

# P I C O L A Y M A N U A L

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[This text is displayed in the help window of PICOLAY]

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[www.picolay.de](http://www.picolay.de)

PICOLAY (from PICTure OverLAY) is a program for Windows (TM) computers designed for the generation of improved images and three-dimensional projections from digital picture series.

PICOLAY is freeware without any warranty. The copyright remains with the author. Please cite the website ([www.picolay.de](http://www.picolay.de)) when you use PICOLAY for the production of published images.

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## Main features

Although PICOLAY is small and very easy to use, it has powerful functions. It is designed for

- Focus stacking: Generate sharp images with increased depth of focus from picture series taken at sequential focus levels (so-called z-stacks).
  - Stacking based on target colours instead of sharp areas.
  - Average images.
  - Add or subtract images.
  - Auto-align positions if images are not perfectly congruent.
  - Auto-adjust brightness of the pictures in a stack.
  - Generate a flat field with adjustable background or white balance.
  - Add text and a scale bar to pictures
  - PICOLAY generates animations of picture series either as slide show or as animated gif file.
  - There are many more functions to improve images by changing parameters like sharpness, contrast, size etc.
- Unique features of PICOLAY are various routines to generate three-dimensional views, projections and animations:

- Generation of three-dimensional images from a single stack taken with a constant perspective.
- Generation of rotating 3-D images, red-cyan overlay images and images for crossed-eyes or parallel viewing
- 'Hologram stacking', a routine that makes items visible that are hidden by the normal stacking process.

Thus PICOLAY gives you a little bit of 'confocal microscopy' for free. It also has several functions for changing general image parameters like sharpness, contrast, brightness, colour saturation, gamma correction, resizing and cropping.

Please look [www.picolay.de](http://www.picolay.de) for more information.

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## Installation

PICOLAY consists of only one file (picolay.exe) with a size of less than 1 MB and does not need installation. It does not change any system files on your computer. Just copy the file into a directory of your choice and generate a link to picolay.exe on your desktop.

In subsequent sessions, PICOLAY will automatically use the last directory for searching images. (The initial directory is saved under 'picolay.ini'.)

If your computer is expecting decimal points instead of commas, (according to the 'Regional settings' of your Windows system) select this under 'Options'.

Tip: Often helpful hints are showing up, when you move the mouse over items on PICOLAY windows.

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#### Quick Stacking for the impatient user

- Select images (File | Select images or Ctr-L)
- Press the 'F1' key to get the start the routine, running with a series of pre-adjusted parameters.
- Select the best one of the resulting files (filename starts with p#sharp and contains the values used for its generation).
- If necessary, vary the parameters 'Preference of upper structures', 'Filter frame size' [1, 2, 3] and 'Minimum contrast' [3 ... 20] etc. to get the perfect result.

Details are described below.

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#### Image formats usable with PICOLAY

PICOLAY can open and save images as Bitmaps (BMP files), JPEG format (JPG files) and gif format. (In principle, TIF files can be opened, too, but there are so many different format variations that problems cannot be excluded.) All images must have the same dimensions (width and height) and the files must be saved in the same directory.

Typically, one will use a series of pictures taken in sequential focus levels, a so-called 'z-stack'. PICOLAY can also be used with frames of video sequences exported as single images. Several functions can also be performed on single files.

Bitmaps (bmp files) are faster processed than jpg files, especially with large pictures. If there are problems with jpg- or tif files, transform them into the bmp format. For this click on 'Options' and select the bitmap format under [Save as=]. Then click on 'Enhance image', (eventually change some parameters,) and carry out 'Apply to all marked images'. The gif format is sometimes useful for anaglyph images (red-cyan), and used for animated gif files. The latter can be opened, but the animation will not (yet) be displayed.

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Files generated automatically by PICOLAY

Most of the PICOLAY routines generate files that are automatically saved and added (unmarked) to the image list (see below). Most of the new files have names beginning with 'p#'. PICOLAY will not overwrite original images, but will overwrite p#-files without prior request!

Therefore it is strongly recommended to copy images of each stack to be analysed into a separate folder.

When you exit PICOLAY you will be asked whether all 'p#' files in your list should be erased (leaving no residues on disk). If you erase them, be sure to have saved before your final result under a name not beginning with 'p#!' (This can be achieved under Image list | Rename marked files.)

Exceptions: clip#, enh#, xy# and b# files

If you decide to crop a square from ALL MARKED IMAGES the results will be saved under 'clip#' + original file name. Similarly, series of enhanced images will be save under 'enh#' + original file name, images processed by the auto-align x-/y- positions or auto-adjust brightness subroutines will be saved under 'xy#' and 'b#' + original filename, respectively.

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### Select pictures

The first step is to select pictures. Click on 'File' and 'Select pictures'. Then click on the first image of the series, keep the SHIFT key pressed, and click on the last image file of your series. In order to select only part of the files from your directory, you can keep the CTRL key pressed and click on each file separately.

Once you have selected images, their names are displayed in the listbox at the left. PICOLAY sorts the files in alphabetical order. Take care that the names have the same sequence as the layers of your z-stack. The sequence can be reversed under 'Image list'.

Tip 1: In order to get familiar with PICOLAY use small images first (and copy them into a separated directory before you start!).

Tip 2: For a quick start, you can also mark a list of images files within the Windows explorer and then move this onto the PICOLAY Icon.

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### Browsing the image list

You can display the selected images by clicking in in the listbox or by means of the up- and down-arrow keys. By DOUBLE-clicking on the names one can mark [X] ... or unmark [\_] ... files for further processing. Alternatively one can toggle all marks, delete selected images, clear the list etc. under the menue item 'Image list'.

One can zoom in and out by clicking on [-] of [+] on the image window. Of course, PICOLAY will not change the original images in this case. A click on the number in the middle brings you back to the 100% view.

A special feature of PICOLAY is the following: When you have zoomed in or you have selected a certain part of an image, the position and size will be kept constant during browsing other images in the list.

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### Handling the 'Image list'

Under the menu item 'Image list' one can reverse the order of the image files. This is relevant because PICOLAY assumes that the first image shows the uppermost layer, and because only the first image can be added or subtracted from the rest of the list. (Eventually you'll have to rename the file to be added before selecting it.)

It is also possible to flip marks (by double-clicking) and to delete some or all of the selected files from disk.

Furthermore, one can rename marked files by replacing characters in the name string. This function is helpful, e.g. to escape the automatic deletion of 'p#...' files when finishing the program.

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### Image list: Slide show F12

With the F12 key or under 'Image list | Start slide show' you can get an animated presentation of all marked images. Under 'Options' it can be adjusted how fast the images are displayed. Furthermore, you can set whether the show goes back or starts from the first image when the end of the list is reached. Clicking on the image or on the list will stop the show.

This function can be used for the original stack as well as for stepwise 3-D rotations generated by PICOLAY, or for any other images series :-)

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### Image list: Generating an animated gif image

The slide show can be transformed to a single animated gif file. Please regard that a large number of images results in large files (file size increasing with the square of the image dimensions!). Don't give it a name without the '.gif' at the end.

Animated gif images can be opened, but the animation will not (yet) be displayed by PICOLAY. Internet browsers can show the animation without requirement of a special driver.

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### Image window: Zoom in or out

Clicking on [+] or [-] on the image window (as well as on the result window) increases or decreases the zoom factor. Clicking on the number in the middle brings the display back to 100%.

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### Image window: Edit functions

Under 'Edit' one can copy an image or parts of it to the clipboard.

Other functions are 'Crop' or paste an image from the clipboard.

'Crop all marked images' can be used to select the interesting area in your z-stack for further processing. In this case the new file names will be 'clip#' + <original name>.

The contents of the image window can be copied to the result window either as 'result' or as '2-D-map'. This functions can be used to make changes to the stacking results or 2-D map, before they are used for 3-D projections.

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Image window: Enhance image

When a selected image is displayed you can change a series of image parameters by clicking on 'Enhance image' on the upper window edge.

First check the result, then apply the changes. There is no 'Undo'-function as in other routines. However, a new file will be generated. If you 'Apply' the enhancement to a single image, the result will be saved automatically under 'p#enhanced-01.jpg' (with increasing numbers at the end). Additionally, one can save the displayed result by selecting 'Save result' or Ctrl-S). The file format used for saving is adjusted unter 'Options | [Save as=]'.)

The selected transformations can be applied to all marked images if the corresponding box is checked. In this case the results will be saved under 'enh#' + <original name>.

Please select the file format of the generated images under 'Options | Save as'.

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Image window: Mouse functions

The standard function is 'Rectangle' used to select areas for cropping or copying to the clipboard.

There are several other 'Mouse functions' selectable:

Tip: Set the zoom factorto 100% to get the fastest diplay of the mouse actions.

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Mouse: Retouching an image with paintbrush

'Paintbrush' is used to paint on your image. RIGHT-clicking on the image will feed the pipette with the colour underneath the tip, which is indicated in the little tool window. Moving the mouse with the left mouse button (or the Ctrl-key!) pressed will paint on the image with fading edges. You can restore the image back to the last mouse-down situation by clicking on 'Undo' or by pressing 'Ctrl-Z'.

The thickness of the brush line indicated by a dashed circle, and is adjusted in the additional tool window. Finally you'll be asked whether the changes should be saved under 'p#enhanced-..' (not overwriting the original).

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Mouse: Stacking by hand

'Clone to result image' copies the area underneath the mouse circle in the image window to the same position of the result window (indicated by a ring in both windows). This allows manual stacking. The depth map will keep the information about the layer used for cloning, thus allowing a correct 3-D projection. (Only the original images used for stacking should be marked during cloning to the result window.) For

exact positioning the brush width can be adjusted as described above, and one can zoom in by clicking on [+].

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Mouse: 'Cloning within image'

This function allows to clone areas inside the same image, e.g., in order to overwrite disturbing items with undisturbed background. First RIGHT-click on the target position. Then (left-)click on the source and gently move the mouse through the source area while observing changes in the target zone, which is indicated by a second ring.

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Mouse: 'Insert text'

This opens a new window for the selection of a text string, font, colour and direction (vertical or horizontal).

A subsequent click places the text on your image. Use 'Undo' and repeat placing the text until you are happy with the outcome. Then 'Save' the result.

You can add the text to all marked images if you check the corresponding box.

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Mouse: 'Insert scale bar'

This opens a new window for the definition of dimensions and colour direction of a rectangle.

A subsequent click places the bar on your image. Use 'Undo' and repeat placing the bar until you are happy with the outcome. Then 'Save' the result.

You can add the scale bar to all marked images if you check the corresponding box.

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Stack operations

Once you have images selected, various tasks are enabled. Stack operations can be started by means of the F1...F12 keys or from the corresponding menu items. Operations are applied to marked images, only.

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Stacking based on sharp areas

F1

The F1-key or clicking on 'Stack with varied parameters' will start a series of routines routine with pre-adjusted parameters.

\* Non-marked entries in the listbox (but not the image files on disc) will be removed.

\* The images of the stack will be corrected for x- and y-displacements (especially required for stacks taken with a stereo microscope).

\* Brightness of the images will be harmonised.

\* Repeated stacking runs with varied parameters will be performed. This generates a list of 'p#sharp...' files with the parameters as part of the name.

\* The files 'p#avmap.bmp' and 'p#avsharp.bmp' will be generated, which contain the average of the stacked results and the corresponding 2-D maps.

Just browse the stacked results to find the best one or to learn which parameters give the best result. Then you might average a number of good results or repeat the stacking routine as described below.

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Edit stacking parameters                      F2

Press F2 in order to adjust the parameters prior to stacking.

A new panel at the right opens, where you can adjust a few parameters. Upon clicking on 'Go' the routine filters out the areas with the highest sharpness, i.e. contrast between neighbouring pixels.

'Filter frame size' defines the diameter of the frame that is screening the images for sharp areas. The value should be small (1 or 2) if you have finely structured objects with frequent changes of the sharp layer, while for large structures and few changes of sharp layers one should use larger values. Just try it out!

'Minimum contrast' is set to a value that allows separation between a smooth background and areas with sharp structures. This avoids that minor disturbances or noise is selected instead of filtering it out. Low-contrast pixels will be averaged, giving a smooth background.

PICOLAY selects original pixels of the highest contrast combining areas of the same layer depending on the selected frame size. If you set the minimum to zero PICOLAY will generate a sharp image composed of original pixels in the stack, only.

One can also smooth the transitions of the 2-D map and automatically enhance contrast and sharpness of the resulting image. If this is not improving your result, switch the functions off and try the 'Enhance' function after loading the resulting image by clicking on its name in the image list.

If you start the stacking routine you can follow the stacking process in the result window. Finally, an unmarked file named e.g. 'p#sharp\_ff3\_mc5.jpg' will be saved and added to the listbox with 'p#sharp' meaning sharpness-based PICOLAY file, and '\_ff3\_mc5' describing the reading frame size (= 3) and minimum contrast (= 5), respectively.

A map showing from which layer a pixel is derived, is generated concomitantly with the sharp result. The information in the map is used to generate three-dimensional pictures and projections (see below).

A click on '[Flip view=] Result' (or '[Flip view=] 2-D map', respectively) switches between the stacked image and the depth map for pixel usage. Yellow indicates uppermost and blue the lowest levels of the stack. If selected under 'Options', the 2-D map is saved automatically as 'p#sharp2Dmap...' and added to the image list

(unmarked). Check the 2-D to find the perfect values for 'Reading frame' and 'Minimum contrast'.

The resulting files are generated in the same directory as the analyzed images, and will overwrite older 'p#'-files with the same name (but not the original images).

Again: One can improve the resulting image by clicking on [Enhance image] as described above.

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#### Colour-based stacking F3

Starting 'Stacking based on colours' in the main window of by 'F2' opens the colour-based stacking routine. A new panel entitled: 'Select target colours' is showing up. You can define a target colour by clicking in the original image with the RIGHT mouse button, or by entering numbers in the corresponding fields on the panel, or by clicking on the red, green, blue, black and white squares.

Especially black and white (R/G/B = 0/0/0 and 255/255/255, respectively) give interesting information about your image stack!

Clicking on 'Go' will produce an image composed of those ORIGINAL pixels from the Z-stack that are closest to the target colour. This feature is especially helpful for phase contrast images and stained objects.

Colour-based stacking also generates a 2-D map and opens the three-dimensional possibilities described below.

If you check the box with 'Strip non-target part', your target colour will be filtered out from the stack while other colours are neglected.

The resulting image is saved as 'p#colour\_r0\_g0\_b0.jpg' (with numbers indicating the RGB values) and added to the image list (unmarked). If selected under 'Options' the 2-D map is saved as 'p#colour2Dmap\_r0\_g0\_b0.jpg'.

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#### Averaging images F4

The mean image is obtained by hitting the F3-key of from the 'Stack operations' in the main window. The resulting image is saved as 'p#mean.jpg' and added to the image list (unmarked). [Again: Older files with the same name will be overwritten!]

To enhance the quality of the result one can increase sharpness and contrast using [Enhance image]. In some cases this gives an excellent output, comparable to sharpness-based stacking.

Averaging might also be helpful, if you want to combine different images that were produced by PICOLAY. E.g., try out what happens if you average the resulting images for the darkest and lightest pixels...

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#### Insert intermediate images F5

This function allows to generate additional images inserted between you originals. This can be useful for smooth transitions in a slide show or for 3-D projections, if you would like to increase the number of layers. The name of the new images will get an 'i' added at the end.

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Auto-align x- and y-positions      F6

If some of your original images are displaced with respect to their horizontal and vertical positions, you can compensate this by using the auto-align function. This routine will especially be helpful for pictures taken through a stereo microscope, where the object appear moving with the focus. The resulting images will be saved under 'xy#' + <original file name>. Use this function prior to stacking.

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Auto-adjust brightness              F7

If some of the pictures in your stack differ in brightness, try 'Auto-adjust brightness' to compensate for that. The resulting images will be saved under 'b#' + <original file name>. Use this function prior to stacking.

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Set white balance                    F8

If you use this function you can correct the white balance of your picture/s. Just click with the RIGHT mouse button into the background and then on 'Go'

The corrected images will be saved automatically with 'p#' inserted in front of the original file name (and can be used for further PICOLAY processing after changing the mark correspondingly).

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Add or subtract an image            F9

PICOLAY allows to add (or subtract) the first image in your list to all following images. Take into account that RGB values above 255 and below 0 are not allowed and will be cut off automatically.

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Set background /flat field correction      F10

If you select this function and a 'Factor' of -1 (a negative factor means subtract) this function can be used to eliminate disturbing elements that are present in all pictures of your stack (e.g. 'Coolpix rings', vignettes or dust particles on your lenses). The function does not simply subtract the first image (which would result in nearly black images) but adds RGB values required to reach the background colour you have selected by clicking in the image with the RIGHT mouse button. The best result is usually obtained when you select the natural background colour.

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Divide by 1<sup>st</sup> image                      F11

This function was implemented to divide alle marked images by the first marked image in the list. This is done colour-based by using the RGB values 0-255 for each of the channels. E.g., if the first image (divisor) has a value of 127 in the red channel then the R channel of the other images will be divided by 255/127 (i.e. multiplied by 127/255).

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Main window: Options

Under 'Options' you can adjust

- which colours should be used for 2-D maps.
- whether colour maps showing the layer from which each pixel is derived are saved automatically or not
- whether your sytem expects decimal commas or points.
- whether automatically saved files should have the bitmap or the jpeg format.
- whether a scale bar should be drawn on the resulting image. You can define the position, size and colour of the bar. (However, you'll have to calculate yourself how many pixels per length your image has.)
- how long images of a slide show should be visible, and whether the show should start from the first slide or go backwards, when the end of the list is reached.

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Main window: Help

Under 'Help | Info' the release date of the PICOLAY version you are using is displayed.

This manual is diplayed under 'Help | Manual'

For further hints and examples check the TUTORIAL at [www.picolay.de](http://www.picolay.de). Please, take care to download the newest version - I am still developing the program. Let me know any comments, problems and suggestions.

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Generating three-dimensional images

As described above the stacking routines (both, sharpness- and colour-based) generate 2-D maps (from blue to yellow, background pixels in grey if averaged), indicating which layer is used at each position. The depth information in this map can be used to produce various three-dimensional projections and true 3-D images.

Introduction to the 3-D functions

While 3-D projections are quite common and easy to look at, true 3-D images require the generation of two different pictures for the right and left eye, respectively, and a special technique for the

observation. However, they give a completely different and fascinating impression!

Some people can stare with parallel eyes (wall-eyed) on the two images presenting the object at slightly different angles ( $2 - 4^\circ$ ). After some time you'll see three images, and the one in the middle gives the 3-D effect. Other people are looking with crossed eyes. Eventually, they also can see three images and that in the middle in three dimensions, however, fore- and background exchanged. While wall-eyed viewing is difficult with images broader than the distance between our eyes (about 6.5 cm), cross-eyed people can see the effect also with larger images. Many people are unable to see the 3-D effect in two adjacent images. In this case red-cyan (or red-green) glasses and an overlay of the different views can be the solution.

Based on nothing but a single image series taken at a constant perspective, PICOLAY generates freely rotatable 3-D projections that can give a natural spatial impression (especially if animated) as well as images suited for wall- and cross-eyed viewing and red-green overlays. A special feature is 'Hologram-Stacking' - the visualisation of structures that are covered by the common stacking operations: If an object has more than one layer with interesting structures usually only that with the highest contrast remains visible. By using 3-D projections with changing viewing angle this problem can be overcome by means of PICOLAY.

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#### Using PICOLAY for the generation of 3-D views

Clicking on 'Generate 3-D views' in the main menu or on [3-D view] in the Result window (after stacking operations) opens a new window that allows to produce different kinds of 3-D images: pairs of true stereo images for cross-eyed or wall-eyed viewing, red-green overlay for the use of red-green glasses, freely rotatable 3-D landscapes derived from the 2-D map, and so-called hologram stacking.

The dimensions (width and height) of the 3-D images will be the same than those of the original images. However, the projections will leave some space unused. This will be filled by the background colour you select at the corresponding panel.

##### - Length of Z axis

The Z axis is defined in comparison to the height of the image (Y axis). If the lowest and top-layer of your z-stack have a distance of 100  $\mu\text{m}$  and the height of your image is 200  $\mu\text{m}$  the correct value would be 50 (%). A value of 0 will not give any 3-D effect. Too large value will result in separation of layers of the object.

##### - Seam between layers

The seam is used to fill horizontal and vertical fissures in 3-D images. Larger values for the length of Z axis, and viewing angle (right panel) require larger seams and vice versa.

##### - Projection based on 2-D map

The following routines will generate 3-D projections based on the two-dimensional depth map generated during the previous stacking process. The quality will depend on the parameters you have used for that.

- Hologram stacking

This will require a new reading of all images as described below.

- Images to be generated

Pictures produced are named 'p#red-cyan\_x0\_y0\_z0.jpg' where the numbers after x, y and z indicate the angles of the three axes.

- Surface

One can adjust whether the images to displayed shows the original pixels, the 2-D map, or a mixture of both. Furthermore, the output can be set to greyscale.

- Background

As background used for pixels below the minimum contrast one can use either the average of all images or a selected colour. After clicking on 'Colour' you can set this either by a RIGHT-click in an original image or after clicking on the colour bar.

- 3-D view

If you check '3-D view', two images with slightly different viewing angles (for your left and right eye) will be generated. The viewing angle is set on the right. The produced images can be overlain as red-cyan images. Alternatively one can produce 2 separated images, 2 or 3 combined images or a panel with 4 half-sized images for the right and left, respectively left and right eye combined. The latter has the advantage that one can see 3-D effects independent of the question whether somebody is staring with crossed or parallel eyes on the panel. The upper images will give a convex impression, the lower a concave - or vice versa.

- Hologram stacking (sharpness-based)

Hologram stacking requires a threshold value to make sure that only the sharpest pixels remain visible while others are suppressed. Therefore, the minimum contrast should be set to a higher value than used for normal sharpness-based stacking.

To make all layers visible in a series of resulting images, 'Stepwise rotation' should be activated.

- Hologram stacking (colour-based)

For colour-based hologram stacking the target colour and tolerance have to be defined. To set the colour, click with the RIGHT mouse button into the original image or use the colour definition function in the colour-based stacking window. If you are selecting the black or white as target colour, a large tolerance value might be necessary. Again, you can also produce pairs of images for stereo viewing or red-cyan overlay images.

Once you have found the perfect stacking parameters for an image stack, you can save these under 'File | Show parameters/batch file | Save'. This will generate (and overwrite (!) if already existent) a textfile named 'PICOLAYparams.txt' (in the current image directory). Later, one can load parameters saved in this file via the same subroutine.

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### Batch operations

The textfile 'PICOLAYparams.txt' also allows to perform batch jobs, i.e. series of PICOLAY procedures with varying parameters. If a job is defined under 'Job(s)' (e.g., 'F1' in the second last line of 'PICOLAYparams.txt'), the stacking routine will start after clicking on 'Set/Run'. If a series of data is added in a parameter line (separated by '/' e.g., 1/2/3 for the minimum contrast) the routines will be repeated to test all of them, and the results will be saved under the normal PICOLAY names. Please use 0 and 1 to check or uncheck parameters like 'Auto-enhance result' etc.

It is also possible to do a series of different jobs by adding several entries to the second last line. The routines started with F-keys (F1 ..F12) are set by F1/F2... The routines defined by control keys under 'Image list' are set by the corresponding characters like R for 'Reverse list top-down', A for 'Mark all' etc. 3-D views can be generated by adding Map or Holo to the Job list for Map-based or hologram stacking, respectively.

Example:

```
Minimum contrast: 0/0/0/0/1/2/3/4
.
.
.
Job(s): F6/F7/F5/F1
```

will give the following actions when started:

```
F6 = Auto-align x- and y-positions
F7 = Auto-adjust brightness
F5 = Insert intermediate images
F1 = Stack the resulting images
```

During the first four jobs, Minimum contrast will be set to 0. Because there are more than 4 parameters under minimum contrast, the last job (F1=Stacking) will be repeated with Minimum contrast values of 2, 3 and 4.

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That's it already!

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