show output log, and then click on CLOSE.
5. Next click DOIT in the Deconvolve window. This will open the DeltaVision Queue Manager window.
6. Repeat this procedure for each raw data set you wish to add to the queue.
7. Once all data sets are queued, click START NOW.
8. Optional : Deconvolutions may be queued to run at a specific time by selecting StartLater and setting the clock for the desired start time. Note that for this to work, you cannot close the Queue Manager window or log out of the dvuser account until the queue is finished.

Step 2:Project the deconvolved image stack

1. From the DV toolchest, choose **View** - **Volume Viewer**.
2. Optional: If you want to project a subset of the entire field, click SELECTREGION and scroll a box around area of interest. If you only want to use a subset of the Z sections, Click DETAILS and enter values for starting and ending Z section.
3. Click LOAD.
4. The new software has three different projection METHODS: Additive, Maximum intensity, and Progressive. Note that he default for DV v2 is Max intensity, while the default for v 1.2 was Additive. (Don't choose the RGB/Opacity option.)
5. There are also three options for projection QUALITY: Good, Better, and Best. In terms of processing time, Best takes longer than Better which takes longer than Good. (All three will take considerably longer than v 1.2!)
6. Ignore the z sampling, xy sampling, and interactive buttons. Click MOVIE to select the series of projections.
7. To choose the angles at which projections will be performed, choose one of the options in the ROTATION button bar menu, or choose custom and enter the desired values in the StartAngles, No. of projections and ChangeAngles pink boxes.
8. If you wish to do projections exactly like version 1.2 default, use the following settings: **Start angle** = 0, -30, 0 ; **#projections** = 13; **Rotation** = Custom; **Change Angles** = 0, 5, 0; Then click DOIT.

Step 3:Export to TIFF

All the files created in the DeltaVision system are of a proprietary file format - in simple terms that means that only the DeltaVision software on an SGI computer can read them. To use the files on other computers, you will need to convert the files to TIFF format (that is the only exportable format presently supported by the software). This conversion must be done on the SGI with DeltaVision software.

Please note that TIFF files come in 3 flavors - which one you choose is determined by whether you want to save the colors or not and by the import functions of the software that you will use to analyze the files on your own computer.

8-bit TIFF - greyscale image, single fluorescent channel. As DeltaVision files are more than 8 bits (they're 12-14 bits), some data will be lost in this conversion.

16-bit TIFF - greyscale image, single fluorescent channel, no loss of data, however, not all programs support 16-bit TIFF files (NIH Image does, PhotoShop on the Mac platform does not)

24-bit TIFF - saves multiple fluorescent channel images (up to three fluorophores) WITH color information (*but no scale bar!*)

1. To convert files to TIFF, first open the dataset you wish to convert (double click on dataset icon).
2. In the image window, choose **File - Save as TIFF**.
3. The input and wavelength(s) will be chosen for you.
4. Leave compression at NONE
5. Autoscale should be checked, we suggest checking Short File Names (will leave off "_w528_w617" notation)
6. Enter the output directory (/usr/people/dvuser/data)
7. Enter a File prefix - *Keep it short (5-8 characters)!* This will default to "DV" if you don't enter anything.
8. Choose what type of file you want to generate (8, 16 or 24 bit). Note that for datasets with multiple wavelengths, 8 bit or 16 bit files can only export one wavelength at a time, so choose the wavelength to export. You can then go back and export the other wavelength(s) separately. Click DOIT.

To save a single image (not a stack):

1. Click CREATENEWFILE in the Resolve3D window. Specify a filename.
2. Click SAVELASTIMAGE.
    a. It will appear as though nothing has happened.
3. Click CLOSEFILE button
4. File will be saved into the default data folder (/usr/people/dvuser/data).

To Save an image with the scalebar:

1. In the DV toolchest, choose **Utilities** - **Image Snapshot**. An IRIS capture window will appear.
2. In the IRIS window, choose **Actions** - **Settings**.
3. Enter your "filename".rgb
4. Click auto increment (this means each capture will use the base filenameX and increment by 1 - file1, file2, etc.) and close window.
5. Next click on SWEEPAREA. Hold down mouse so that the + in the middle of the camera is at one of the corners of the image. While holding down mouse, drag until the red box encloses the desired area and release the mouse. Upon release, the image will be saved in the dvuser folder.
6. Next open the rgb file by clicking the icon. Choose save under the File menu. Select TIFF. The .rgb will be changed to .tif in your file name when the conversion is made.

File management:

1. You only need to save 3 files when finished:
   *.r3d (raw dataset)
   *.r3d_d3d (deconvolved dataset)
   *.r3d_d3d.log (log of deconvolution process)

2. Any file with the suffix .sh or .log (other than d3d) should be deleted.
   a. To remove a file, place the cursor on the filename, click with left mouse button to highlight files, hold the right mouse button down and select remove. Then from the SGI Toolchest in the top left corner of the screen, choose File - Empty Dumpster.

Transferring files to your Jaz disk:

About the disks:

A Jaz disk fits 1 GB of data - it is recommended that you leave 4-5 MB of free space on each disk.
Use Mac formatted disks, as the SGI Operating System (IRIX 6.2) does not recognize IBM format.

At the SGI computer:

1. Make sure green light is lit on Jaz drive.
2. Insert Jaz disk into drive *slowly.*
3. WAIT until orange light stops blinking.
4. Jaz icon (top right of screen) should have disk inserted.
5. Double click Jaz disk icon - directory will appear.

6. Open window for directory where your files are placed (Desktop - HomeDirectory; fill in path).
7. Use drag & drop to copy files to Jaz drive.

To Eject disk (very important)

1. Close all open windows for /jaz path.
2. Click jaz icon with left button - icon should turn yellow.
3. With cursor on jaz icon, hold down right button - choose eject Jaz command (near bottom of list).
4. Wait until orange light stops blinking.
5. Manually eject disk using black button on the face of the drive.

Note: If you do not "software eject" (step 3) before "hardware eject" (step 5) you may lose all of the data on the disk!!

Jaz disk technology is still new to the SGI platform, so occasionally things go wrong. Here are some tips gleaned from past errors:

Don't perform actions on datasets located on the Jaz drive (i.e. don't project a d3d dataset on the Jaz, don't export to TIFF with the output going to the Jaz). Copy the data to the SGI hard drive, process the dataset, copy the output to the Jaz, erase data from the SGI hard drive.

If you put a Jaz disk into the SGI and when you open the /jaz directory nothing appears, eject the disk from the SGI (software, then hardware). Bring the disk to one of the Macs in the lab - put it in the drive, double click the disk icon and open a window, make sure files appear, close window and eject disk. Now go back to the SGI and try again. Sounds like voodoo, but it's worked in the past.

If that does not solve the problem, take the disk to one of the CSIF Mac computers and run Norton Utilities on the Jaz disk. Usually, it finds an error with volume bit maps and such. Have it correct any errors it finds. Then take it back to the SGI.

If you try to eject the Jaz disk from the SGI and you get a "drive busy" error, close the DeltaVision program, log out and log back in to the dvuser account. Try ejecting again. Sometimes it's just that the program has recorded the /jaz directory as the current directory and you need to erase that from memory.

Aligning DIC on Olympus IX-70 inverted microscope

Setup:

Put 20X objective (or 10X if you have it) in position
have polarizer OUT
Analyzer can be in
focus on specimen
use open position of the condenser (not BF or 60X DIC, etc.)

Align condenser:

Wheel condenser down ALL THE WAY
Close down field iris (just below DV emission wheel chart)
Focus on the leaves, using the adjustment wheel on condenser (above polarizer and below condenser mount), NOT by raising the condenser. Tighten screw when done.
Center condenser with centering screws on condenser mount
Open field iris to just illuminate entire field

Align DIC (rough adjustment):

Push in U-DICT (should be UPSIDE DOWN in inverted microscopes - this part was made for uprights, so the label will be on the underside). Turn screw clockwise all the way, then back off about 1/2 of a turn.
Put CT/Bertran lens in
push in polarizer and turn until / is in sharpest focus. Note that the line will still be fairly fuzzy. Polarizer wheel will be approximately at the white dot. Lock down polarizer with black screw.

Align DIC (fine adjustment):

Move 60X or 100X objective into position
Move condenser to O (from CT)
Put 60X (DPO 60) or 100X (DP100) prism into place on condenser
Fine tune DICT screw for contrast and even illumination of field

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