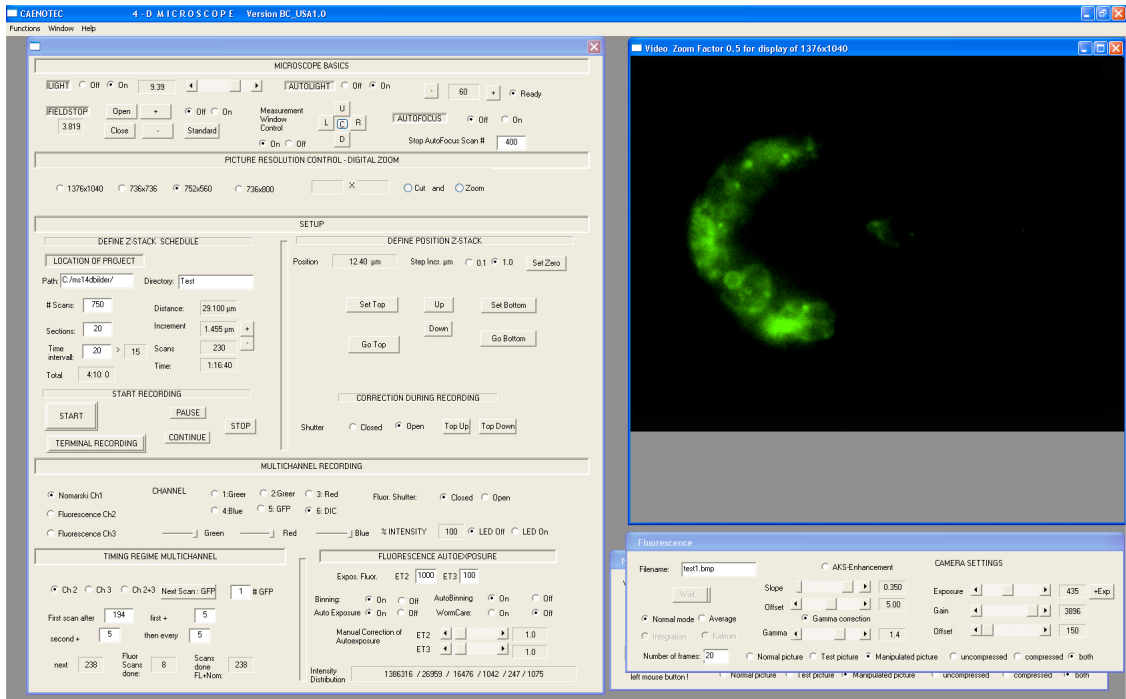
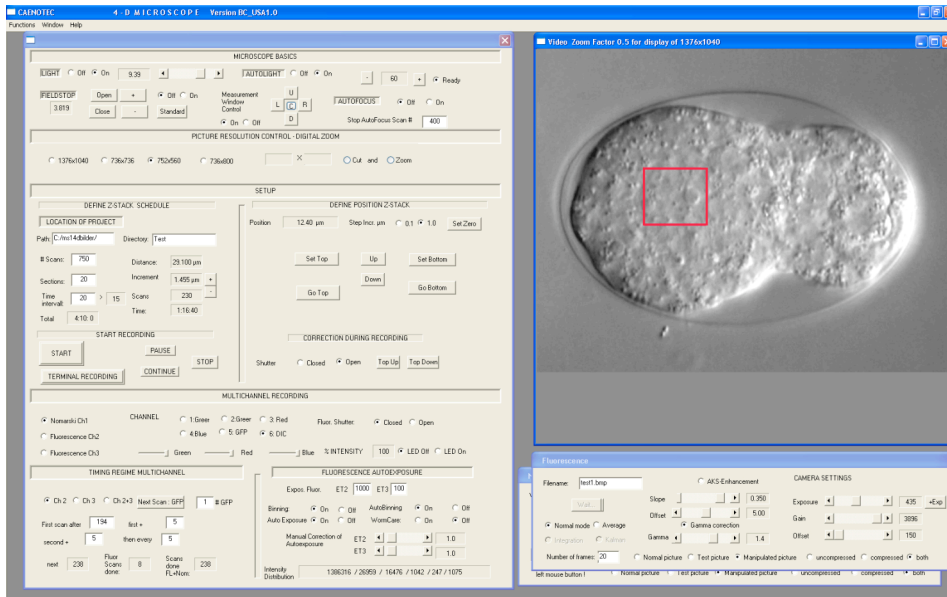


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## Time to live

## A Multi-Channel 4D-Microscope Software



Control surface of *Time to live* in the "bright light" (DIC) and fluorescence channel (GFP) mode.

## Résumé

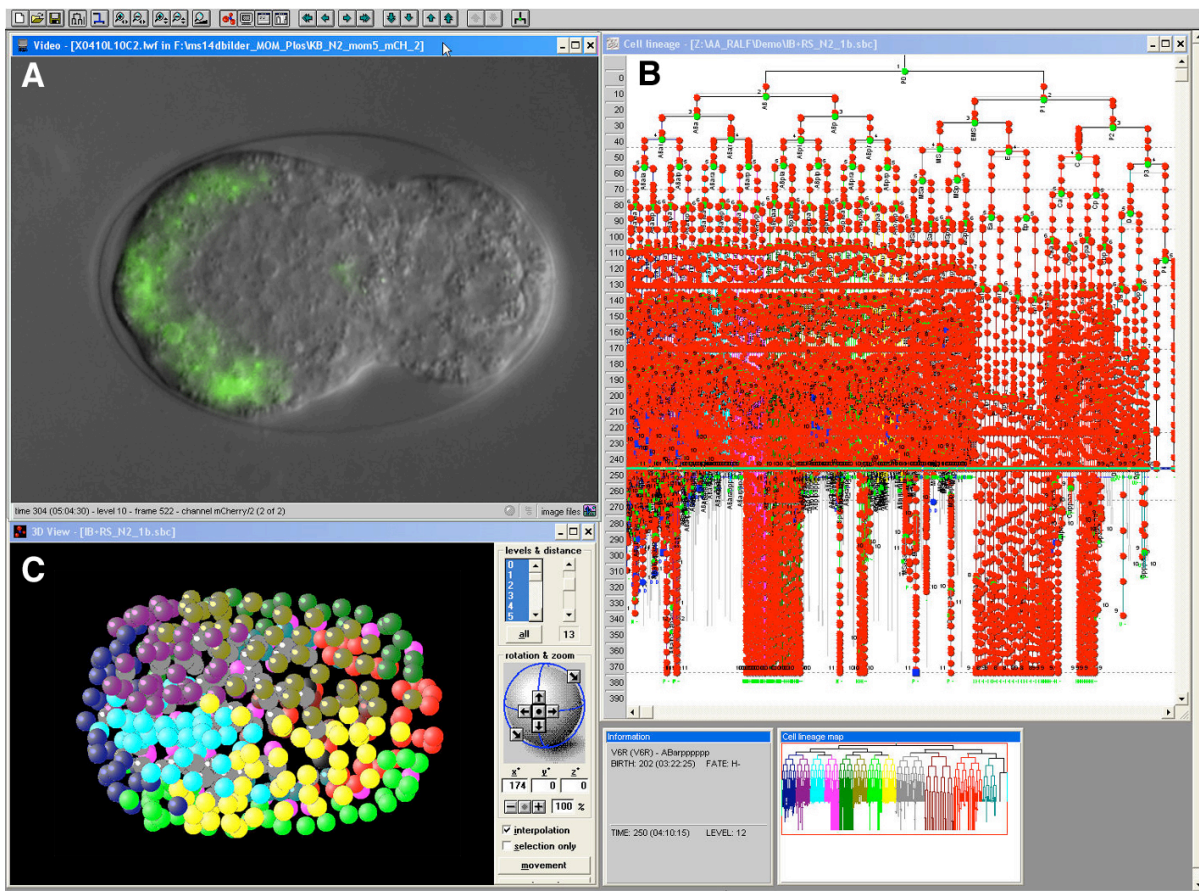
*Time to live* is a software to control the acquisition of 4D-multi-channel microscopic records. The setup of recordings is extremely convenient and fast. The control software in combination with very fast high resolution light microscopes (Zeiss Imager M2), modern LED fluorescent light sources (Zeiss Colibri) and extremely sensitive video cameras (PCO Sensicam) allows now the recording of many thousand fluorescent pictures without damaging cells by photo bleaching. Modern biology more and more turns to system approaches requiring intensive screening of cell, mutants and molecules. The implemented automation of the recordings allows now a 24h a day use of the microscope and thus a high turn over of experiments.

### **Long term analysis of cellular processes by live imaging (4D microscopy) Biological relevance and technical problems and solutions**

Modern biology currently experiences a revolution in the analysis of processes governing the differentiation, specialisation, adaptation and homeostasis of cells in culture, embryos or even organisms. Instead of only analysing the starting and then terminal states of processes in "still pictures" – by taking and "killing" samples for analyses" - the continuous analysis of the whole process comes now into focus. This is technically a major challenge, since it requires the live imaging of molecular markers and sensors in cells with extreme resolution and sensitivity, for long periods of development.

The development of fluorescent proteins now allows the analysis of the expression and even the subcellular localisation of proteins in cells using microscopes with fluorescence optics. This resulted in an unprecedented advancement of the field, but still mostly using more or less "still pictures". The problem is that classical video microscope setups, using either mercury (Hg) or Xenon lamps for fluorescence, kill cells in less than 60 sec observation time (50 to 100 pictures). This precludes live imaging over significant time periods. Of course it is today possible to record videos with hundreds of pictures using very expensive instrumentation like (two photon) confocal or spinning disc microscopes. The disadvantage of these methods, however, is that the general context of cell or embryonic development gets lost, since it is not possible to follow for example the genealogy of cells in fluorescent pictures showing only a limited number of cells or few structures within cells.

Differentiation of cells in culture takes several days. The development of a *C. elegans* embryo needs 10 hours,. The complete documentation of these processes requires the acquisition of ten to twenty thousand pictures. In Figure 1 it is shown, using a *C. elegans* embryo, how records like this - including fluorescent pictures of the expression of a GFP marker gene - can be analysed using the data base SIMI°Biocell (SIMI Unterschleißheim, Germany [www.simi.com](http://www.simi.com)).



### Fig. 1 The data base SIMI°Biocell

(A) The video window shows the overlay of the expression of a fusion gene of MOM-5::GFP (Frizzled) with the corresponding DIC picture of the *C. elegans* embryo just when morphogenesis of the embryo is initiated. This is one of 18.000 pictures documenting the whole development of the embryo. (B) The lineage analysis of all cells at that stage shows the position of the nuclei (C). With such analysis the identities of all cells expressing the gene can be determined throughout development.

As already mentioned it is not possible to acquire so many pictures only with fluorescent optics. Therefore, we developed the strategy to document the general development with a low level of normal light using DIC optics. The expression of the GFP marker gene is assed approximately every 10 to 15 minutes during the 8 h of development of the worm embryo by switching to the fluorescence optics. This requires 25 to 50 z-scans with 25 pictures each, thus up to 1250 fluorescent pictures. This results in the precise determination of the expression and behaviour of the marker gene in the context of the development of the cells and the embryo (Fig. 1). This is also possible when the expression of the marker gene produces only a signal just above the natural autofluorescence of cells, which represents the worst case scenario. It also shows that the setup as the highest theoretically possible sensitivity. A "save" upper limit should be 4000 fluorescent pictures or 160 z-scans with 25 levels each. A relatively strong expression of the marker gene (visible by eye) will still allow a much higher number of pictures. However, a second limit to the number of pictures, which make sense to be recorded, is the bleaching of the fluorescent signal itself which gets usually very faint after approx. 2500 seconds light time.

Such schedules are possible, since we discovered that photo damage of cells is non-linear in relation to the light dose used for fluorescence. We determined the "survival time" (exposure time in seconds) to assess the photo damage caused by a certain light intensity. The survival time first increases slowly, from approx. 100 sec, and linearly when the light intensity of the Zeiss Colibri LED lamp is reduced from the highest intensity available (100%). When the light intensity is reduced from 15% to 7% it increases from 1300 sec to 1800 sec but jumps up to 8000 sec if it is reduced further to 3% intensity. This intensity still suffices to detect any fluorescent signal above the autofluorescence of cells when a high quality video camera is used (we recommend the sensicam from PCO, Kehlheim, Germany). These very low signals cannot be seen by the eye. Cells (embryos) exposed to a light dose corresponding to 50% of the killing time will develop normally.

A very important aspect for increasing the analysis time for the fluorescent signal was not only the reduction of light intensity, but also the reduction of the time cells are illuminated but no picture is recorded. Two time components of a recording are important in this respect. First the time while the microscope is in action, for example the focus is changed and light is shining but no picture is taken and then the process of taking the picture itself. The first problem could be principally solved by using the "fast" light shutters (<200ms) of modern microscopes like the Imager M2 from Zeiss. However, they work only with a frequency of 2 Hz which would mean that 25 pictures in a z-stack would require at least 25 sec for controlling the shutter. This would cause a delay of approx. 60 sec when a z-stack is taken with the fluorescence optics. This delay is too long to match the normal 35 sec cycles of the DIC recordings and thus cells cannot be followed any more unambiguously. Here is the capability of the LED lamps (Zeiss Colibri) to flash synchronously with the exposure of the camera (TTL control) a very nice feature helping to save time. Soon we will also implement an automatic light management in *Time to Live* to make sure that the lowest possible fluorescent light intensity is used for the recordings.

When we used the first LED illumination for live imaging we observed a much longer surviving time of cells than with a Hg lamp. We attributed this to the much purer light emitted by LEDs (for eGFP  $470\pm 15$  nm) compared to Hg or Xenon lamps. However, careful investigations now showed that it is only the much lower light intensity of the LED causing the survival. However, LED lamps are still very useful because of the flashing mode, the very easy intensity control (software controlled) and the very low costs in the long run.

## **Summary of important features:**

**Easy and fast setup of recordings.**

**Assisted set up of optimal DIC brightness and contrast.**

**All relevant parameters of the recording can be changed easily**

**All parameters used during a recording, including those changed automatically, are saved in a log file.** This permits semi quantitative evaluation of for example expression intensities of marker genes.

**Loss free compression of pictures by a factor of ten.** A full recording can be stored on a CD.

**Automatic focus control.** Top level remains constant.

**Automatic light intensity control.**

**Multi-channel fluorescent optics recordings.**

**Automatic exposure of fluorescent pictures.** The algorithm exposes properly also in the extreme cases that all cells or only one is expressing a marker.

**Schedule for timing and use of different channels automatically executed.**

## **Long term live imaging**

**Preset parameters for low/high expression of marker genes**

**Automatic control of camera sensitivity to save light time in case expression is changing during development.**

**Flashing mode in conjunction with the Colibri (Zeiss) illumination.**

**Automatic management of fluorescent light intensity.**

**Automatic calibration of fluorescent light intensity for specific specimen (upcoming)**

**We are very flexible in implementing reasonable changes of the program for special needs.**

## Some selected references

- Bischoff M, Schnabel R (2006) A posterior centre establishes and maintains polarity of the *Caenorhabditis elegans* embryo by a Wnt-dependent relay mechanism. **PLoS Biol** 4: e396.
- Bischoff M, Parfitt DE, Zernicka-Goetz M (2008) Formation of the embryonic-abembryonic axis of the mouse blastocyst: relationships between orientation of early cleavage divisions and pattern of symmetric/asymmetric divisions. **Development** 135: 953-962.
- Hejnal A. & Schnabel R. (2005) The embryo of the eutardigrade *Thulinia stephaniae* has an indeterminate development and the potential to regulate early blastomere ablations. **Development**, 132, 1349-1361
- Hoeppner DJ, Hengartner MO, Schnabel R (2001) Engulfment genes cooperate with *ced-3* to promote cell death in *Caenorhabditis elegans*. **Nature** 412, 202-206
- Hunt-Newbury R. et al., (2007) High-Throughput In Vivo Analysis of Gene Expression in *Caenorhabditis elegans*. **PloS Biology**, 5: e237
- Kaletta T., Schnabel H. & Schnabel R. (1997) Binary specification of the embryonic lineage in *Caenorhabditis elegans*. **Nature**, 390, 294-298
- Kinchen JM. et al. (2005) Two pathways converge at CED-10 to mediate actin rearrangement and corpse removal in *C. elegans*. **Nature**, 434, 93-99
- Ravin R, et al. (2008) Potency and fate specification in CNS stem cell populations in vitro. **Cell Stem Cell** 3: 670-680.
- Schnabel R. & Schnabel H. (1997) *Hox*-genes misled by local environments. **Nature**, 385, 588-589
- Schnabel R., Hutter H., Moerman D. & Schnabel H. (1997) Assessing normal embryogenesis in *C. elegans* embryo using a 4D-microscope: variability of development and regional specification. **Developmental Biology**, 184, 234-256
- Schnabel R. et al. (2006) Global cell sorting in the *C. elegans* embryo defines a new mechanism for pattern formation. **Developmental Biology** 294: 418-431.