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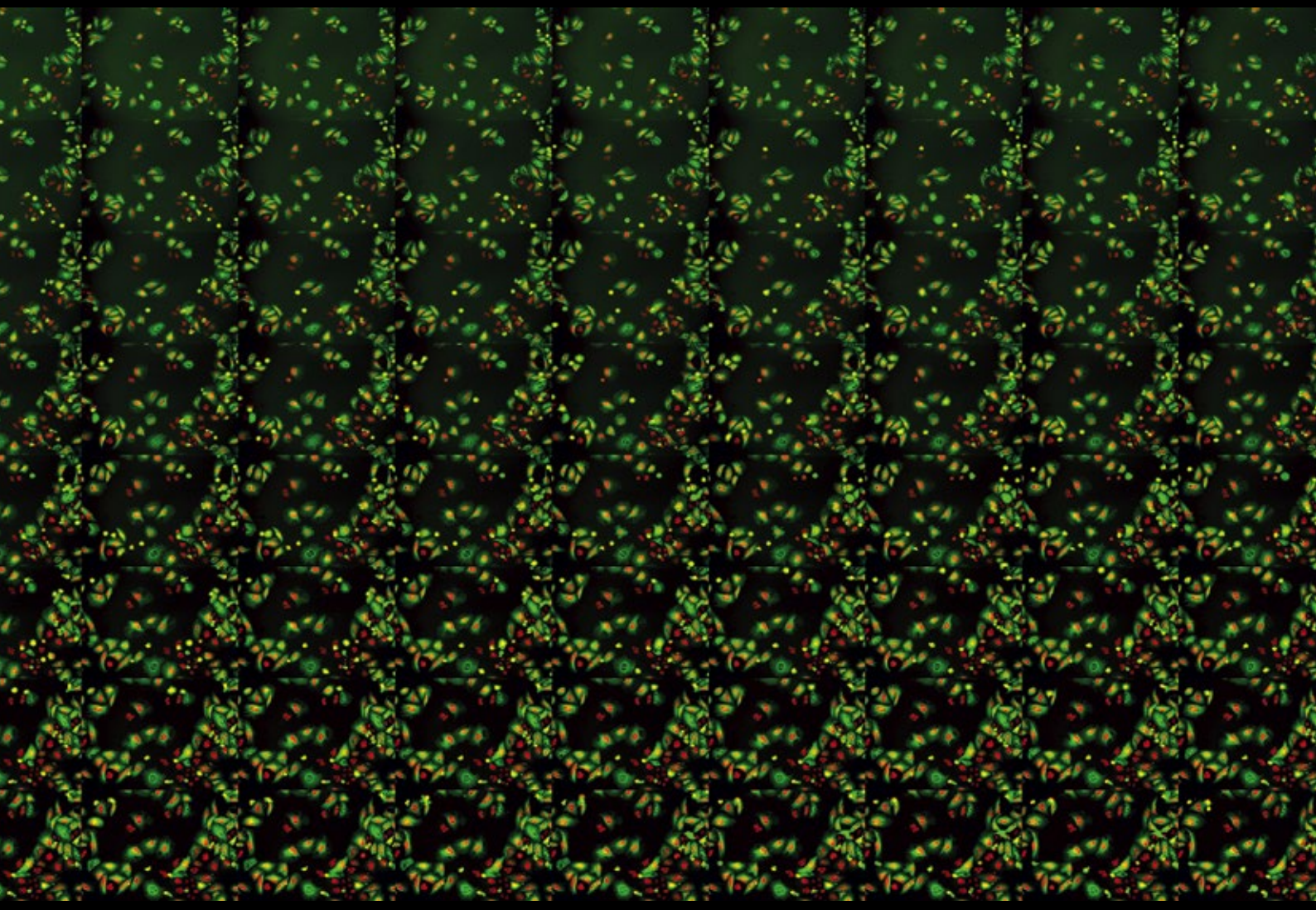
Automation in MICROSCOPY



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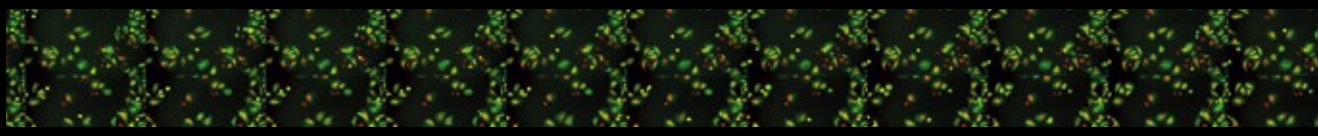
Dear Reader,

Often in life sciences research, the data you are after will only be revealed through multiple runs of experiments or complex assays. Automation and parallelization of your imaging can be the only way to get there. Observing live samples over a number of days or imaging lots of multiwell plates really puts your microscope through its paces. To get reproducible, unbiased data, you must control environmental conditions such as light, temperature and CO_2. Especially for demanding long-term timelapse fluorescence imaging, you need gentle illumination and a stable platform. This collection of White Papers aims to give you insights into the challenges and recent developments in the ever-expanding world of automated microscopy solutions. Automation can simplify your laboratory setup and make your work more efficient and comfortable.

Dr. Bernhard Zimmermann
Senior Director Business Sector Life Sciences



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Microscopy and Automation – The Challenge

The Long Way from Motorization to True Automation in Imaging

Horst Wolff

Rapid developments at all levels of microscopy, such as contrast, illumination, resolution, signal detection and data processing have occurred over the last decades and there is reason to expect that these advances will continue. However, severe limitations in accuracy, reproducibility and throughput are caused by the involvement of humans in all steps of the imaging workflow. It also poses a significant burden and workload for the researcher. To improve this situation is the biggest challenge in automation.

Image: ZEISS/GraphicStock

Why Automation?

Biological microscopy and light microscopy in particular, is an essential tool of modern molecular, cell and developmental biology laboratories. It spans applications in basic research, preclinical and even clinical domains. Microscopic applications in imaging are constantly growing and enable insights that are impossible without microscopic imaging. Recent additions to the biologists´ toolbox like CRISPR/Cas9 and related methods, will continue to drive new discoveries. Rapid developments at all levels of microscopy, contrast, illumination, resolution, signal detection and data processing have occurred and there is reason to expect that these advances will continue.

However, for almost all of today´s imaging systems, one aspect is still the same as 30 years ago, the human factor. To limit human labor to the areas where it is really useful and adds value is the biggest challenge in automation. Limited human resources and human error cannot only be problematic in clinical and diagnostic workflows, but do also pose problems in research environments, which automation can help to resolve.

What does automation mean? Are all fully motorized microscopes of today also automated imaging systems? This article attempts an approach to what automation in imaging really means. It is more than just motorization and enhanced throughput.

A Well-Known Situation

Imagine you have spent days, or even weeks or months, to get a hold on the sample that now is in front of you on a slide or petri dish. You may have had to generate transgenic animals or stably transfected cell lines to obtain the tissues or cells you want to inspect with your imaging systems. Optimizing the cultivation conditions of your cells or model organism might have taken months during your project, and treatment with special supplements or substances could be expensive and cumbersome.

You have set up your imaging system to observe your specimen overnight and manually screened and selected a position within the sample carrier that seemed promising. Then you start the imaging experiment. An experiment that just has to go well, because you need the data. You may need the results either for your lab meeting, as the group leader is putting pressure on you to eventually generate valuable results. Or it is an integral part of a scheduled talk on a conference, or of a manuscript or grant application, that has to be submitted soon.

What you experience on the next morning when you review the results from the overnight time series will often tear you up. Although you had configured a multi-position time-lapse experiment, none of the positions yielded suitable results. In some cases the cells of interest just died after several hours, in others they went into a resting state, and some of them have left the field of view due to unusually high cell motility. Let alone the numerous cases, where the system has kept some debris in focus, but not the objects of interest.

Almost all researchers know these issues. Although the situation has improved over the past few years (e.g. with today´s state-of-the-art multi-position imaging, compared to single position-experiments 10 or more years ago), the demands, expectations and standards for publications have risen even more during the same period.

What can be the solution to this and similar problems? Sleeping next to the microscope, to supervise and adjust the imaging? More motorization, or faster imaging methods to capture even more objects of interest? Or rather intelligent automation that does not just generate huge amounts of data, but exactly the data you need?

SETUP	
1	*Identify and calibrate the sample carrier (e.g. slide, dish or multi-well plate)*
2	*Configure channels/tracks, according to your dyes, your fluorescent stainings or contrasts*
3	*Adjust additional imaging parameters (e.g. z-stack, laser-power, time intervals)*
4	*Spot and mark the right location(s)/object(s) of interest in your sample or define an area*
ACQUISITION	
5	*Find and keep the focus automatically*
6	*Stop acquisition when imaging does not yield predefined quality criteria*
7	*Identify events that follow certain criteria and adapt imaging parameters*
8	*Continue acquisition until a statistically relevant number of events have been acquired*
PROCESSING/ANALYSIS	
8	*Quality inspection of images and appropriate correction (e.g. for drifts, shifts or spectral cross-stalk) and processing (e.g. background correction, deconvolution)*
9	*Meaningful image analysis based on information about sample type, stainings, morphology and imaging dimensions*
EXPORT/REVIEW	
10	*Sort and categorize images and data based on quality criteria (including discarding them, if they do not meet predefined criteria)*
11	*Report generation containing images, analysis results and meaningful meta data*
12	*Generate movie clips, 3D renderings and even publication-quality figures*

Table 1: *A selection of workflow automation-targets in imaging-experiments.*

Before we approach the answer to these questions, let´s have a look on what Automation is, and what it is not.

What is Automation?

Automation or automatic control, is defined as the use of control systems for operating equipment and driving processes, e.g. in factories, at home, or in office environments.

In contrast to motorization, which just describes any item that contains a motor, automation implies, that minimal or reduced human intervention is required. The biggest benefit of automation therefore is, that it saves labor. Furthermore, it can be used to save energy and materials and to improve accuracy, reproducibility, quality and precision.

As for other automated systems, Automation in Microscopy is achieved by various means, including mechanical, electrical and electronic devices, sensors and computers, usually in combination.

What Does Automation for Microscopic Imaging Mean?

In the mid-1990s, microscopes evolved into so-called imaging-systems, which were mainly characterized by a switch from analogue to digital cameras, by step-by-step motorization of components, and advances in software-control of components and detectors/cameras.

During the last one or two decades, advanced data processing and storage, as well as robotic integration (fig. 1) was often added to motorized microscopes to further boost throughput and efficiency of imaging systems.

This did already help in achieving some of the automation goals, at least partially. These imaging systems save the researcher labor and time and improve accuracy, quality and precision of imaging experiments, and last but not least reproducibility. When Nature quizzed about 1500 scientists towards reproducibility, the majority of participants agreed that "there is a ‚crisis' of reproducibility". "Low statistical power" or "poor analysis" were among the considered most responsible factors underlying the reproducibility problem [1].

But is a so-called "fully automated imaging system" really fully automated, when you have to interact with the system at many points? During setup and conduction of experiments the operator is often still required and much time has to be invested to acquire, process, analyze and export images and data that fulfill high research and publication standards.

What are the steps during a typical workflow at the imaging system that need a high degree of automation to perfectly support the researcher?

Some of the more advanced microscope systems on the market will offer features from Table 1. An example for a proper realization of many of these features is ZEISS Celldiscoverer 7, which is fully controlled by the ZEN software, and exhibits a variety of automation features, that render it a truly automated system. It can, besides many other capabilities, identify the type of sample carrier, measure bottom type and thickness and also calibrate the carrier. All without the user having to interact with the system or even knowing what steps are currently automatically undertaken to set the stage for the imaging experiment. The software will then for example carry out screening of a large area at low magnification, for specific objects (rare events), that are automatically acquired at high magnification in 3D over a longer time-period (fig. 2).

Of course automation does not stop at this point, but the above steps alone save the researcher several minutes or even hours each time a new sample is inserted, let alone the hassle that is avoided.

Desirable Automation Features

There is a number of features that a researcher should look out for, when aiming for an increase in automation.

First and foremost a proper integration of all motorized components, sensors and input devices into a software environment is essential. State-of-the art machine learning, object recognition and advanced processing that can interact with, and influence, the acquisition engine, is the basis for successful imaging experiments of the next generation. Ideally the software does not only allow pre-made configurations, but also has well-documented interfaces to include new executables and code snippets, and lets the user edit and complement databases for dyes, hardware etc. Only then, the most recent innovations from the scientific community, can boost intelligent automation when needed.

Apart from the software, some well-known options can be of high value, such as a sample preview- or overview-camera, a hardware-based autofocus and automatically adaptive optics. Integrated barcode-reading can be a real game-changer, when barcoding has already been introduced in the research lab environment.

What is true for software, also applies to hardware: interfaces to interact with newest hardware and accessories, such as trigger out- and input and well-documented optical connections, are crucial and will make any automation effort more seamless and successful.

Fig. 1: *Proper integration of robotic loaders into imaging systems can boost the degree of automation and thereby throughput. However, this is only one automation aspect and robotics must work seamlessly together with all other components of the system.*

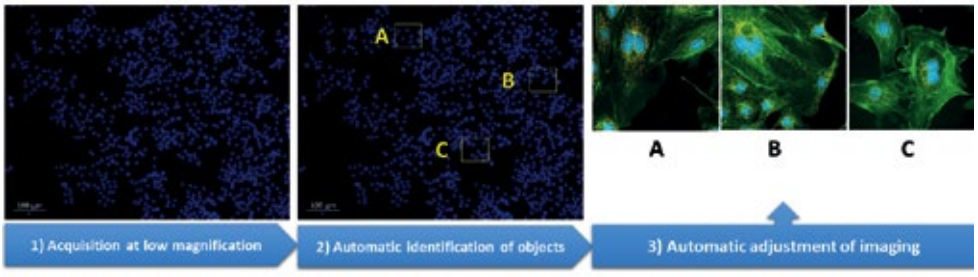

Fig. 2: *Rare event detection. A large field is acquired at low magnification and with only one channel (in this case DAPI-stained nuclei). Based on automated image analysis and adjustment of imaging parameters, objects of interest are acquired at high magnification and multiple colors (or time points and z-sections). With this targeted image acquisition, the researcher saves a lot of time and does not have to find spots in the sample by visual inspection anymore.*

VITA

Horst Wolff

Horst Wolff has studied biology and obtained a Ph.D. in cell biology and virology. As a researcher he has focused for almost 10 years on developing cell-based assays for high-content analysis and screening projects.

At Carl ZEISS Microscopy he has started as a product manager for imaging software and has since worked in several positions. Currently he is responsible for managing a part of the ZEISS Life Sciences Research portfolio.

Any Threats?

A maximum of automation in every aspect might not always be desirable. In some cases, it will be tempting for the researcher to not think about the experiment and leave it all to the machine. However, machines are developed and programmed by humans who can also make mistakes. A wrong entry in a dye database can for example lead to an imaging configuration that produces crosstalk that is mistaken as a valid signal by the researcher. A system might be properly configured for one kind of experiment to discard and even delete useless images. However, for another kind of experiment or another user, this could result in loss of precious data, if not adapted in the right way. The same is true for image analysis. Automated exclusion of certain objects from the analysis will be essential for one type of assay, while it will remove all of the information from other assays.

Despite all trust in technology and automation, reviewing configuration and setups and carrying out quick plausibility checks from time to time can therefore be very valuable.

Outlook

Certainly automation does not just begin at the imaging system and does also not stop after images have been acquired. The challenge to remove the need for human intervention in imaging are very similar to the challenges in laboratory automation in general.

Some 20 years ago, millions of samples per year and per lab were required, that investment of a laboratory automation environment could be justified. With increasing standards in documentation and reproducibility, even more interdisciplinary research projects and continued short-term researcher contracts, automation also enters the realm of smaller labs. Recent technologies like Smart Connected Products and the Internet of Things have the potential to change the game completely. Microscopy must then be ready to play this game new.

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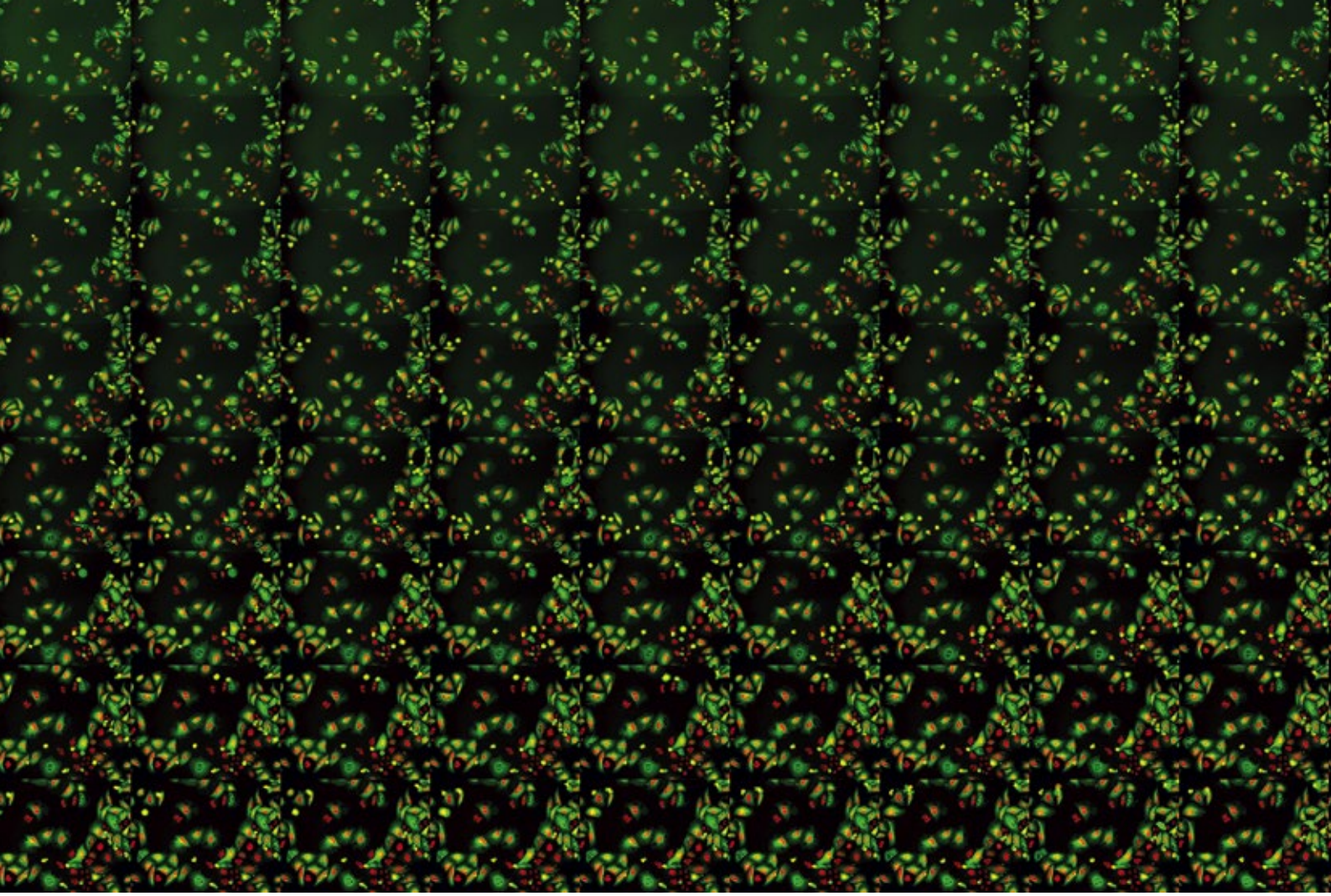

High-Throughput Live Cell Imaging

New Opportunities and Challenges

Aliaksandr Halavatyi[1], Beate Neumann[1], Rainer Pepperkok[1], Sabine Reither[1]

High throughput microscopy of fixed samples has been extensively used in the past to characterize gene function at the genome scale with excellent single cell resolution. High throughput live cell imaging, which can provide essential information about system dynamics in single cells bears additional challenges and has thus been used in only a few cases for genome scale experiments. Here we describe latest developments of the technology focussing on high throughput live cell imaging and feedback microscopy with ZEISS Celldiscoverer 7 and LSM 780.

Introduction

Since the sequencing of the first human genome, life sciences has undergone drastic changes resulting in exciting opportunities surrounding a more comprehensive understanding of the basic molecular mechanisms of life and disease. Genomes and their variations can now be determined at affordable prices in a relatively short time. Together with the latest genetic technologies to perturb cells and organisms in a gene-specific manner, this has now opened the possibility of conducting large scale genome-wide projects aimed at understanding the function(s) of each and every human gene in the context of the physiological processes under investigation. Besides genetics and biochemistry, microscopy based approaches have started to become essential tools in such projects. They can provide information with regards to the dynamics and molecular regulation of protein specific reporters with excellent spatial and temporal resolution at the single cell level. This, however, requires imaging of up to millions of single cells at high quality and in a quantitative manner. In order to achieve these aspirations light microscopy techniques have been automated over the past decade and a number of commercial systems are now available for such high throughput studies. Besides these hardware developments, progress in automated sample preparation and most importantly image data analysis have rendered high throughput automated microscopy as a powerful tool to achieve single gene functional analyses at the genome-wide scale [1,2].

High Throughput Live Cell Imaging with ZEISS Celldiscoverer 7

High throughput microscopy has been used in a number of excellent studies in the past few years. However, up to now mostly fixed and fluorescently stained samples have been utilized. While this has led to many new insights into how genomes are functionally organized these fixed cell studies could not explore the dynamics of the systems under investigation, despite the fact that these dynamics are of utmost importance in order to gain a comprehensive understanding of the studied processes. Very few live cell genome-scale analyses have been reported so far [1], most likely because of their increased complexity and the challenges that imaging live cells pose on the experimental microscope set-up, sample preparation and data analysis.

In order to achieve reliable and robust live cell automated fluorescence microscopy analyses without compromising the physiology of the samples, a number of essential challenges have to be overcome. First, the imaging system must be sensitive in order to achieve sufficient image quality whilst at the same time exposing the samples to as little excitation light as possible; a key factor in the survival of the sample. Second, in order to obtain sufficient temporal resolution in multi-labelling experiments, excitation and detection optics should be rapidly switchable, ideally without moving parts of the microscope. Thirdly, the system should provide the flexibility to use culture systems (e.g. coated plastic dishes), which are optimized for the cells of interest. However, in our opinion one of the most important challenges in live cell imaging is stable environmental control, for example when tissue culture cells are under investigation. In our experience, temperature deviations from 37°C or fluctuations of only one to two degrees centigrade already lead to a significant delay in cell cycle progression and thus any studies related to this process become difficult to evaluate. Several commercial solutions exist to keep the samples at the appropriate temperature. Since these have limitations for various reasons, custom built environmental control systems have been used for critical experiments (e.g. [1]). In this respect, automated live cell analyses with ZEISS Celldiscoverer 7 have shown that this system provides an excellent commercial environmental control to a wide-field microscope system. Cells expressing FP-tagged H2B to monitor nuclear DNA can be followed for several days without any apparent delay in cell proliferation compared to cells kept in a tissue culture incubator (fig.1). At the same time the system offers the sensitivity to detect fluorescent nuclei with an image quality that allows the quantitative monitoring of cell cycle progression through mitosis. Phenotypes characterized by temporal or spatial distortions, e.g. induced by gene knock-down or drug treatment, can be reliably detected (fig.1, and fig.2). The optics of this system allow efficient detection of fluorescence even with low magnification, long working distance objectives (fig.2), which is typically challenging due to the low performance in collecting fluorescent signals with standard long distance objectives. This enables efficient imaging of fluorescence in cells for which culture on plastic support is an advantage or even essential. The use of powerful long working distance air objectives in this system has the additional advantage that multi-position imaging can be efficiently performed without the use of immersion media. These typically become limited during extended periods of imaging at 37°C due to evaporation, thus necessitating the use of immersion media dispensers. Although such dispensers can to some extent cure the problem, they typically run the risk of instabilities in imaging conditions during live cell imaging experiments that last for several hours or days.

VITA

Rainer Pepperkok

Rainer Pepperkok is the head of scientific core facilities at EMBL in Heidelberg including the Advanced Light Microscopy Facility. He has developed quantitative microscopy based approaches to study membrane traffic and organelle biogenesis in the early secretory pathway with a particular focus on high content screening approaches.

Pepperkok's laboratory also applies the developed technology to disease related questions investigating molecular mechanisms of cholesterol homeostasis and cystic fibrosis. Pepperkok is one of the founders and current scientific coordinator of the European Light Microscopy Initiative (ELMI) and an active member of EuroBioimaging.

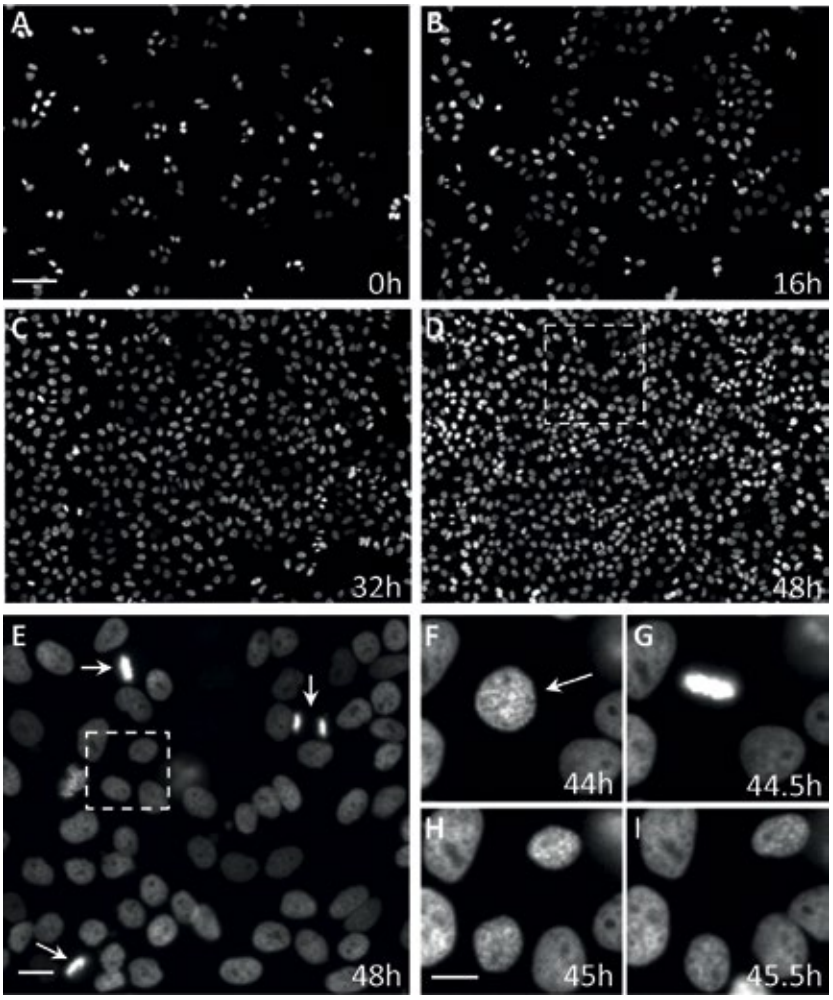

Fig. 1: *High throughput time-lapse microscopy with Celldiscoverer7*
HeLa-Kyoto cells stably expressing H2B-mcherry were seeded in a 96 well plate and imaged with the Celldiscoverer7 at 37°C with a 20x/0,95NA objective with magnification changer 0.5x. Images of the same fields were acquired every 30 minutes for 48 hours. (A-D) Different time points of one example field of view. (E) Zoom in of the rectangle in D, arrows point to cells in mitosis. (F-I) Zoom in of the rectangle in E showing the cell of interest (arrow in F) in (F) prophase , (G) metaphase, (H) telophase (I) interphase. Scale bars 100 µm (A-D), 20 µm (E), 10 µm (F-I)

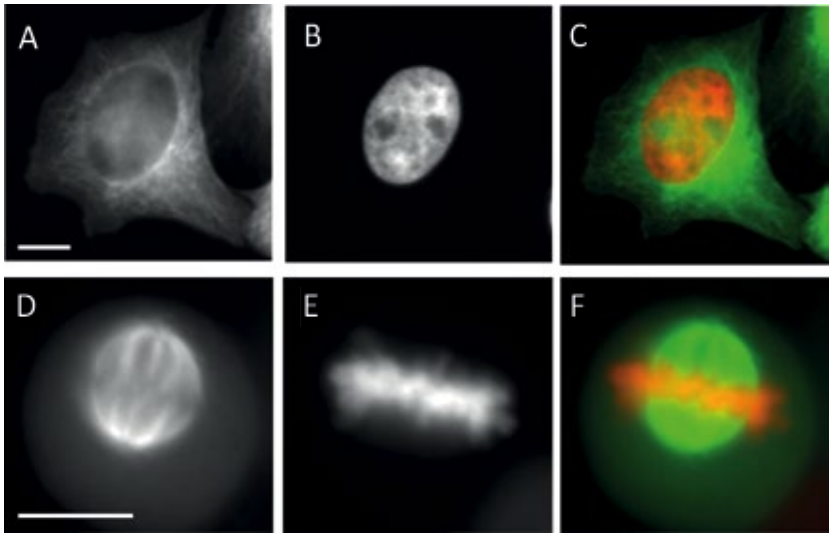

Fig. 2: *Double colour imaging in plastic cell culture plates.*
HeLa-Kyoto cell stably expressing H2B-mcherry and GFP-tubulin were cultured in a plastic cell culture plate from NUNC, fixed and subsequently imaged using a 20x/0.75NA objective with a magnification changer 2. (A,D) GFP-tubulin, (B,E) H2B-mcherry, (C,F) overlay image (GFP-tubulin shown in green and H2B-mcherry in red). Scale bar 10 µm.

Advanced Live Cell Imaging by Feedback Microscopy

Many systems used in high throughput microscopy experiments have been optimized for handling specific tissue culture formats such as 96 or 384 well plates. In addition, imaging modes offered by these instruments are optimized for speed and robustness and are thus often limited to standard wide-field or confocal microscopy imaging modes. More complex live cell imaging protocols requiring parameters such as: changing the objectives during the experiment; identifying specific objects or cells in the sample and then imaging them for the duration of an extended time-lapse; or more advanced techniques such as fluorescence recovery after photo-bleaching (FRAP) are not possible with such systems. We have overcome these limitations in our laboratory by developing automated feedback microscopy protocols where images acquired by rapid, low resolution automated fluorescence microscopy are analyzed online to identify objects of interest which are then subsequently imaged with more complex microscope settings (fig.3). Realization of such feedback microscopy requires the possibility of remotely controlling the microscope settings, e.g. via a command interface that also allows communication with common and open source image analysis packages such as Fiji or CellProfiler. Several wide-field and laser scanning confocal microscope systems including the new Celldiscoverer 7 offer this possibility and thus enable in principle the automation of complex imaging protocols, which require several analyses and decisions before the final image data is acquired (fig. 4). Feedback microscopy has allowed us to completely automate fluorescence recovery after photo-bleaching (FRAP) in order to determine the kinetics of membrane turnover of vesicular coat complex COPII components at highly mobile structures such as endoplasmic reticulum exit sites (ERES, fig. 4, [3]). In this way several hundreds of recovery data sets can be automatically acquired in an overnight experiment, thus drastically increasing the data significance compared to manual data acquisition where at best several tens of useful datasets can be acquired in the same amount of time. One reason for this disadvantage of manual acquisition is that it suffers from the fact that ERES often move out of the field of view due to the length of time required to manually conduct each step described above in a photo-bleaching experiment. Complete automation by feedback microscopy considerably shortens the time between object selection, photo-bleaching and recovery data acquisition such that movement of the ERES becomes less problematic. In our laboratory, feedback

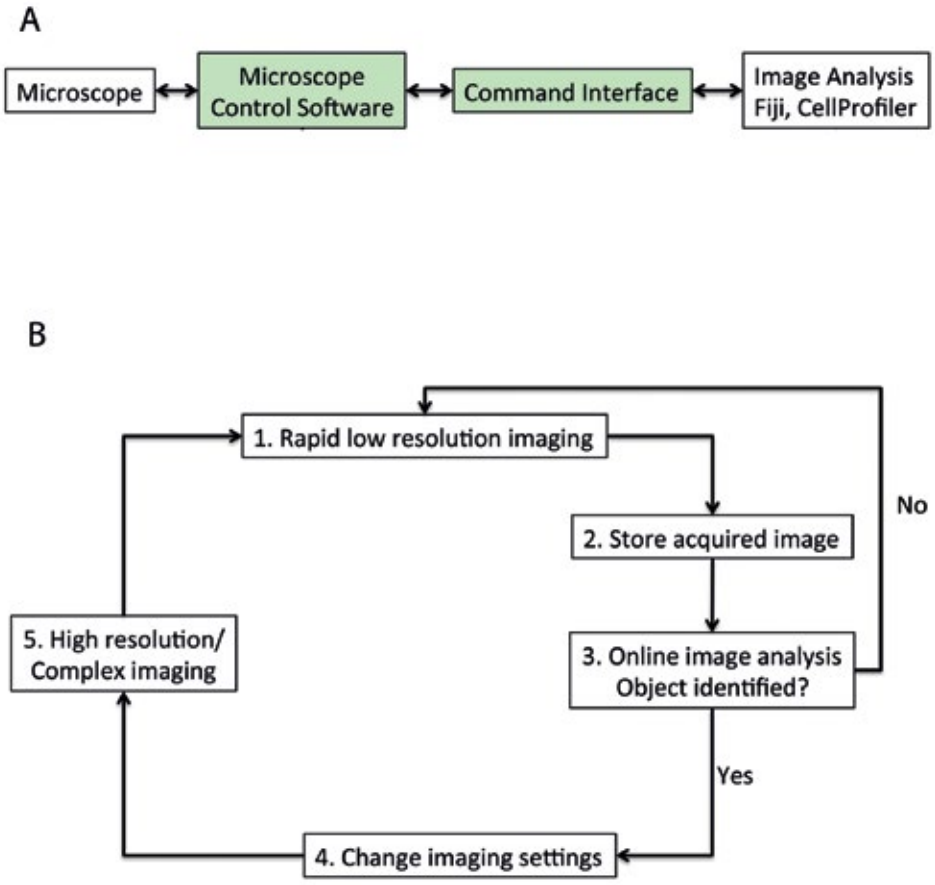

Fig. 3: *Components and their communication in adaptive feedback microscopy*
(A) Components and their communication required for adaptive microscopy. The command interface enables communication between the microscope control software and image analysis packages and needs to be developed in a system specific manner. For feedback microscopy on a ZEISS LSM 780 a Visual Basic Macro as described earlier [5] is used as command interface.
(B) Flow chart of a basic feedback microscopy experiment.

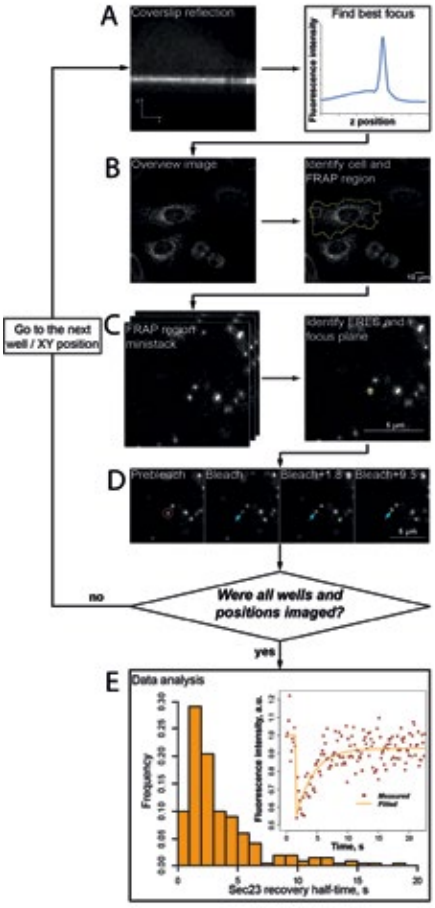

Fig. 4: *Steps in automated FRAP acquisition and analysis by feedback microscopy on a ZEISS LSM 780*
After automatic identification of the best focal plane for imaging (A), cells of interest expressing the ERES-associated and GFP-tagged vesicular coat component SEC23 are automatically identified by rapid scanning at low magnification and image analysis (B). Subsequent more detailed imaging at higher magnification within the selected cell, including the acquisition of 3D image stacks, identifies the position of ERES that do not touch neighbouring structures (C). Finally, the selected ERES are analysed by FRAP (D). The cycle is repeated until the predetermined number of data sets has been acquired. Automated analysis is used to extract recovery times (E).

microscopy approaches have also been developed to enhance efficiency when conducting multicolor time-lapse imaging of cells during mitosis [1] and to automate fluorescence cross correlation (FCCS) experiments [4].

Conclusion and Outlook

In this article, the latest developments in high throughput live cell imaging have been presented. Excellent environmental control combined with efficient fluorescence detection with long working distance objectives work together to enable high quality, high throughput live cell imaging even in plastic bottom culture dishes. In our view, these are the hallmarks of the new ZEISS Celldiscoverer 7 and together these represent significant improvements in this technology area.

Feedback microscopy, which requires a command interface to control the microscope settings from e.g. open source image analysis packages, is possible with both ZEISS Celldiscoverer 7 and the confocal microscope ZEISS LSM 780. This enables the complete automation of even complex imaging protocols such as FRAP (exemplified here) and FCCS measurements [4]. With this technology to hand, integrating and automating advanced imaging with follow-up analyses such as single cell harvesting followed by transcriptome analyses or automated correlative light and electron microscopy may come within reach.

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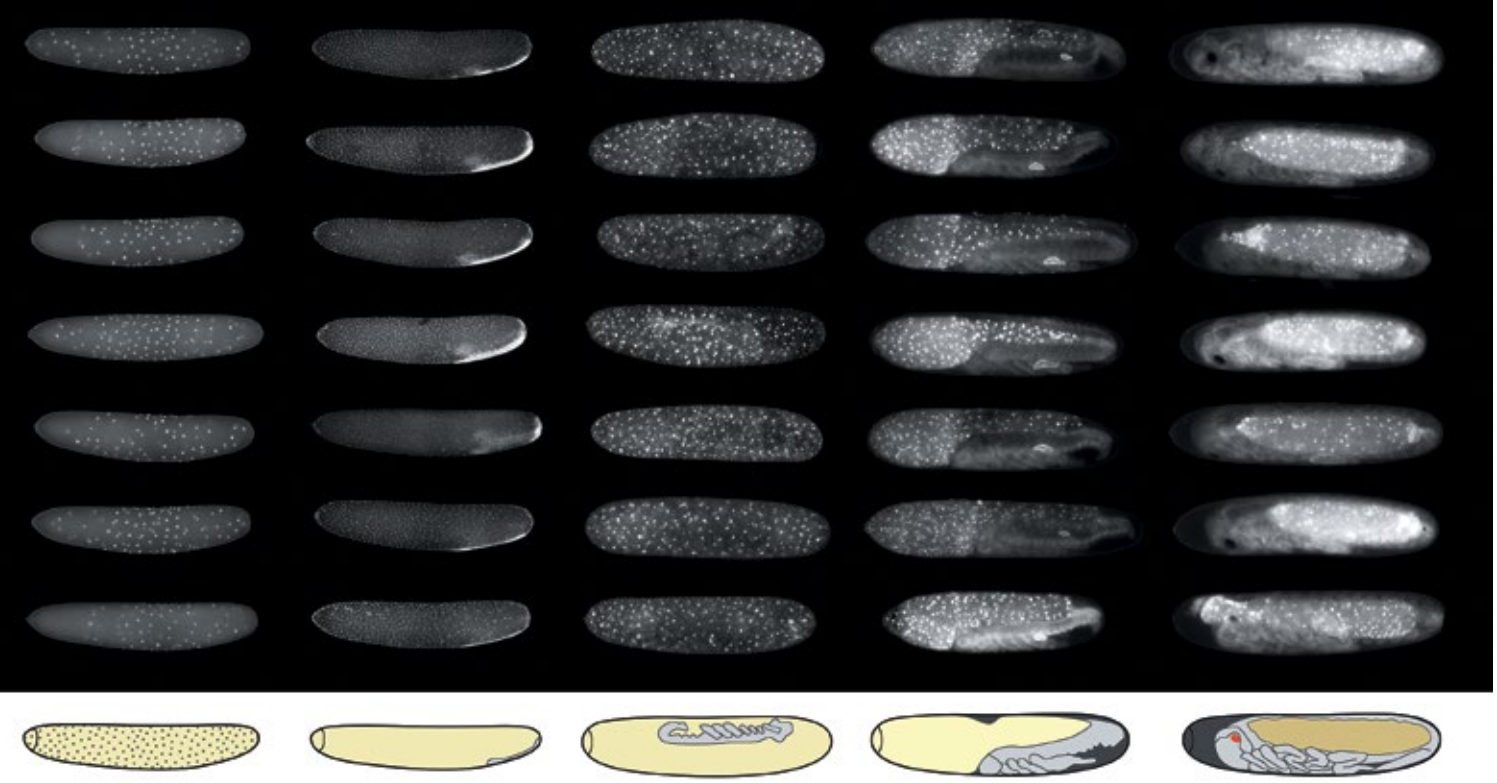


High-Throughput, Long-Term Live Imaging

Automated Microscopy of Insect Development

Seth Donoughe[1] and Sebastian Gliem[2]

The study of arthropod embryogenesis can provide insight into the evolution of development mechanisms. Many aspects of development are most effectively studied by live-imaging the development of many embryos. Historically, this has been difficult, but new tools are making the collection of such datasets easier than ever.

Fig. 1: *ZEISS Celldiscoverer 7. The right module of the system contains the main imaging unit; the left module hosts units for environmental control and an additional camera port.*

Introduction

With regard to the well-studied animal model systems such as zebrafish, nematode, and fruit fly, the last few decades have been enormously fruitful for our understanding of the process of animal development. For those animals, much is known about how cells divide and move in the process of forming an organized body. However, comparatively little is known about how developmental mechanisms evolve. The research group led by Cassandra Extavour at Harvard University aims to uncover aspects of development evolution by characterizing arthropod embryogenesis in a number of emerging model species [1, 2, 3, 4]. By comparing among these species, it is possible to infer the course of evolutionary events that generated some of the great diversity seen in nature.

An ongoing project of the Extavour Lab and its collaborators is to describe embryogenesis of the two-spotted field cricket *Gryllus bimaculatus* (fig. 2A, B). Recent technological advances, such as light sheet and confocal microscopy, have made it possible to explore the dynamics of cellular-level phenomena in emerging model organisms – like the cricket – by sensitive time-lapse imaging. However, although the resulting data have relatively high spatial resolution in three dimensions, these imaging techniques are well-suited for recording at most a few embryos at a time. With such small sample sizes, it is difficult to assess developmental variation and timing [5]. Moreover, pharmacological treatments and functional genetics approaches often produce a broad range of phenotypes (e.g. [6]), which are difficult to characterize without imaging dozens of treated embryos.

This raises the need for a complementary approach in which many samples can be mounted at once, and then recorded during a long-term live imaging experiment with high sensitivity and precise environmental control. A new microscope system, Celldiscoverer 7 (Carl Zeiss Microscopy, Jena, Germany; fig. 1) has provided the opportunity to address these demands. It has been very useful for imaging dozens or even hundreds of samples in parallel, in several different sample carrier types. Therefore, microscopy of this type will potentially provide better insight into many aspects of insect development.

Mounting Embryos

An acrylic mold is used to create low-melt agarose troughs in a glass-bottom dish or in each well of a glass-bottom 6-well plate. All imaging is done on embryos from a trans-

VITA

Seth Donoughe

Seth Donoughe is a PhD student in the lab of Cassandra Extavour in the Department of Organismic and Evolutionary Biology at Harvard University. His research focuses on the evolution, development, and cell biology of insects.

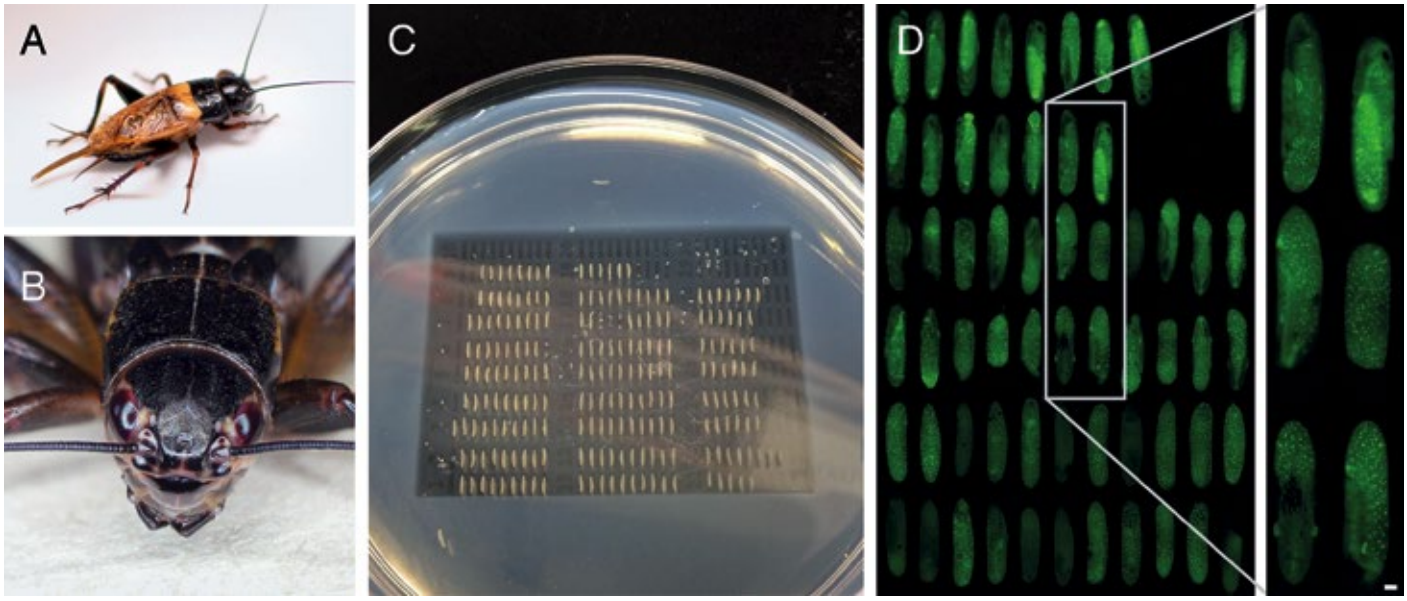
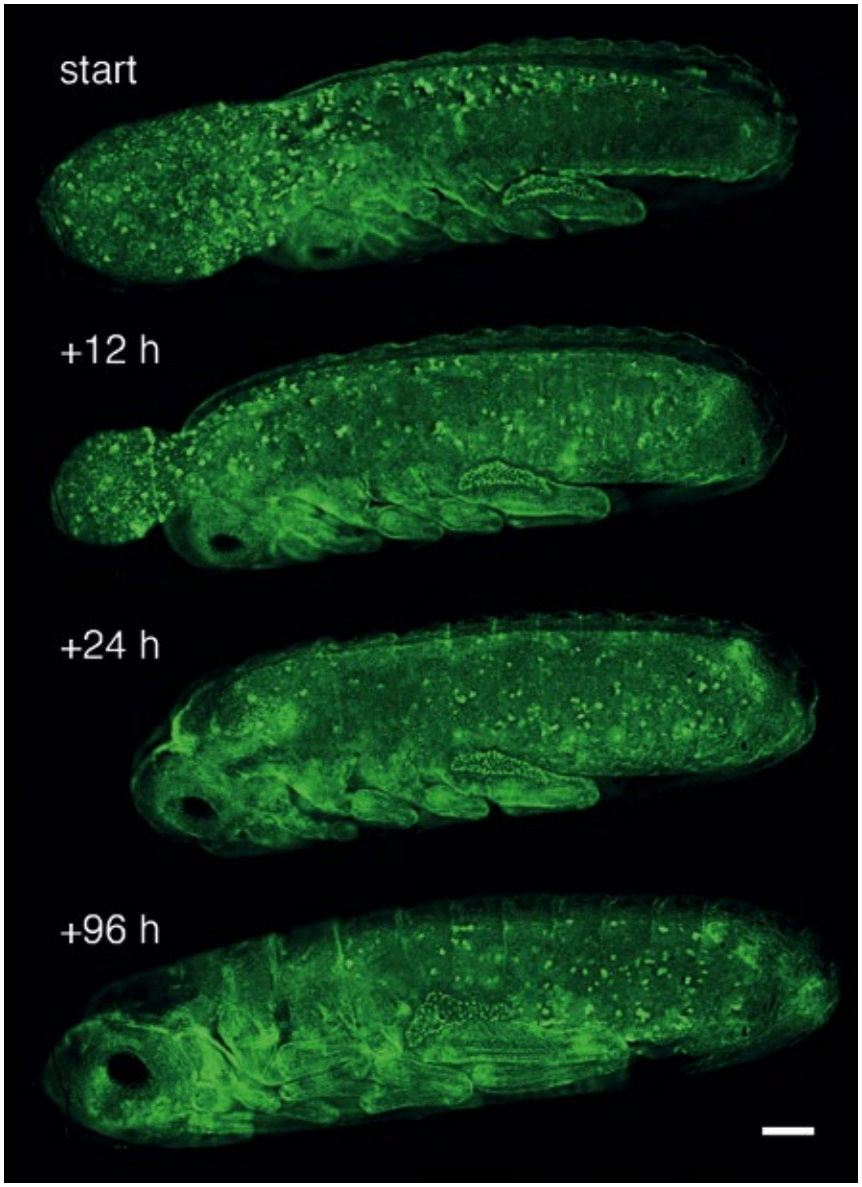


Fig. 2: *Mounting an array of cricket eggs for imaging. A, B: Adult two-spotted field crickets. C: A customized mold insert was used to make a plate of low-melt agarose microwells. After eggs were placed into the microwells, additional low-melt agarose was used to hold eggs in place. D: This technique was used to mount transgenic eggs containing embryos from several different developmental stages. This image was generated from 15 individual tiles that were acquired at 2.5x magnification on Celldiscoverer 7. Scale bar: 200 µm.*



◀ ***Fig. 3:*** *A developing cricket embryo. During the development from egg stage 15 to egg stage 21, dorsal closure as well as limb and body growth can be observed. All images were acquired at 2.5x magnification. They were extracted from a multi-position time series (5-minute sampling interval) and deconvolved from a 130 µm thick z-stack. Scale bar: 200 µm.*

genic cricket line that ubiquitously expresses the cricket Histone2B protein fused to Enhanced Green Fluorescent Protein [7]. Freshly laid cricket eggs are placed into the troughs, and then a few drops of low-melt agarose are added to the troughs to hold the eggs in place (fig. 2C). Water or Robb's Saline is then poured into the dish/well to keep the eggs hydrated. The sample carrier is then loaded into the microscope. The microscope automatically detects the carrier type, measures the cover glass thickness, and adjusts the correction collars of the objectives to minimize optical aberrations. Using a 2.5x magnification, many embryos can be easily imaged at each timepoint by tiling an array over the dish (fig. 2D). At each (x,y) position a z-stack is captured. This approach results in less z-resolution than a confocal or light sheet microscope can provide, but in exchange it is possible to image a much larger sample size while still retaining cellular-level resolution in the x-y plane (fig. 2D).

Long-Term, High-Quality Imaging

The microscope provides precise environmental control for the imaging chamber. Cricket embryos at several different developmental stages could be live-imaged for more than five days with no loss of fluorescence intensity.

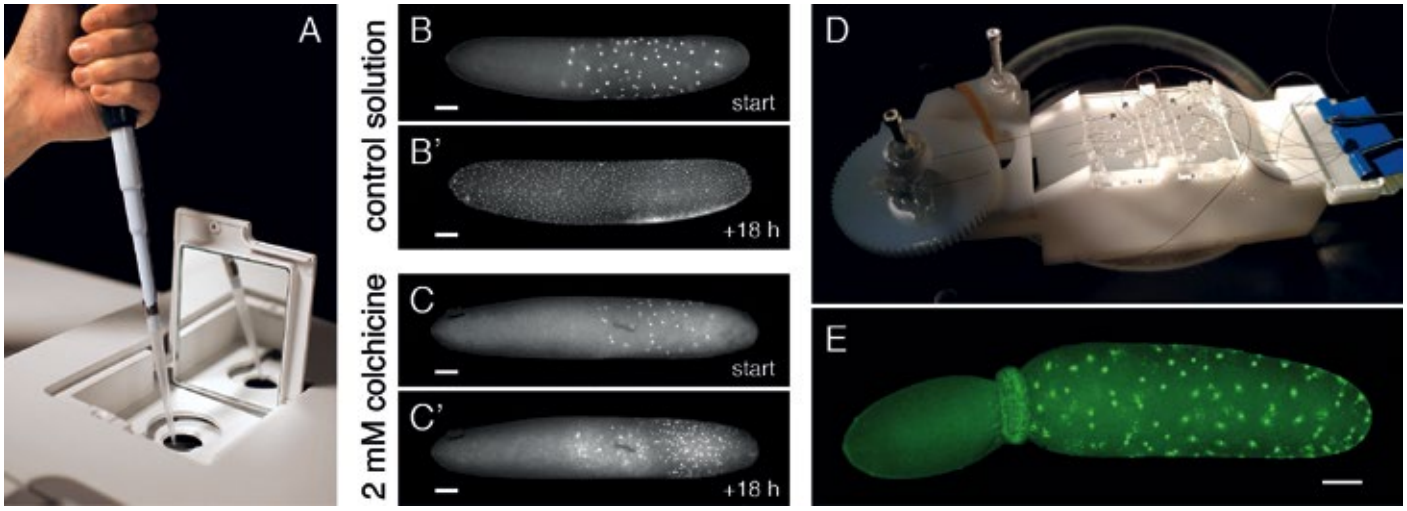


Fig. 4: *Investigating the effect of chemical treatment and physical manipulation on cricket development. A: Celldiscoverer 7 has a fluid handling chamber that can be used to add chemical compounds to the embryos without interrupting incubation. B: Robb's Saline with DMSO was added to embryos as a control condition. This resulted in normal embryonic development up through 18h. C: Robb's Saline containing 2 mM colchicine (a disruptor of microtubule polymerization) inhibited nuclear movements within the egg. D: Device for embryo constriction. A ratchet mechanism applied incrementally higher tension to a human hair wrapped around a cricket egg. The tension was applied while watching the embryo under a dissection microscope. The hair was then fixed in place for imaging. E: By imaging the fluorescently labeled nuclei, it is clear that the constriction impaired movement of nuclei into the tip of the egg. Scale bars: 200 µm.*

Moreover, the survivorship of the embryos was >90% (similar to non-imaged controls). Even with low magnification, the optics are sufficiently sensitive to detect and track nuclei from egg fertilization through the formation of the germ band (see micrographs at top of of the article) and in the extraembryonic tissues throughout development. It is also possible to observe appendage elongation and dorsal closure, an important process during embryonic development in all insects (fig. 3). The large field of view reduces the number of tiles that is required to cover the full array of embryos, thereby decreasing the sampling interval of the time-lapse. For instance, an array of 30+ embryos could be imaged at 2-minute intervals.

Investigating Chemical and Physical Treatments on Embryo Development

Experimental manipulations are an especially valuable application of high-throughput embryo time-lapse recording. Colchicine is a small molecule that inhibits microtubule polymerization. It was dissolved in dimethyl sulfoxide (DMSO) and then diluted in Robb's Saline to five different concentrations, ranging from 2 µM to 20 mM. An array of 20 cricket embryos were mounted in each well of a 6-well glass-bottom plate. Each well was filled with a different concentration, plus a control treatment of DMSO only. By recording time-lapses over 18 hours, a presumptive arrest of nucleus movement within the embryo could be observed. This indicated that cricket cell movements during the formation of the blastoderm are sensitive to microtubule disruption (fig. 4B, C).

Mechanical manipulation is another way to probe the physical mechanisms at work during embryogenesis. A custom ratchet device was developed to wrap a human hair around an individual egg, and then slowly increase tension on the hair, thereby constricting the egg (fig. 4D). This device was used to constrict many embryos, and then live-image the effect on their development. Such manipulations produce effects with a high variance. Some embryos fail to develop, while others may eventually burst from the strain. Only by imaging in a high-throughput manner it is possible to capture numerous instances of embryos that continue development within their artificially truncated eggs (fig. 4E). This technique will be used to assess whether a change in the density of cells/nuclei causes them to change their divisions and movements.

Conclusion

An epifluorescence microscope such as Celldiscoverer 7 with its large field of view but high quality optics, precise environmental control, and sophisticated stage control system serves as a useful complement to confocal and light sheet microscope systems. In the example case of cricket development, it was straightforward and advantageous to conduct the fast, high-throughput time-lapse recordings that facilitate the characterization of developmental variation and high-variance experimental manipulations of embryogenesis.

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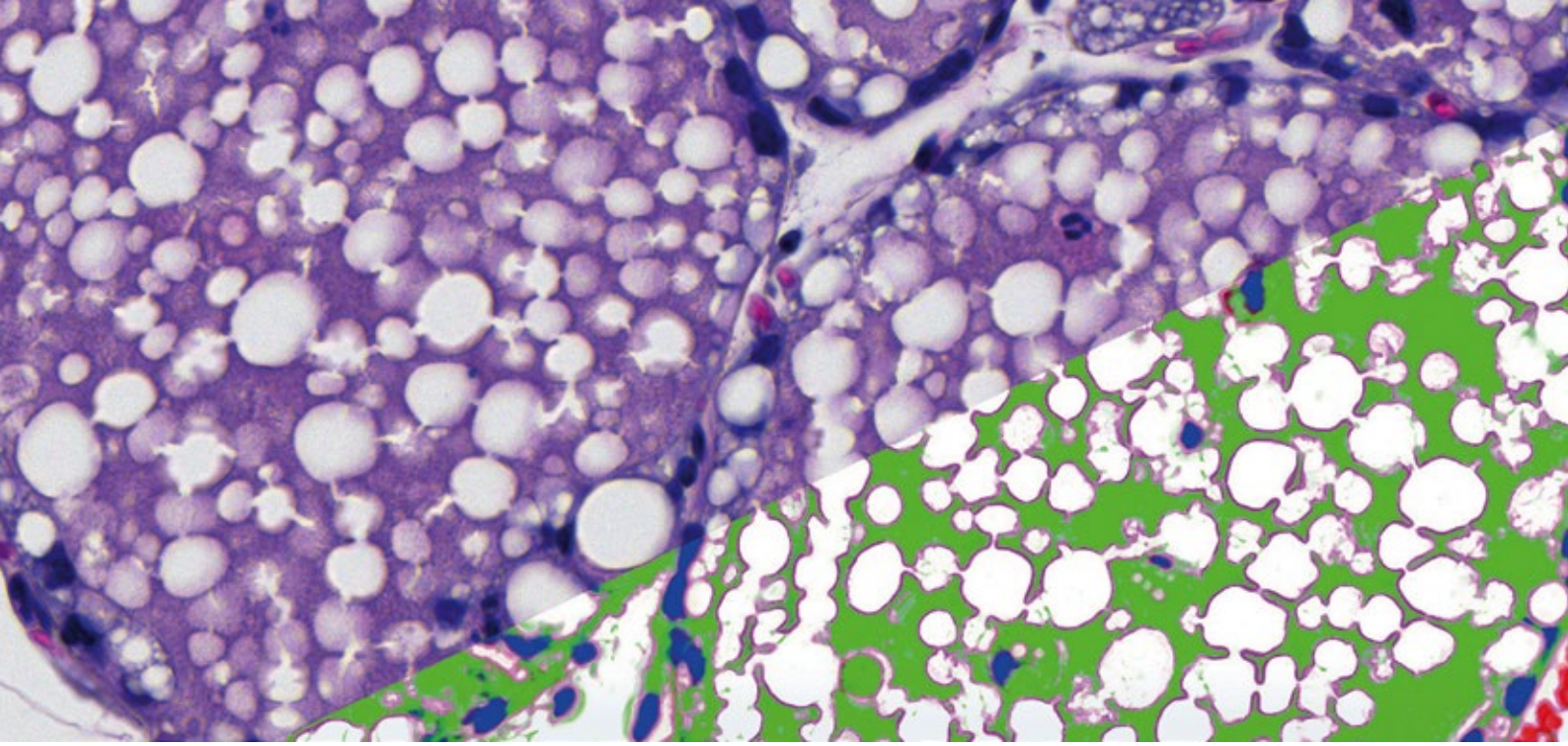


Big Microscopy Dataset and File Management

Examples of Three Workflows Implemented at the FMI in Basel

Laurent Gelman

New microscopy modalities, e.g. live-cell imaging, slide-scanning, high-content screening and 3D-electron microscopy, associated to biological projects aiming at more quantitative data, generate datasets which are one to several orders bigger than before. To cope with this exponential growth, new workflows and important investments in IT solutions are needed. Unfortunately, there is no single workflow, nor a single computer configuration that can do it all. I present here three different workflows to exemplify the issues and some solutions that have been found in our institute.

Slide-Scanning Workflow

There are several slide-scanners since many years on the market, piloted by user-friendly software, which can scan hundreds of slides in an automated manner. The challenge in slide-scanning is the management – naming and ordering – of the hundreds of files generated, and their processing – visualization, transfer and storage, and analysis.

To avoid confusion between slides and files, long copying times of the files (blocking access to the slide-scanner) and loss or duplication of data, we developed a largely automated workflow, from histological staining to imaging, schematized in figure 1 and detailed below.

Step 1: To access a resource from our facility, users fill a form on the intranet, which needs to be approved online by their group leader by replying to an automatically generated e-mail. Upon authorization, a project is automatically created in our home-made project management system (PMS) [1], to which a list of instruments is added, enabling the user to book these instruments in our home-made reservation system. The creation of a project in the PMS automatically triggers the creation of a new project folder with the same name in our image database (IMS, Imagic).

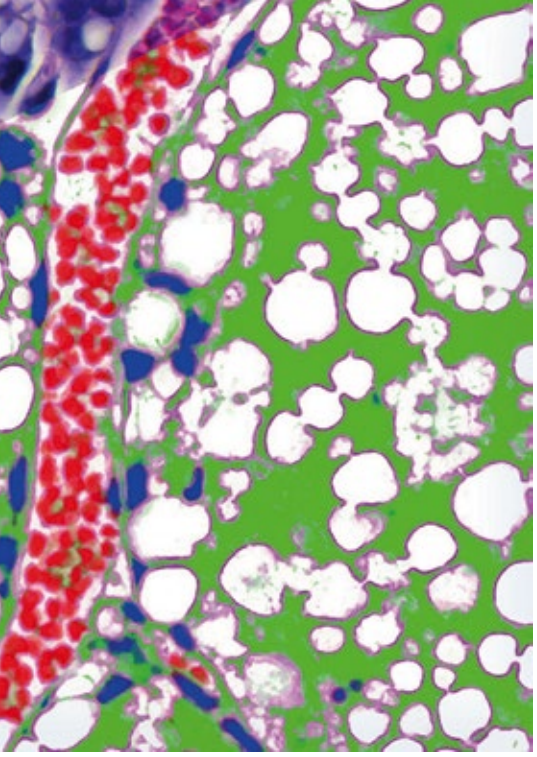
Step 2: Within the project in the image database, the user describes each slide to be scanned in a separate experiment subfolder, with the relevant information for the staining of the sample (e.g. antibody types and dilutions, buffers), which is thereafter used by the Histology Facility staff to program the robot for slide staining (Ventana). After completion of the staining, the Ventana machine generates a protocol and a run report and the staff of the Histology Facility imports these files into the image database in the user's project.

Step 3: The user loads the slides into the slide scanner. To avoid the tedious and error-prone naming of each slide in the software driving the slide-scanner (ZEN Blue 2.0 and Axio Scan.Z1 respectively, from ZEISS) a script has been developed in-house to export from the Image Database the names of the experiments in an .xls file, which can be directly imported into the slide-scanner software. A unique tag is added to the experiment name both in the database and at the export to identify the slide unambiguously. Each slide can then be traced back from the staining in the histology facility down to the scanning and archiving of the pictures without any confusion.

Step 4: Slides are scanned automatically and images are saved locally on solid-state drives (4 TB capacity). A Robocopy script mirrors automatically this data folder on a server linked through a 10 Gb connection.

Step 5: A home-made script running in the image database searches regularly for new files on this server, and when the name of a file matches that of an experiment within a project, a thumbnail picture of the label of the slide and the link to the file itself on the server are added to the database.

Step 6: The user can review the pictures acquired from any computer from the



facility or using virtual machines accessed by remote desktop connections, all being linked to the server though a dedicated 10 Gb network.

Step 7: If the user estimates that the image is worth keeping, the mask for the experiment in the image database presents a small button, which, when selected, triggers every night the copy of the files from the server to the archiving system of the institute, consisting of drives and tapes with a maximal capacity of 8 PT, where 3 redundant copies of the data are made. We introduced on purpose this additional "click to archive" step/action for the user, to avoid the automatic import of unsuccessful experiments into the image database.

High-Content Screening

Similar to slide scanning, the most challenging part of HCS is the review and the processing of the data. How to get a quick overlook of an experiment typically containing hundreds of thousands of single-plane files? How to process and quantify all these files in an amount of time compatible with an error and trial approach for image processing where many parameters need to be optimized?

In collaboration with the group of Prisca Liberali we established a workflow schematized in figure 2 and detailed below.

VITA

Laurent Gelman

Since 2010, Laurent Gelman is co-heading the Facility for Advanced Imaging and Microscopy at the Friedrich Miescher Institute in Basel, together with Dr. Christel Genoud.

Dr. Gelman obtained Engineering and Master degrees in Immunology in Paris, a PhD in molecular biology from the Pasteur Institute in Lille, and worked as a post-doc at the IGBMC in Strasbourg, and as a lecturer and post-doc at the University of Lausanne.

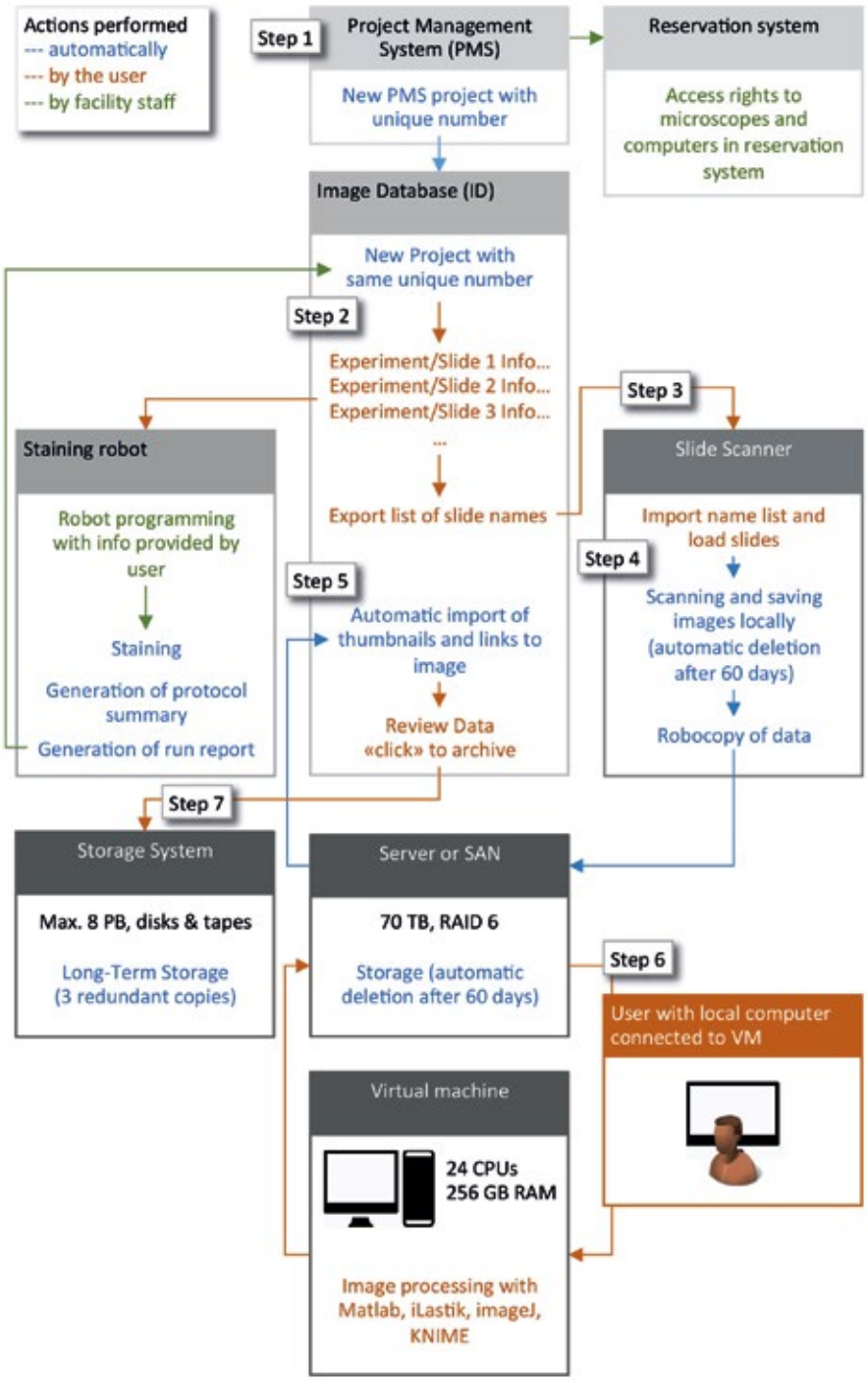


Fig. 1: *Workflow for the scanning of slides stained at the histology facility.*

Step 1: After optimization of the assay, the user sets the acquisition parameters on the HCS machine (CV7000S, Yokogawa). For multi-plate screenings, a robotic arm loads a new plate as the previous one is scanned. When appropriate, the "Search First" machine learning option of the Wako Software Suite (WAKO) is used to pre-scan the plates with a low magnification in 2D only to specify the regions with objects of interest that will be imaged thereafter at higher magnification in 3D. This reduces tremendously the amount of data collected and the time of acquisition. Files are saved on a Network Attached Server (NAS) linked physically directly to the CV7000S computer. The NAS has a capacity of 60 TB in a RAID6 configuration.

Step 2: A Virtual Machine (VM) linked to the NAS via a 10 Gb network automatically pre-processes the files (see details below) and saves results to a Storage Area Network (SAN) linked directly via a 10 Gb connection to the VM infrastructure. The SAN has a capacity of 130 TB. Before the screen starts, the user needs to enter some important parameters about the format of the experiment and the type of pre-processing desired in a text file saved locally on the VM, which is used thereafter by a Matlab or a Python script to process the images. The pre-processing, done on-the-fly, consists first in the generation of tiff files compressed in a lossless format, but which still retain the 16-bit format of the original tiff images. This step reduces by 30 to 50 % the size of the dataset, a crucial step when one considers the average size of a full screen, typically 130 TB. The pre-processing generates also well- and plate-overviews out of the numerous tiles acquired. The user has also the possibility, when relevant, to merge the different channels (multi-color imaging) and to realize maximum intensity or sum projections of the stacks before stitching the tiles. Montages are saved as jpeg files to save space as their purpose is merely a quick inspection of the run. This quality control step is important, as the final processing of the huge dataset produced is very computer-intensive and time-consuming and must be only done when the data are satisfactory. A good software or database for immediate or even online previsualization of the data, circumventing their time-consuming pre-processing, would be interesting to implement in the future.

Step 3: After review of the pre-processed images, the data are typically analyzed either with a second more powerful VM (typically 24 CPUs and 256 GB RAM) or sent to a cluster (Brutus, Eidgenössische Technische Hochschule Zurich, typically 500 to 1000 CPUs). Image analysis workflows using home-made Matlab, ImageJ, and KNIME scripts are used on the local VM. On the ETHZ cluster, the iBRAIN software is used. iBRAIN is a computational platform for large-scale image analysis with super-computing facilities developed at the University of Zurich in the group of L. Pelkmans [2]. One can create inside iBRAIN workflows with CellProfiler and home-made Matlab modules. It has also pixel-based and object-based machine learning classifiers and links to gene and protein annotation databases. iBRAIN is not only an image processing pipeline editor but also a job scheduler for cluster computation: it manages the different projects (queuing system) but also automatically re-submit jobs that failed on the cluster. A web-interface enables the user to follow the progress of the workflow, as intermediary reports are saved as pictures or pdf files.

Step 4: As the analysis of the data is running, the compressed images generated during the pre-processing are backed-up by a member of the Facility Staff to the archive system of the institute. These data will be also manually deleted from the SAN after completion of the analysis, which may take several months. The original non-

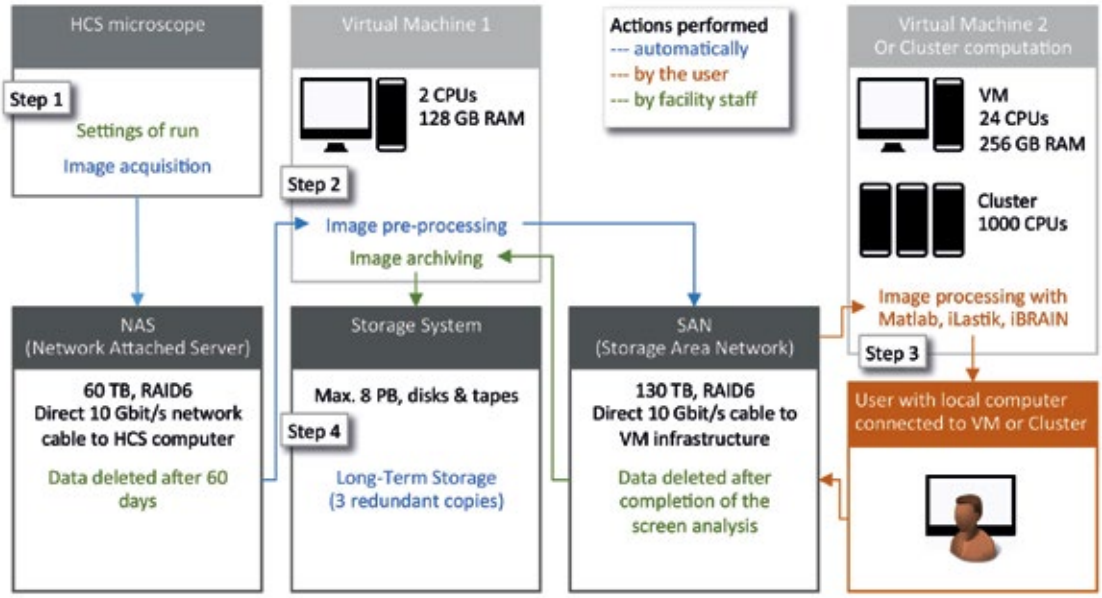


Fig. 2: *Workflow for High-Content-Screening.*

compressed images on the NAS are always manually deleted after 30 days.

3D-Electron Microscopy

Many projects in neuroscience require the reconstruction of neuronal wiring diagrams, so-called connectomes. One of the key techniques offered by our facility is 3D-EM, a technique generating Z-stacks of EM images through the combination of a scanning electron microscope and a microtome installed inside the microscope, in our case a QUANTA (FEI) or a MERLIN (ZEISS) microscope equipped with a 3View (GATAN) microtome.

The challenge here is the large-scale reconstruction and annotation of neurons and ultra-structural features such as synapses. This must be done to a large extend manually, a tremendously time-consuming task. If one considers that tracing and annotating one single neuron takes approximately 6 hours, tracing 1'000 neurons per data set, several times (for reproducibility) and for several datasets (animals) is an impossible task for a single person. Only an "army" of annotators can perform the job in a reasonable amount of time, in a crowdsourcing approach.

Fortunately, such an army exists: in 2014, Adrian Wanner, a Ph.D. student in the group of Rainer Friedrich at the FMI, created ariadne-service GmbH, a company providing a link between scientists and annotators [3]. Ariadne-service hires and manages annotators, trains them with known reference reconstructions, supervises work quality, and carries out payroll and accounting. Currently more than 30 annotators work for ariadne-service, each of them having more than 1'000 hours of experience.

Step 1: The datasets are pre-processed at the FMI. Image stacks are registered, eventually stitched, and reformatted into small "cubes" (image stacks) to be annotated, usually with three-fold redundancy.

Step 2: The cubes are sent to the annotators, either by FTP or on hard-drives per express mail, together with a set of instructions.

Step 3: Annotators work from home on their own PC and send back the results by e-mail or FTP.

Step 4: Ariadne-service keeps track of the working hours by monitoring the annotation behavior of each annotator and accordingly meets payrolls and sends the bill to the neuroscientist.

Thanks to this workflow, it was possible to reconstruct the entire interglomerular projectome in the Zebrafish olfactory bulb [4].

Conclusions and Perspectives

Each project requires a specific workflow. The possibility to exchange data between software is crucial (many thanks to the LOCI/Bio-Formats reader!). There is still an urgent need for a new image standard for big datasets, which could be based on the HDF5 format. Software companies must also develop more server and cluster compatible versions of their products as well as reliable floating license management systems. Finally, most of the efforts will come from IT Facilities, which have to provide large and backed-up storage devices with high I/O performances and fast networks, as well as powerful servers or cluster computation infrastructures with user-friendly interfaces.

Acknowledgements

The workflows presented here have been developed together with many people in different facilities or research groups at the FMI. We would like to thank Steven Bourke, Jan Eglinger, Dean Flanders, Christel Genoud, Stefan Grzybek, Saadia Iftikhar, Risto Milani, Roger Schmidt, Raphaël Thierry, Sjoerd van Eden, Katrin Volkmann and Yauhen Yakimovich for their collaboration.

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