

High-Speed Microscopy

Scanning of Microtiter Plates at Unprecedented Speed

A high-speed scanning solution for transmitted-light microscopy which allows scanning of microtiter plates at unprecedented speed was developed at the Fraunhofer-Institute for Production Technology IPT in Aachen, Germany. The novel solution is based on a continuously moving scanning stage with synchronous z-position adjustment and flash illumination.

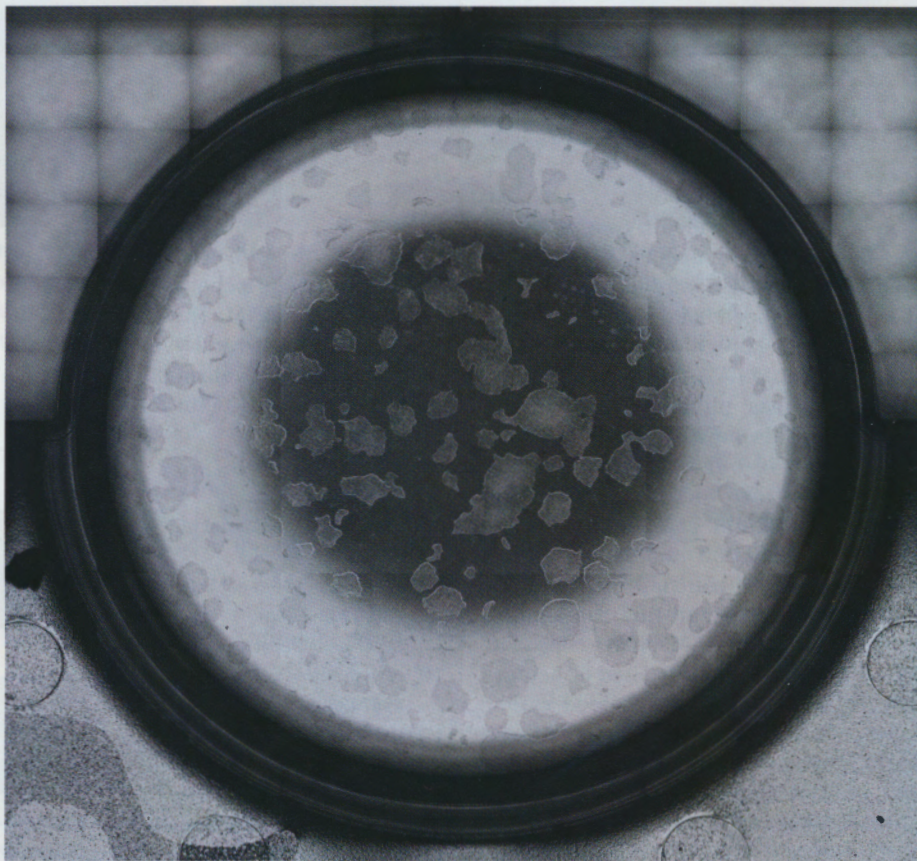


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Introduction

In nearly all cell culture applications it is critical to control cell growth and cell status regularly. Due to the tiny dimensions of cells the morphologic assessment has to be assisted by microscopy. A very common and widespread technique for making cells that are almost transparent visible is phase contrast microscopy. Cells are usually cultured in nutrient medium on transparent plastic material for example microtiter plates (MTPs). Such a plate which has a footprint of about 128 mm × 85 mm contains several wells which are separate compartments for the cells to grow in. Microscopic analysis directly takes place in these microtiter plates on inverted microscopes.

In order to get a good overview of the cell culture status it is helpful to image the whole content of an entire well of a microtiter plate. This is especially true because cell colonies tend to grow preferably in the border regions of a well. Usually it is not just one single well on a MTP that needs to be imaged but the whole plate. As the wells are closely adjacent to each other the well bottom area covers almost the whole plate's footprint. As a consequence, to examine a completely filled microtiter plate under a microscope means imaging



Stitched phase contrast overview image of a 6 well MTP with human iPS cell colonies on Matrigel cultivated in mTeSR. Captured at a speed of 34 mm/s with 4x magnification.

an area of about 10,000 mm². Due to the small field of view of a microscope's objective of less or maximum a few square millimeters depending on the magnification and the camera chip size, thousands of single acquisitions are necessary to capture such a big object. When using a 10x objective and a standard size camera chip of 2/3" almost 19,000 individual images are required. The single images are subsequently stitched together to form the desired overview images of the single wells with microscopic resolution.

The problem with microscopic acquisitions of large areas is that the time required is usually very long. In lab based cell culture work this might be tolerable, in large scale production processes this is a severe problem. When hundreds of microtiter plates have to be scanned every day for process and quality control in automated units, high-throughput microscopy is indispensable.

State of the Art

The required scanning time is mainly influenced by the number of images and the acquisition time for each image. The number of images can only be slightly reduced by increasing the field of view using a bigger camera sensor. However, this is practically limited to 4/3" because the optics of most microscopes cannot illuminate sensors beyond that size.

The acquisition time is mainly determined by the positioning time that takes much longer than the actual exposure of the sensor. Modern fully automated microscopes use motorized stages that position the object before it is imaged. Then the stages moves on to the next position and stops again before the next image is taken. This process repeats several thousand times for a whole MTP. In order to cope with the position uncertainties, the separate positions are

often displaced by less than the dimensions of the field of view. A blending algorithm later matches the tiles to an exact stitching result using this overlap. This further increases the required number of images. But the main challenge remains the positioning time that sums up to hours for thousands of single acquisitions. Therefore short positioning times are necessary for fast scanning of microtiter plates. Although acceleration, deceleration and speed of the stage can be tuned to a certain extent, the augmentation of these parameters is limited by the fact that the liquid culture medium shakes when being moved abruptly. The shaking of the medium induces varying heights of the water column that attenuates the transmitted light on its way to the sensor. This heavily decreases the image quality of the stitched image as the single tiles appear in different brightness rendering the whole image very inhomogeneous and hard to be processed by automated image processing methods. That is why smooth acceleration values and waiting times between the single image captures are indispensable when using state-of-the-art microscopy for MTP scanning. The achievable frame rate is practically limited to 1-2 fps. Imaging a whole microtiter plate even with a relatively small magnification of 4x on conventional microscopes therefore takes up to 20 minutes. It is quite obvious that state-of-the-art microscopy cannot provide the necessary throughput for automated cell culture examinations.

New High-Speed Approach

To close this gap researchers from the Fraunhofer IPT have developed a high-throughput microscopy solution that is able to scan whole microtiter plates in seconds. It is used within the research project StemCellFactory (www.stem-

cellfactory.de) which aims for the automated generation of induced pluripotent stem cells (iPS cells). Thanks to the new high-speed microscope hundreds of MTPs - for example with automatically generated iPS stem cell colonies - can be examined in that facility every day.

In order to speed up the microscopic image acquisition process, the conventional "stop-and-go" mode was replaced by a high-speed mode where the object is imaged

while moving uninterruptedly. In order to avoid motion blur, a flashing LED as stroboscopic illumination is used. Without the need to stop before taking an image, much higher frame rates can be achieved which tremendously reduces the overall imaging time. Furthermore the undesirable shaking of the liquid inside the MTPs is very much reduced due to the continuous movement resulting in a superb image quality in the final stitched images.

The breakthrough in imaging speed is based on a precise synchronization of the LED flash with stage position and camera acquisition. This is realized in real time by a high-resolution stepper motor controller (Märzhäuser Wetzlar) which features position dependent triggering, a novelty in microscope controllers. For the hardware triggered image capture a 4/3" sCMOS camera (pco.edge) is used.

To further reduce the scanning time by continu-

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Fig. 1: High-speed microscope inside an automated production facility for iPS cells.

ously moving the object under investigation, the number of stops is reduced to a minimum by scanning meander like along the whole MTP instead of imaging it well by well. This way, only two stops per line are necessary to scan a full MTP.

The achievable processing rate of the customized LabVIEW software that uses the computer's RAM to buffer the images reaches up to 50 fps. This allows moving the MTP at the full speed of the stage (120 mm/s) when using a 4x magnification. The new high-speed microscopy solution reduces the acquisition time from hours to seconds. A whole MTP can be scanned in less than two minutes using 4x or 10x objectives. The positioning and triggering accuracy of the system provides perfect alignment and allows overlap free stitching of the single images. The solution is currently implemented on a Nikon Ti-E inverse microscope but can be applied to almost any system on the market easily.

Focus Measurement and Adjustment

Prerequisite for sharp acquisitions is that the object is located in the focal plane during observation. Although the adherent cells – as the name suggests – adhere to the transparent bottom of the microtiter plate, they do not lie in a perfectly even plane. The geometric deviations of the plate bottom caused by the injection molding manufacturing process are in the order of 200 microns. This is far more than the depth of field of a microscope objective can tolerate. Therefore the plate to lens distance has

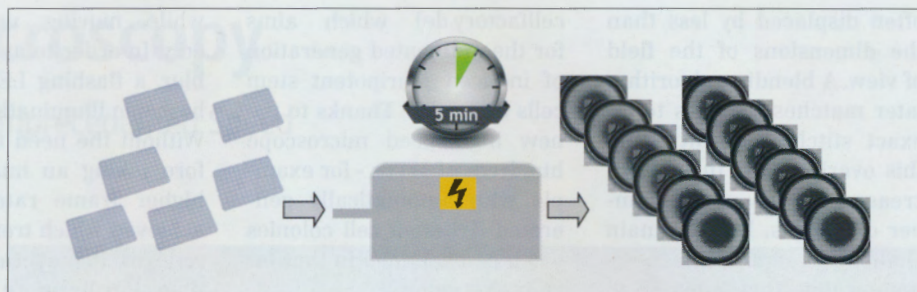


Fig. 2: The challenge in automated cell culture: Scanning plenty of microtiter plates in the shortest possible time.

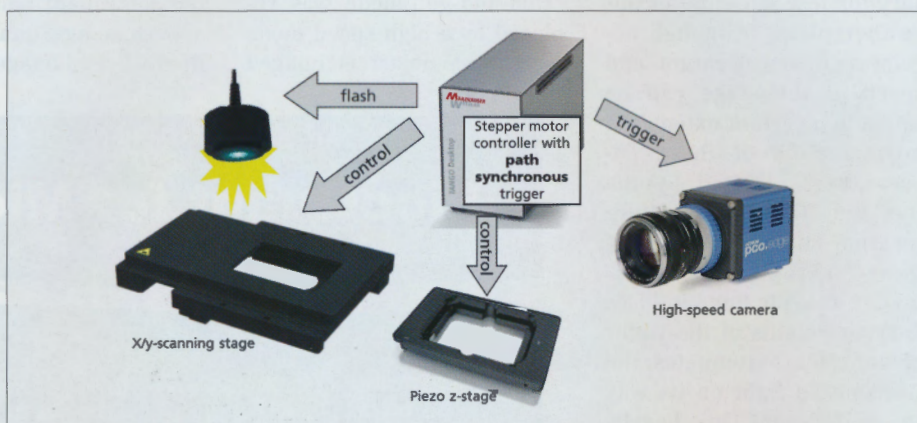


Fig. 3: Core components of the high-speed microscopy solution.

to be corrected constantly during the scan. One very common way to determine the correct working distance is to compare multiple images taken at different distances regarding a certain focus criterion (i.e. sharpness). This image processing based autofocus procedure not only takes long but also requires that the object must not move during measurement. That is why only a few positions are measured this way and the complete focus map is calculated using interpolations. Currently a much faster and more robust hardware-based focus measurement method is under development which will allow a real-time measurement of every focus position even during stage movement. Following focus measurement the adjustment of the focus position is achieved using a synchronized piezo z-stage with a moving range of 300 microns.

As a next step the high-speed acquisition method is planned to be extended to fluorescence microscopy. If that is successful, this widespread microscopy technique will benefit from a considerable increase in speed as well.

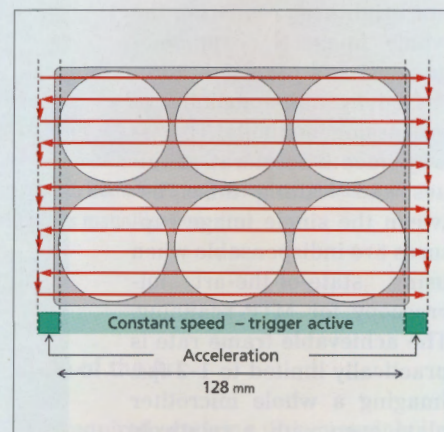


Fig. 4: Meander like scanning along the whole MTP with the least possible stops

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