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4D-Microscope software (by Christian Hennig and Ralf Schnabel)

Handbook (Version, 11_2010) 3-channel version

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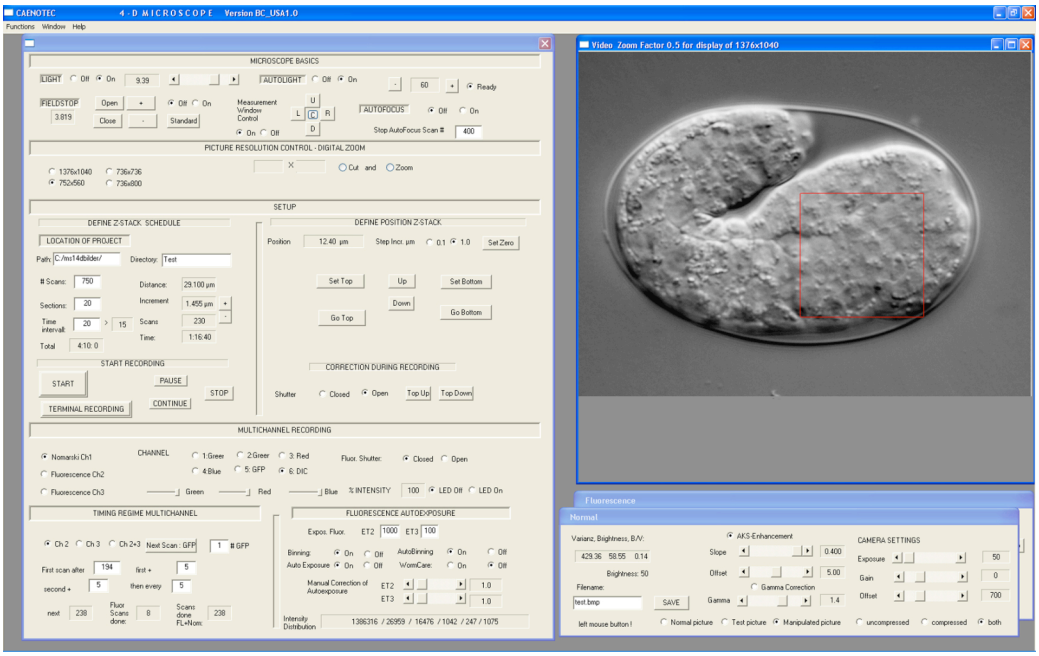
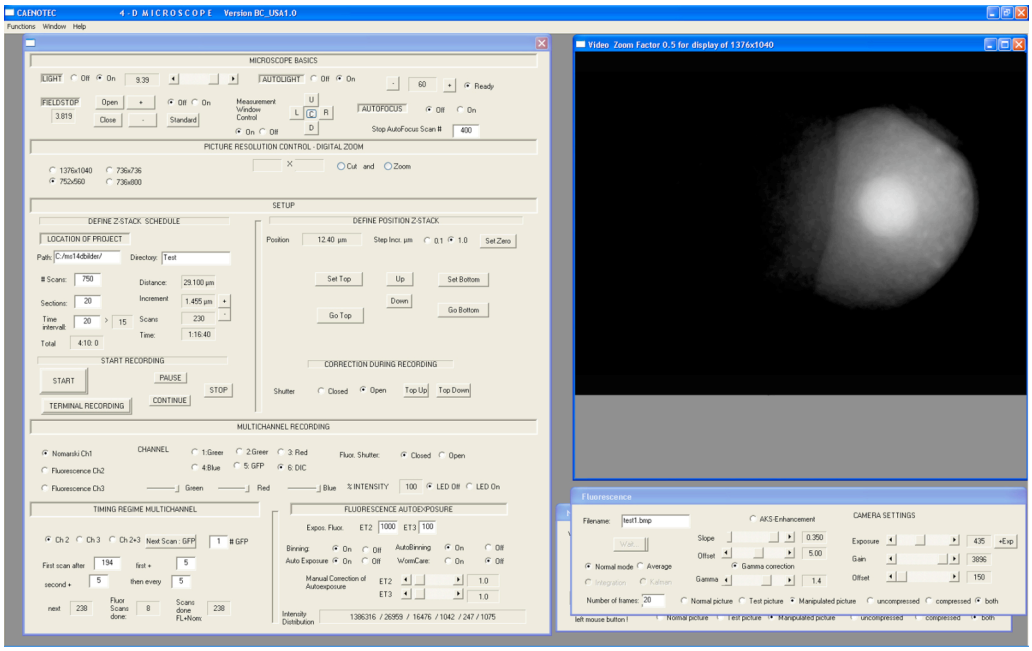




Installation

The installation CD (or Zip-file in case of email) contains the following files:

- steuerprg.exe (This is the 4D-program. Make a new folder in "Programs" on your computer and copy the exe into it. Put an alias on the desktop)

- hdd32.exe (This program installs the dongle driver when started from any location).

For Windows NT or XP the following DLLs must be dropped into the System folder C:/Windows/System32 and for Windows2000 in C:/WINNT/System32

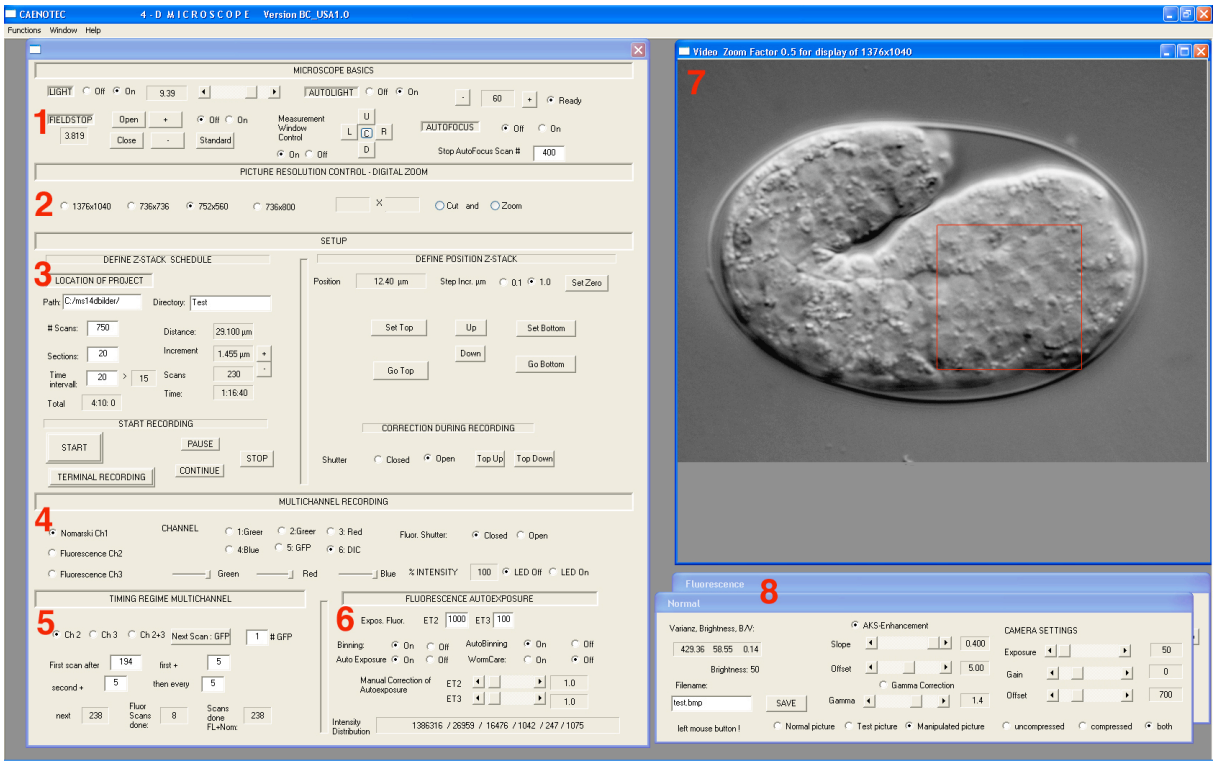
-lwf215p.dll
-MFC42D.dll
-MSVCRTD.dll
-MFCO42D.dll

To get started
Connect your microscope to the computer (Imager USB) and make sure that everything is working with the installation programs of Zeiss (Imager MTB 2004 Configuration MTB Test). Make sure that the camera was installed properly and is connected to the computer.

Copy the 4D-program into the "Program" folder of your computer and make an alias on the surface of your desktop. Install with the hdd32.exe the driver for the dongle Also install the DLLs from the CD into the system folder as specified above. Set the resolution of the graphic display on 1280x1024 and plug in the hardware dongle. Make folders "Pictures" and "MS14dbilder" on hard drive C:, or if a different location was desired in the corresponding partition in which the single picture (Pictures) and the 4D-recordings (4D-Recordings) are saved. Allow network access to these folders. Please note that the 4D-program crashes when pictures should be saved and these folders are not existing in the right location. Finally restart your computer.

The program may crash (or may take for ever to start) if the microscope was not switched on or if the camera is not switched on.

Please note that the computer should not be used for any other purpose than for controlling the microscope. Overloaded computers tend to get slower, which may compromise the performance during the recordings. It is also advisable to use a computer with 2 GHz for the 3 channel version of the 4D-program since the overlaying and saving of the pictures takes a lot of calculations. Especially do not try to put the program in the background during a recording to work in parallel on another task.



The main windows

Video camera

The program is currently designed to function with the sensicam or pixelfly (PCO) cameras since I found that these cameras are very good. On request we can also implement other cameras. However, we learned that this is a lot of effort and we have to have the camera in house to do so.

Using the 4D-program for normal microscope control

See next chapter for starting the program and setting up a specimen. The 4D-program can be also used to manually view and document specimen with DIC and fluorescence optics. Select the appropriate channels and filters in box 4 (MULTICHANNEL RECORDING). After choosing a channel the appropriate camera control window ("Normal" for bright light or "Fluorescence" will be activated (for handling the camera see below). Follow the procedures described below to set up the specimen. If you want to take a picture just click with the left mouse button in the video window. The picture will be saved with the system time down to the second as file name. This creates an unique document in the laboratory. Alternatively you may enter a file name in the field "file name" in the Camera windows 8 and save the file into the folder Pictures. You can save uncompressed or compressed or both types of pictures by selecting the corresponding buttons in the active camera windows. Compressed pictures are set as a default. Single pictures can be uncompressed with the software Irfanview-Plugin 4.22 (freeware) or other software e.g. Photoshop with a Lurawave plug in or Lurawave`s LuraSmrt.

Using the 4D-program for 4D-recordings

First the typical flow of the procedure to start a recording will be described the details of the supplied functions will be explained later.

Place a specimen under your microscope and set the Köhler illumination.

Köhler illumination for DIC

Focus the specimen in the mid-plane. Close the field stop and centre it in the viewing field. Turn the polariser for the darkest position. Turn the DIC prism in the objective to darkest position. Open the field stop that it does not touch the embryo anymore. Regulate the light intensity that you can see the specimen nicely with the eye. Turn the polariser out of the darkest position to get an optimimal DIC contrast. Turn the DIC prism to improve the DIC picture further. The optics produces a black and a white rim at the borders of the embryo. Try to minimise those.

Start the recording program

Double click the alias for the 4D-program. (Always use a newly started program before starting a new recording!) The program opens and you will see the main window (Fig 1), however, the Video window will be white. To open the capturing of the video camera pull down "window" in the top panel and activate "*win1"*. Open the shutter for the camera and now you should see the specimen, if the light intensity and the camera are set properly in boxes 1 and 8. The specific functions of the boxes will be explained later.

In Box 1 (MICROSCOPE BASICS) you can switch the light on and off and regulate the intensity of the light source by pulling the slider. You can also regulate the light on the knob at the microscope. The new program also controls light intensity automatically if you wish. See MICROSCOPE BASICS for details. In box 2 (PICTURE RESOLUTION CONTROL DIGITAL ZOOM) the desired resolution of the recorded picture can be chosen.

The digital camera has a very high resolution (1376x1040 pixels), which can not be used properly with high magnifications when the specimen is not large enough. Thus huge pictures, which do not contain much meaningful information would be collected. Therefore, smaller windows were created, which cut a section out of the large window. This causes an apparent magnification in the video window without compromising the resolution, which is still the highest available according the rules of optics. Now we also feature a "pull and draw frame" function, with which one can select freely the picture size around the specimen.

Recenter your specimen, turn the camera until anterior of the embryo is to the left and optimise the picture again using the DIC prism and the light control.

It is important to record in a standard orientation since later during the lineage analysis anterior should be to the left on the screen. One gets used to this orientation and makes "automatically" mistakes during lineaging when embryos are recorded in unusual orientations. If two embryos are recorded side by side in the large window

anterior should be also to the left. If a different orientation of the embryo is unavoidable use anterior to the top.

If necessary adjust the exposure time of the camera in Box 8 if you need too much light to see a proper picture (light should be less than 5 optimal is 3). The picture can also be improved by altering the setting "Gain" and/or "Offset" 8. I believe the pre-settings I chose are optimal but may be you are able to still improve the picture Get your own experience. The feature "Variance, Brightness, B/V" in Box 8 will assist you to get an optimal picture by reading out the variance of the brightness values of the pixels and the mean brightness of a field (200 x 200 pixels) in the center of the measurement window.

Pictures will be exposed properly if the brightness is around 40 to 90 (get your own experience, this depends on the brand and settings of the computer screen used). The ratio of Brightness/Variance represents the contrast of the picture in the measurement window. It thus helps to set the DIC contrast properly. A value of B/V of < 0.2 corresponds to a very good DIC picture, if you look at an upper plane of the embryo. If you focus up and down you can see how the variance changes. If you move above the embryo the blurred picture will result in a high value.

The camera is preset to 50 ms, which is the normal video rate. The updating timer of the program is set on 5 ms, thus a "life picture" can be seen in the video window. Long(er) exposure times slow down the refreshing of the video picture and also the collection of the z-series if the recording has been started.

Box 1 also controls the Field Stop (which can be also set manually). You can open or close the aperture completely, move it with the smallest possible increments or "Standart" will set the aperture in an optimal position for DIC if a 100x objective is used. Please try to optimise the contrast yourself after setting up the DIC during the Köhler procedure. Setting of the Field Stop is part of optimising the DIC.

Now everything is ready to set up a 4D-recording.

Some training is required to be able to set up the specimen fast enough to start a recording with one to four cell stage embryos, which facilitates later to identify the starting cells during the setup of the Biocell analysis.

4D-recording with DIC only (all buttons box 3)

-Enter a name for the recording in box 3 (SETUP) box (DEFINE Z-STACK SCHEDULE; LOCATION OF PROJECT) in the field "Project" (as a default the date is set). This name for the recording is later the name of the folder found in the directory specified in the field "Path" in which the single pictures are saved during the recording. The pictures are named with the system X0000L00C1 (X"number of scans, "L" number of levels "C" number of channel).

Important: never push return after an entry, since this will close the corresponding window. If it happens anyway you can reopen the window by choosing it again in "Functions".

It is recommended to structure the project name as follows:

RS (initials of operator)_**strain name** (or whatever)_**1** (for first recording, 2 for second.....)

In principle you can enter 256 characters for the path and the project name. However it may be a good idea to restrict the project name to 50 characters. Only use letters and numbers and – o r_.

Now use box "DEFINE POSITION Z-STACK"

- Focus on the surface of the specimen using the microscope focus control or the "UP" – "DOWN" buttons.

- Press "Set Zero" and "Set Top" to set the top level

- Focus down by pressing "Down" until you are seeing the lower edge of the specimen (for *C. elegans* embryos "Distance" should be at least 25 *µ*m). If you moved too far you can move back up by pressing "Up". Above these buttons you can choose the step size executed at each click and read out the distance the stage moved. Click "Set Bottom" and return to top by using "Go Top". You can now check the setting of the bottom plane by using "Go Bottom" and return again to the top level by pushing "Go Top" again.

-Change the preset recording parameters if desired.

In my experience at 25 ºC the time interval should not be longer than 35 seconds and 25 levels for a z-series are enough. A rule of thumb is that the increment between two focal levels should be around 1*µ*m. An increment of 0.5 *µ*m is too small. With Biocell you do not see a significant change of focus changing the z-level later. If you use values larger than 1.5 *µ*m you may miss small cells in late development. If an embryo develops slower the number of scans can be increased or if you want to leave and you know 600 scans are enough you can enter 600 and the recording will stop automatically after 600 scans and keep the shutter closed to save the embryo. As described later, the program helps you to avoid the situation by estimating how long a z-series with n focal levels will take (Box 3 time interval.

Please note that if the time (recording) interval is too short the microscope will just keep going and you may not be able anymore to type a longer time in window, please try. We are afraid if the situation does not improve you have to stop the program using the task manager.

Activate the desired picture format compressed or uncompressed in Box 8. If SIMI Biocell is used always use compressed (otherwise one recording will collect pictures to fill 10 Gigabyte on your hard disk). If the Hard disk gets full during a recording the program will crash. Please check the available space once and a while.

Now everything is ready to press **Start**

In the box START RECORDING

The recording is now running and the shutter closes after a series is taken and reopens for a new one.

The progress and quality of the recording can now be checked "online" by opening the project with Biocell on a second computer.

After starting a recording the agar pad will usually shrink so it looks as if the embryo sinks down. You can observe this using Biocell or by opening the shutter in the box CORRECTION DURING RECORDING. The shutter can also be closed again. If the embryo has sunk down you press button "Top Down" several times until it is again in focus. A warning; you should not press it more than five times (which corresponds 5 times 0.1 μm) in one go because it will cause a jump in the records, which makes lineaging difficult. "Top Up" will move the embryo down if the top level has moved into the embryo. As a new feature there is now also an automatic Focus available (see below Box 1).

If it turns out that the lower parts of the embryo are not part of the z-series during the recording you can increase the increment by clicking in box DEFINE Z-STACK at "+" which will cause the z-series to move further down. Using the button "-" you can decrease the increment if the series runs below the embryo.

All parameters can be altered for any new requirements. In case the number of sections is increased the program will just add new focal levels with the increment displayed in the field "increment" thus also the "Distance" from the top to the bottom level will increase.

Please note that if you once have started a project in SIMI to analyse the recording while it is still running you have to change the number of levels in the *project.sbc* file using an editor (e.g. endnote). Otherwise you will not be able to view the new planes although they are there.

I choose this strategy because otherwise a big "jump" of focal levels would occur during lineage analysis. If you want to increase the number of levels but keep the distance please use the "-" button to lower the increment as described before.

If you want to execute changes within the program or at the microscope and you need more time than the 20 seconds or so, which remain in between two scans, you can pause the recording by clicking at "Pause" in the box START RECORDING. If you are done just press "Continue" and the recording will start immediately again with the old or altered settings.

This feature is especially useful at the beginning of a recording when embryos have still large cells, which do not move significantly. At the bean stage (400 cells) a pause for more than 60 seconds will usually prevent lineaging after the pause.

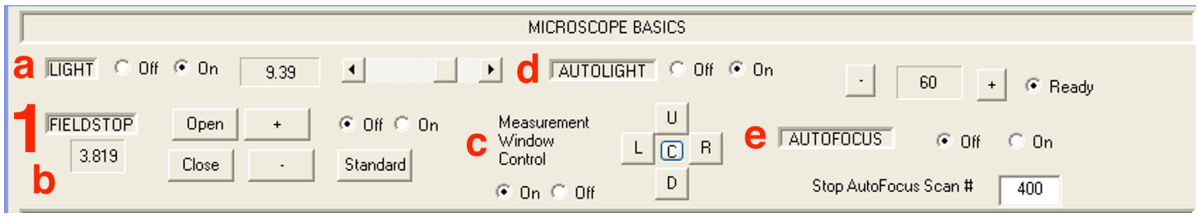
You stop the recording by pressing "Stop".

Normally you should stop the recording when the embryo starts to move because you will not be able to follow cells anymore once the embryo started moving.

In case you record mutants do not forget to record a z-series of the terminal phenotype after some hours by pressing the button "TERMINAL RECORDING". This will create a new folder with the name "project name"_t. Also with normal embryos it is a very good idea to write down in your lab book that the L1 larva hatched and looked normal.

All relevant parameters of a recording are now stored in a file named recdata.log in the Project folder.

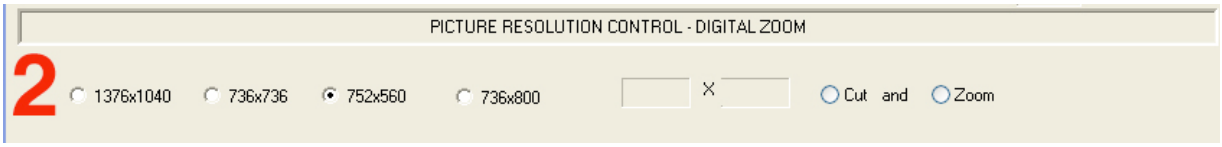
Detailed description of functions and some advice



Box 1 MICROSCOPE BASICS

This box controls the light and field stop and allows you to activate an automatic focus control. (a) Light can be switched on/off manually and intensity is regulated with the slider. Intensity is displayed showing the voltage applied to the lamp. (b) The field stop can be either completely opened (OPEN) or closed (CLOSE), which is useful to Köhler the optics. +/- opens/closes the field stop with small steps. The field stop contributes contrast to the DIC pictures. It is usually optimal to move it just outside the picture. "Standard" should be the optimal position for a 100x lens. Before activating AUTOLIGHT or AUTOFOCUS the "measurement Window control" has to be switched on. A small frame (200 x 200 pixel) will pop up in the video window (7). Using the navigation cross you can move it into an optimal position, which should be the center of the embryo. If the frame gets lost you can centre it again with the button "C". The frame is used for the analyses of the picture in real time. Variance Brightness and the quotient of those is displayed in the video window "Normal" (**8**). Pictures will be exposed properly if the brightness is around 40 to 90 (get your own experience, this depends on the brand and settings of the computer screen used). The ratio of Brightness/Variance represents the contrast in the picture. It thus helps to set the DIC contrast properly. A value of B/V of < 0.2 corresponds to a very good DIC picture, if you look at an upper plane of the embryo.
If the AUTOLIGHT function is on the mean pixel brightness within the Measurement Window can be set. Ready indicates when the desired brightness was reached. The microscope will compensate any change of the brightness during a recording. The measurement window is also used to keep the top level, you set up earlier, constant during the recording. It can be switched off later in development when morphogenesis starts with specifying a scan number in the field "Stop autofocus Scan #". This is necessary since the autofocus will inexorably follow the surface of the embryo. This is not useful anymore when morphogenesis creates holes in the embryo. Usually the top level is anyway constant after 300 to 400 scans or so. These features now allow recording over night.

Box 2 PICTURE RESOLUTION CONTROL DIGITAL ZOOM



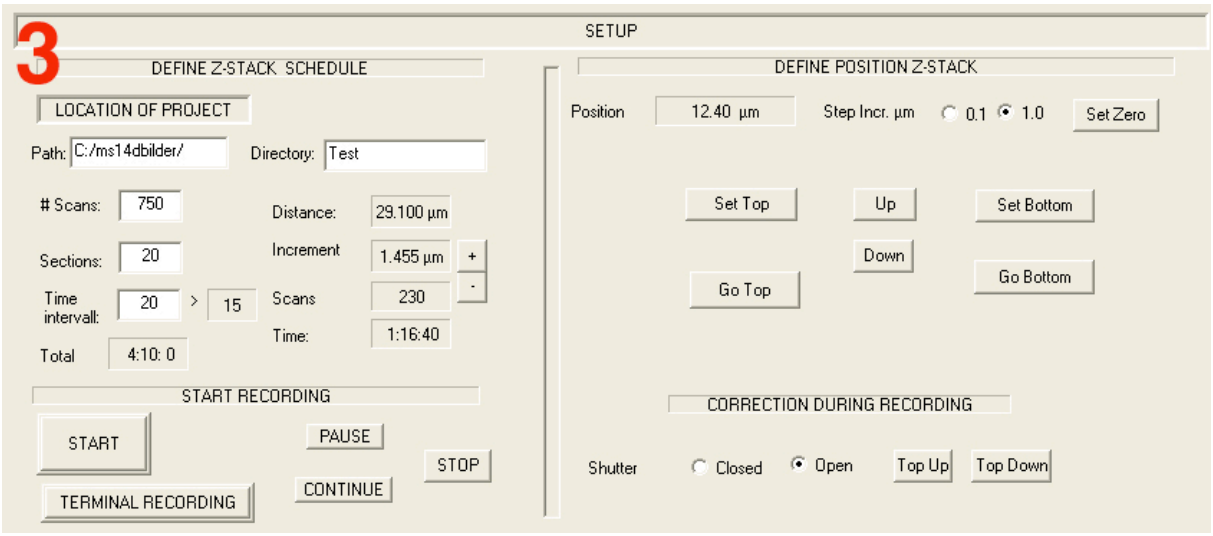
This box controls the resolution of pictures of a recording or single picture taken. Left are some preset sizes. 1376x1040 is the full resolution of the camera.

We now introduced a feature which permits to create a freely designed resolution matching the needs for a specimen. To enable this function the masurement window has to be switched on. Hold the right mouse button and draw a frame around the specimen. Activate "Cut" and you see the picture, which will be recorded. Activate "Zoom" and the picture will be zoomed into the Video window. This function is now extremely popular in my lab. It spares the user from moving the stage into a position that the specimen fitts exactly into the center of the preset window. But please stay close to the optical centre of the microscope.

Please be aware that using a 100x lens it does not make any sense to use the full resolution of the camera. A window of 640x480 pixels for one embryo will still guarantee that the full optical resolution is saved in the picture. Please match embryo size and the resolution of the pictures using the optovar of the microscope. The maximal magnification of the optovar multiplied by the magnification of the lens should nor exceed "aperture x 100". Thus, a 100x lens with an aperture of 1.3 allows a further magnification of 1.3. A 40x lens with an aperture of 1.3 allows further magnification of 3.25. To use the full resolution of the camera makes sense if you fill the complete field seen with the 40x lens with embryos, or several ones with the 100x lens. It does not make any sense to blow a single embryo so much up that it fits the full resolution of the camera.

Please be aware that using fluorescent light in conjunction with the optovar needs special care. The optovar consumes a lot of light if it is set to high magnifications. Thus it favourable to use magnifications below 1.0 in conjunction with small pictures e.g. 640x480.

Box 3 Setup



A recording consists normally of thousands if single pictures stored in a folder, which is used later with Biocell to analyse the embryo. In the left part of the box (LOCATION OF PROJECT) the location and name of the folder for the files for the project is defined. The path for the location of the project folder is set by default to the folder ms14dbilder on the hard disk of the computer. You may type in a different location if you wish. If you want a different path as default please tell me. In the field "Directory" (as a default the date is set) you must enter the name of your project/recording. This name for the recording is later the name of the folder found in the directory specified in the field "Path" in which the single pictures are saved during the recording. The pictures are named with the system X0000L00C1 (X"number of scans, "L" number of levels "C" number of channel).

Important: never push return after an entry, since this will close the corresponding window. If it happens anyway you can reopen the window by choosing it again in "Functions".

It is recommended to structure the project name as follows:

RS (initials of operator)_**strain name** (or whatever)_**1** (for first recording, 2 for second.....)

In principle you can enter 256 characters for the path and the project name. However it may be a good idea to restrict the project name to 50 characters. Only use letters and numbers and – o r_.

On the left of the field DEFINE Z-STACK you specify the number of z-scans you anticipate to be necessary, the number of focal planes and the time interval between the scans. As a default the values for a scan of a worm embryo are set.

In my experience at 25 ºC the time interval should not be longer than 35 seconds and 25 levels for a z-series are enough for a *C. elegans* embryo. A rule of thumb is that the increment between two focal levels should be around 1µm. An increment of 0.5

μm is to small you do not see a significant change of focus later with Biocell. If you use values larger than 1.5 μm you may miss small cells in late development. If an embryo develops slower the number of scans can be increased or if you want to leave and you know 600 scans are enough you can enter 600 and the recording will stop automatically after 600 scans.

Please note that if the time (recording) interval is too short the microscope will just keep going and you may not be able anymore to type a longer time in window, please try. We are afraid if the situation does not improve you have to stop the program using the task manager.

To save you from this situation the program estimates the time necessary for the number of scans you specified and the estimate is displayed to the right of the "Time interval field".

Then move on to the field DEFINE POSITION Z-STACK.

- Focus on the surface of the specimen using the microscope focus control or the "UP" – "DOWN" buttons.

- Press "Set Top" to set the top level. "Position" should now be automatically be reset to zero. If this is not the case you may use the button "Set Zero".

- Focus down by pressing "Down" until you are seeing the lower edge of the specimen (for *C. elegans* embryos "Distance" should be at least 25 μm). I usualy ad 2 μm, since later the embryo extends further down than in the beginning. If you moved too far you can move back up by pressing "Up". Above these buttons you can choose the step size executed at each click and read out the distance the stage moved. Click "Set Bottom" and return to top by using "Go Top". You can now check the setting of the bottom plane by using "Go Bottom" and return again to the top level by pushing "Go Top" again.

Now the increment and the depth of the z-stack is updated in the field DEFINE POSITION Z-STACK. Also the expected length of the recording and the time lapsed after starting are reported.

Activate the desired picture format from compressed to uncompressed or both in box The camera window Normal 8. If SIMI Biocell is used always use compressed (otherwise one recording will collect pictures to fill 10 Gigabyte on your hard disk). **If the hard disk gets full during a recording the program will crash. It will also crash during the first scan if you chose a very very long name for the project or the location of the ms14dbilder folder is not correct, or the folder is missing**.

Now everything is ready to press **Start**

in the box START RECORDING

Please note that you see a scan twice. First live and again when the pictures are saved after the shutter was closed. This saves "light time" to protect the specimen.

The progress and quality of the recording can now be checked "online" by opening the project with Biocell on a second computer.

After starting a recording the agar pad will usually shrink so it looks as if the embryo sinks down. You can observe this using Biocell or by opening the shutter in the box CORRECTION DURING RECORDING, the shutter can also be closed again. If the embryo has sunk down you press button "Top Down" several times until it is again in focus. A warning; you should not press it more than five times (which corresponds 5 times 0.1 μm) in one go because it will cause a jump in the records, which makes lineaging difficult. "Top Up" will move the embryo down if the top level has moved into the embryo. AS a new feature there is now also an automatic Focus available (see below).

In case you use the AUTOFOCUS function the microscope will take care of the Top position of the z-series but not of the depth of z-scan.

If it turns out that the lower parts of the embryo are not part of the z-series during the recording you can increase the increment by clicking in box DEFINE Z-STACK at "+" which will cause the z-series to move further down. Using the button "-" you can decrease the increment if the series runs below the embryo.

All parameters can be altered for any new requirements. In case the number of sections is increased the program will just add new focal levels with the increment displayed in the field "increment" thus also the "Distance" from the top to the bottom level will increase.

Please note that if you once have started a project in SIMI to analyse the recording during it is still running you have to change the number of levels in the *project.sbc* file using an editor (e.g. endnote). Otherwise you will not be able to view the new planes although they are there.

I choose this strategy because otherwise a big "jump" of focal levels would occur during lineage analysis. If you want to increase the number of levels but keep the distance please use the "-" button to lower the increment as described before.

If you want to execute changes within the program or at the microscope and you need more time than the 20 seconds or so, which remain in between two scans, you can pause the recording by clicking at "Pause" in the box START RECORDING. If you are done just press "Continue" and the recording will start immediately again with the old or altered settings. Be careful! In case you use START again the program starts again with numbering the pictures and will thus overwrite what you recorded before.

This feature is especially useful at the beginning of a recording when embryos have still large cells, which do not move significantly. At the bean stage (400 cells) a pause for more than 60 seconds will usually prevent lineaging after the pause.

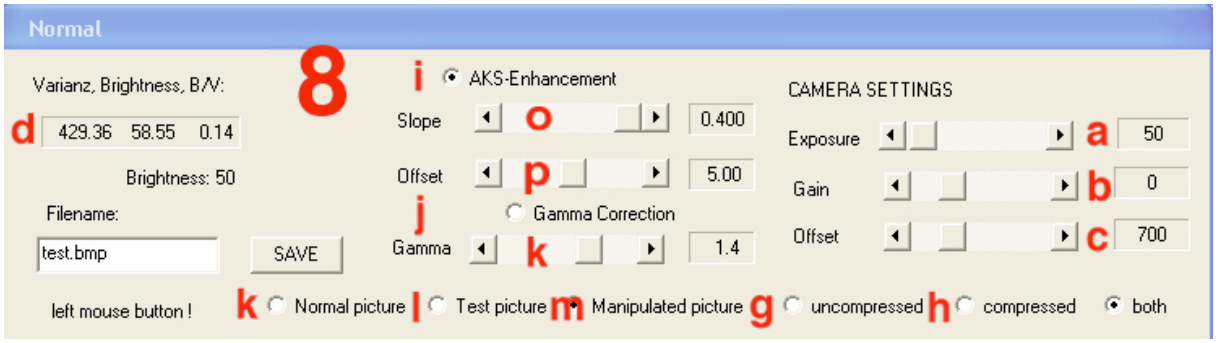
You stop the recording by pressing "Stop".

Normally you should stop the recording when the embryo starts to move because you will not be able to follow cells anymore once the embryo started moving.

In case you analyse mutants do not forget to record a z-series of the terminal phenotype after some hours by pressing the button "TERMINAL RECORDING". This will create a new folder with the name "project name"_t. Also with normal embryos it is a very good idea to write down in your lab book that the L1 larva hatched and looked normal.

All relevant parameters of a recording are now stored in two folders. One named recdata.log in the Project folder relevant for you. The second one "autofocus" goes in the ms14dbilder folder. This file is relevant for us in case you have complaints about the autofocus function.

CAMERA control and advanced video functions (Boxes 8)



If necessary adjust the exposure time of the camera in Window 8 (a) if you need too much light to see a proper picture (light should be less than 5, optimal is 3). The picture can also be improved by altering the setting "Gain" (b) and/or "Offset" (c). Get your own experience. The feature "Variance, Brightness, B/V" (d) will assist you to get an optimal picture by reading out the variance of the brightness values of the pixels and the mean brightness of the "measurement Window" (200 x 200 pixels). Pictures will be exposed properly if the brightness is around 40 to 90 (get your own experience, this depends on the brand and settings of the computer screen used). The ratio of Brightness/Variance represents the contrast in the picture. It thus helps to set the DIC contrast properly. A value of B/V of < 0.2 corresponds to a very good DIC picture, if you look at an upper plane of the embryo.

Taking pictures

If you want to take a picture just click with the left mouse button in the video window. The picture will be saved with the system time down to the second as the file name, which thus creates an unique document in the laboratory. Alternatively you may enter a file name in the field "file name" in the Camera windows 8 and save the file into the folder Pictures. You can save uncompressed or compressed or both types of pictures by selecting the corresponding buttons in the active camera windows.

Real time picture enhancement

Usually *C. elegans* embryos can be seen really well using just the proper Nomarski, light level, camera settings and an optimal stop field width. However, there may be situations where you are unhappy with the picture quality (bad mounting or if you use a different animal), therefore the program offers two "real time" picture processing routines. A gamma function (j) and a function I (RS) call "AKS Enhancement" (i) since Anja-Kristina Schulz made the algorithm.

This functions cope with the problem that an 8 Bit grey value picture has a much smaller dynamic range than a classical video picture on a video screen. The gamma function suppresses brightness (value >1) or darkness (value <1), which may be useful to dim down very bright or to brighten up very dark structures. The AKS function is much more flexible and better than the gamma function, with the two

parameters you can manipulate the representation of the pixels like in the "graduation curve" of Photoshop. For example you can make an S-curves with different shapes. Anyway the theory may be not that important here you will see that you may get very nice improvements of the pictures. Please be aware that these manipulations require complex calculations on every pixel of the pictures and that this slows down the picture representation and increases the saving time during the recording. So if you use the functions during he recording check before that the scan does not take longer than the length of the recording interval (for disaster management see above).

Since it is sometimes very difficult to decide whether a picture manipulation indeed improves a picture we made a function "Test picture". When the corresponding button (l) is activated the picture is split in the middle and the manipulation is only applied to the upper part. Thus the consequence of the manipulation can be compared to the unmanipulated picture. Now you can use the gamma or the AKS function to find out which one gives the better result. With the gamma function you pull the slider (k) to alter the picture. With the AKS function you may play with both sliders (o and p). In my experience pulling the slider (o) to the left improves the clarity of the picture very much. Please play with both functions around to get your own experience. If you want to use the manipulation to take a picture or a recording activate button "Manipulated picture" (m) to apply the manipulation to the whole picture. If you could not improve the picture significantly return to the "Normal picture" (k).

Multichannel Recordings (Boxes 4 to 6 and window 8)

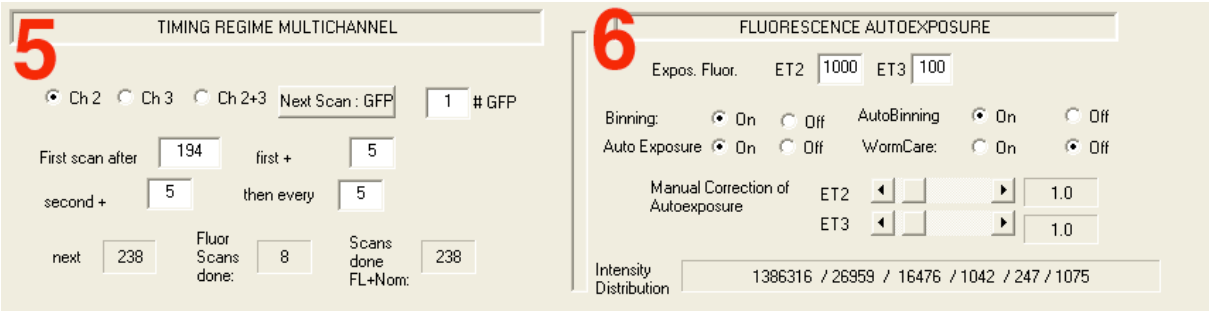
The program was especially designed to determine the expression patterns of fluorescence labelled marker genes on the single cell level. The principle is that 4D Nomarski recordings are made to document development and then, when the marker gene is expressed, single z-series with the fluorescent optics are inserted in the recording. Since we learned that it is very difficult to assign fluorescent signals to specific cells SIMI°Biocell has now a multi channel capability. This allows to look at multi channel records in many different ways but most importantly channel overlays can be crated in real time, which solves the problem to identify fluorescent cells (see Tutorial SIMI° Biocell).

This "Dartmouth version of the program is designed to operate a LED light source in this case the Zeiss Colibri illumination.

In Box 4 channels are selected and the fluorescence filters are assigned. Channel 1 is reserved for DIC optics. Channel 2 automatically uses a GFP filter, channel 3 a red filter, which we assign(ed) according to your choice. Of course you can assign filters also freely to the two channels. If you select a certain filter manually this will be memorised by the program until it is started again. The program links the filters automatically to the appropriate LED colour. For viewing specimens manually the sliders for the appropriate colour has to be activated with the mouse pointer to switch the lamp on. Afterwards intensity can be regulated with the slider. Picture can be optimised using the same features introduced for the camera window 8 "Normal" but which have to be used now in the "Fluorescence" Window. The two windows exist to allow the program to memorise different settings for different channels. They automatically come to the surface when channels are changed. However, you can also activate them with the mouse pointer. This allows to manipulate the settings of an fluorescence channel during a recording using the DIC channel. In this window the fluorescence shutter can be opened and closed manually and the LED can be switched on and off and the intensity can be regulated.

Easy change of channels

A special feature, which will be available soon in an update, makes it very easy to search for embryos expressing a marker gene looking through the microscope. When the mouse pointer is located in (will be specified later) of the main window, the microscope will change channels back and forward if you click the right mouse button.



TIMING REGIME MULTICHANNEL (BOX 5)
This box allows to either trigger a z-series in a fluorescent channel manually (Next Scan GFP) while a 4D recording is running or to set up an automatic regime. In the field # GFP you can specify how many z-series will be recorded in the activated channel. Of course any colour will function here, the names is a left over from old times, where only GFP was used. You can space the automatic fluorescent recordings in the beginning unevenly. Also Next Scan GFP still works when a regime was set up. The box also informs you about the number of scans already taken ...

Depending on the strength of the expression the Exposure time may have to be increased considerably. For recordings the exposure is limited to 1000 ms per frame. However, for manual viewing you can extend it to 4 seconds.

If you work manually you can take **pictures** as described before.

FLUORESCENCE AUTOEXPOSURE (BOX 6)

When I started to work with two channels it took not long to realise that expression of marker genes does not only change as development proceeds, but also from embryo to embryo. Thus, it was almost impossible to determine the expression pattern during a 4D recording. Pictures were always under or overexposed. Therefore, an automatic exposure algorithm had to be implemented. Some programs use a very simple method in which the exposure time is used, in which the first pixel is completely white (value 255). This may work sometimes but not if there is fluorescence in many or all cells - the embryo will be completely white. Analysing pictures I learned that in good pictures the signals range lies between grey values of 120 to 200 or so and the background is "black". Thus I designed an algorithm which measures the ratio of dark pixels to "white" pixels. And to my astonishment it exposes embryos always "right", independently of the number of cells which express the marker and the intensity of the expression. New features now even handle the camera settings automatically. Very important is the setting of the gain for the camera. A gain of zero will result in a nice "soft" picture but the sensitivity is very low. Therefore, a gain of 2600 is usually appropriate for fluorescence. The camera is very sensitive but still pictures differences in intensities. This setting is still appropriate if the observer can see some signal by eye. A setting of 3800 makes the camera still ten times more sensitive and it is possible to see the embryo with an exposure time of one second because of the auto-fluorescence in the GFP channel. Thus this setting is the practical limit for the sensitivity, which, however, is approx. 100 times better than a classical confocal

microscope. With this gain one can record expression patterns even when the human eye sees only a black hole in the microscope. Therefore, we implemented two new functions (not shown yet in Box 6). The button Autogain will switch the gain from the normal setting for fluorescence (2600) to 3800 after an exposure time of 1 sec did not produce a signal. This will then be used in the next fluorescent scan. If expression increases significantly the machine will return to the lower gain. By activating the button "Low Expression" the microscope automatically uses the high gain. But now a more detailled description of the functions in the Box 6,which only apply during recording with the exception of the Binning on/off, which can also be used for manual observations.

Binning activates the merging of 2x2 pixels, which increases the sensitivity by a facto of four. The resolution drops to 688x520 pixels. We rescale the picture to match the full resolution again and - to be honest - we never saw much of a difference.

Autobinning will activate binning if the exposure time exceeds 500 ms, when binning is switched off.

Autoexposure ,when activated, instructs the microscope to determine the proper exposure time before each z-scan using fluorescence optics.

The microscope will start with the time (ms) you entered in the fields behind **Expos. Fluo. ET2** is the time for channel two **ET3** for channel three. 100 ms is the default setting. After an automatic exposure the new exposure time will be displayed. In case **Autogain** is switched off you can produce the Fluorescence Camera window (8) and manipulate the gain. For very short exposure time you may decrease for very long ones increase the gain.

Wormcare was introduced for the Colibri version of the program. It limits the radiation damage when short exposure times are used further. It takes care of the rather slow electronics for the LED control.

Autogain will increase the sensitivity of the camera after an exposure of 1 sec failed to see a good fluorescence signal.

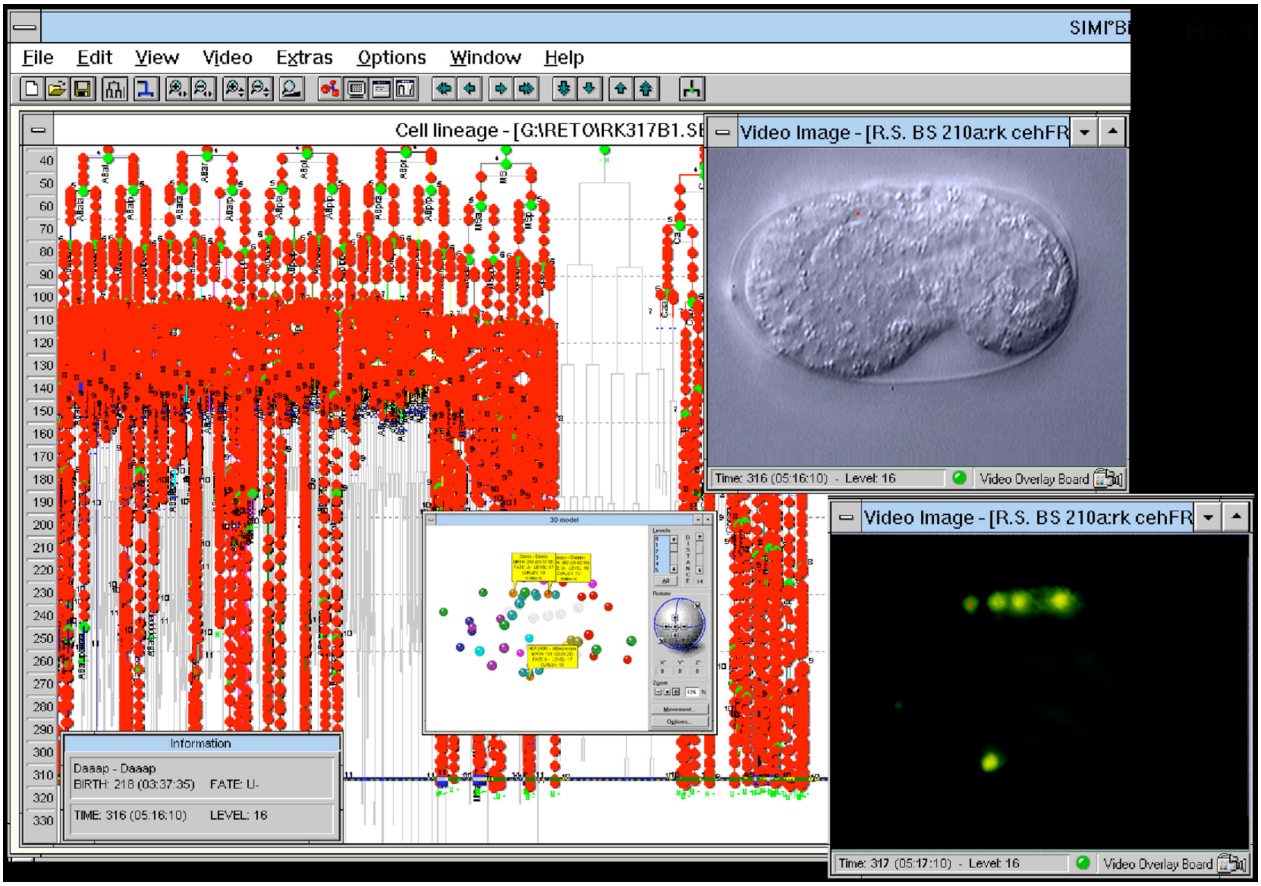
Low expression (will be available next update) presets the gain of the camera to 3800. Thus, it is avoided that the first scan will be underexposed till the autogain function takes control for the next scan.

Manual Correction of Autoexposure. In case, that in contrast to my expectations, a certain transgene is notoriously under or overexposed you can correct the exposure time determined by the microscope. We never used it so far.

Intensity distribution. The field displays the pixel intensities during the automatic exposure procedure. Ask me about the details in case you care.

Do not forget to monitor and correct the recording while it is running as described before. Although a log file now exists useful to write a protocol containing

all relevant parameters used for the recording. I hope you will soon have an analysis of an expression pattern as shown below using Biocell.



Please do not hesitate to contact us if you have problems or to suggest improvements of the 4D-program.

Good luck!

Ralf Schnabel and Christian Hennig