Cell-based Genome Wide RNAi Screening with the novel scan^R Screening Station

Detection, Visualisation and Analysis – In collaboration with EMBL, Olympus have developed a fully automated and highly flexible microscope-based screening platform for life science.

Dr. Konstantin Joanidopoulos, Olympus Soft Imaging Systems GmbH, Gerrmany

The increasing need for scientific methods that allow large-scale functional studies of molecules in their natural environment is becoming more and more apparent. Automation, the handling and analysis of large sets of data all have to be considered. For this purpose Olympus have developed the scan^R Screening Station, on the basis of a custom built screening set-up that was the result of a close co-operation between Olympus and European Molecular Biology Laboratory (EMBL)[1]. scan^R accommodates both the demand on throughput and reliability for functional cell-based assays and the flexibility for leading edge assay development.

scan^R is a modular microscope-based imaging platform for scientific screening and large scale data analysis. Based on advanced microscope technology, scan^R can perform automated image acquisition and analysis with speed and precision. It is ideally suited for a wide range of applications and can be adapted and optimised for very different requirements. Moreover, it is the perfect system for the analysis of biological samples on a variety of formats such as multi-well plates, slides or any custom built array. The scan^R analysis module provides complex image analysis and advanced data evaluation tools.

Different particle and object detection functions can be selected and combined with segmentation for efficient and robust image analysis. From these detected objects further in-depth cytometry-orientated analysis can be carried out. Complex multi-parameter data analysis schemes can be set up easily by gating and classification.

Here we present two very different cell-based assays that show the broad application range and adaptability of the scan^R Screening Station.

Intracellular analysis of protein transportation in fixed cells

With the sequencing of the human genome and the notification of 20 – 30 thousand open reading frames (ORFs), modern biomedical science now focuses on the identification, function, regulation and interaction of these potential genes. At the EMBL, Heidelberg, the scan^R Screening Station is used to perform a genome wide RNAi screen, developed in the group of Rainer Pepperkok[2], with the aim of identifying, all human genes that are involved in and/or interact with the intracellular transport machinery.

1 *scan^R screening system with plate-loading robot*

Automated transfection and immunostaining

The human siRNA library is spotted onto glass slides, together with transfecting agents in an array of 384 spots per slide[3]. HeLa cells are seeded onto the glass slides, where they are subsequently transfected. The spot diameter matches the field of view of a CCD chip when used with a 10x objective. The advantage of this spot array is that 384 experiments, each corresponding to a siRNA-spot, can be performed on one 3 x 1 inch standard glass slide. 4 of these slides are placed into a holder of the dimensions of a standard 96 well plate to allow for optional robotic loading.

2

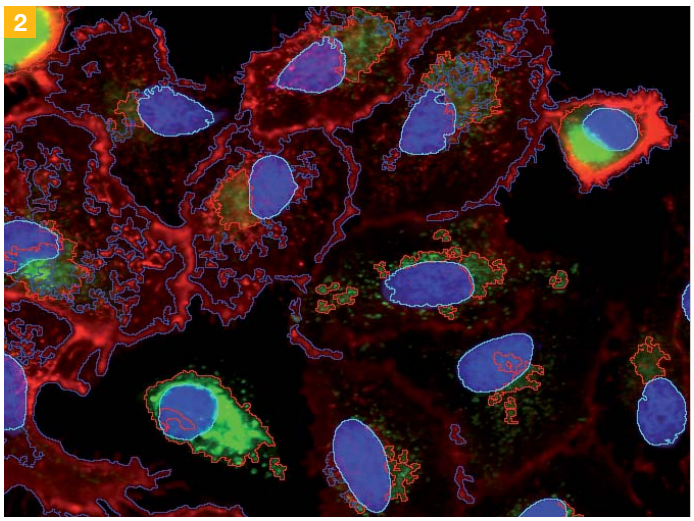


Image screen shots following data acquisition using scan^R, demonstrating the detection and separation of labels. The blue DAPI-stained nuclei are circled in cyan; the detected CFP tagged VSVG in the Golgi are circled in red and the detected Cy-5 VSVG antibodies at the cell surface are circled in blue.

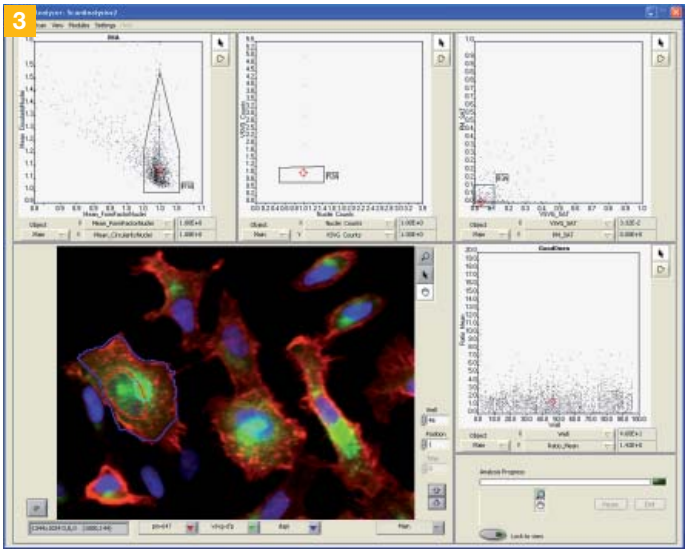
The secretory marker protein ts045 vesicular stomatitis viral G protein (VSVG), tagged with cyan fluorescent protein (CFP) is used as an intracellular transport indicator and can be visualised as it is released from the endoplasmic reticulum (ER) and transported through the cell to the plasma membrane where it is detected on fixed cells by a Cy5 conjugated antibody3. Cell nuclei are stained with DAPI. The scan^R screening platform is thus used to observe deviations in distribution and localisation of intracellular and membrane bound VSVG. High resolution 3-colour image acquisition is performed by the motorised Olympus IX81 inverted microscope. The powerful, object-oriented software enables rapid auto-focusing to maximise the number of cells in focus using a CCD camera. The system also incorporates the advanced MT20 illumination system with parallel operation capabilities, an 8 position filter wheel, to switch between chosen filters and a 14 position attenuator shutter for illumination intensity control. The MT20 offers stabilised intensity for quantitative measurements and fibre-coupled optimised illumination. A novel real time controller allows precise synchronisation and the deleterious effects of bleaching and photo-toxicity, are reduced to a minimum.

Visualisation, Detection and Segmentation

The Olympus scan^R software is comprised of an acquisition and hardware control module and an image and data analysis module. The modules are completely independent and can run on the same PC or on separate PCs. In both cases image and data analysis can be performed “on-line” in parallel to image acquisition.

The scan^R analysis module excels in data analysis and evaluation with a multi-step procedure that can extract multi-parameter image contents. For the automated analysis of the transport assay described above, DAPI-stained nuclei, are identified via an object-oriented detection algorithm. Starting from the nucleus a second and a third particle detection algorithm are applied. One to the cyan fluorescent protein (CFP) colour channel for the detection of the CFP-tagged VSVG protein and the other to the Cy5 colour channel, for the detection of the membrane-associated Cy5-anti-VSVG antibody. (Fig. 1) From the detected object, parameters such as intensity and geometric information are extracted for analysis. Calculations can be performed on these values and “derived parameters” are created. Subsequently these parameters can be plotted on 1D-histograms or 2D-scattergrams and data populations can be selected and gated. This powerful approach is commonly used in flow cytometry for the analysis of large data sets and was adapted to the analysis of image data.

“By gating,” only cells that match specific criteria are selected for further analysis. For the analysis of the VSVG assay, in a first step elliptical to round nuclei are selected for further analysis to exclude overlapping nuclei and irregularly shaped staining artefacts. The second gate dictates that cells with just one single area of VSVG labelling are considered, thus preventing quantification from neighbouring cells. The final gate removes cells with pixel saturation greater than 10%, to prevent biased results. Finally, the ratio of the intensity of the CFP-tagged VSVG intracellular signal and the membrane-associated Cy5-anti-VSVG antibody is calculated and plotted for every cell on every spot. The quantification summary for the entire plate or array is finally displayed. Spots with altered VSVG distribution will be detected immediately. Each data point is linked to the respective image. This allows immediate “quality control” of the applied image and data analysis procedures. (Fig. 2)



Gating, classification and data evaluation by multiple selection of data sub-populations according to specific criteria.

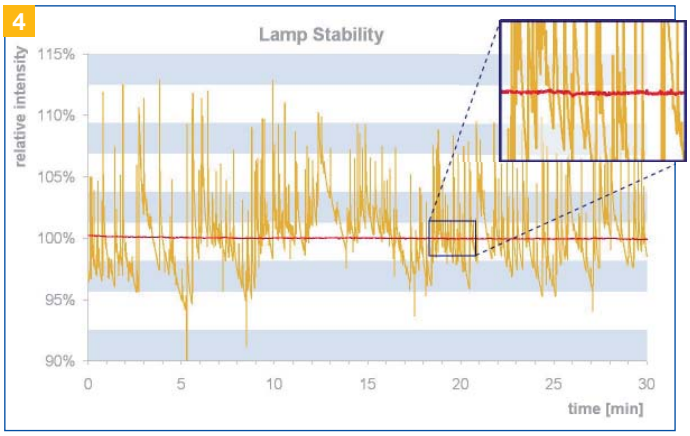
The challenges of live cell analysis

There has been a steady progression from the use of fixed cells for end point analysis, to live cell research, where it is possible to study dynamic events and follow the fate of specific molecules and cell components in vivo.

Rapid image acquisition to capture mitotic events

Mitosis is one of the most dramatic events in the life cycle of a cell. To test all presently known human genes for their involvement in mitosis the Mitocheck project group at EMBL (coordinated by Jan Ellenberg) has worked out, using scan^R ,an image based live cell screening protocol to analyse the frequency and dynamics of mitosis in siRNA transfected cells. Cell division is observed in a HeLa (Kyoto) H2B-GFP reporter cell line which is growing on siRNA arrays.

Since cell division occurs only within a frequency of 24 hours and lasts about 60 minutes, the cell cultures are observed for 48 hours and images captured from 1536 spots, every 30 minutes. This allows a quantitative observation of cell behaviour. Initially a 3D-focus map of the slide arrays is taken and then repeatedly applied to the slides with maximum speed, without re-focusing. Such accuracy is achieved through a specially modified and highly stabilised incubator, developed by EMBL, in addition to the high precision hardware of the scan^R screening station. High frequency image acquisition is an important requirement for long term, live cell experiments. The scan^R hardware is perfect for such applications as it incorporates the Olympus illumination system MT20. The MT20 features a highly stabilized Xenon or Xenon-Mercury burner for



Burner Stability. Comparison of the burner light output normalized to the average intensity (100%). Red: MT20; yellow: standard illuminator with mercury burner.

constant illumination intensity, a precondition for quantitative imaging and automated image analysis (Fig. 3). Additionally, the 8 position filter wheel and 14 position attenuator shutter, with millisecond closing time, are fully synchronized with the CCD camera. This ensures that exposure of the sample with light is reduced exactly to the exposure time of the CCD camera and bleaching and photo-toxicity are reduced to the absolute minimum.

Flexibility and Performance

The scan^R screening station for life science is a highly integrated, comprehensive modular system that can incorporate additional hard and software components for assay specific tasks covering a multitude of disciplines. scan^R can be used to investigate a plethora of cellular functions via single or multi-colour assays including; gene expression, intracellular transport and location, bacterial infection, cell proliferation and cell cycle analysis. It is therefore also possible to carry out extensive drug screening in complex cell cultures.

In addition to the advanced imaging capabilities previously discussed, scan^R boasts comprehensive software that allows detailed experimental design and analysis, therefore cell based assays can be rapidly developed and tested using the associated software. The format manager contains pre-stored plate formats for editing, providing arbitrary screening patterns for use on plates, slides and arrays. Plate loading robots can also be incorporated to increase throughput in a fully automated environment and long term observation of living cells can be achieved through the addition of the Olympus cell^cubator with sensitive temperature, humidity and CO2 control as well as a HEPA sterile-filtered closed airflow.

Conclusion

In conclusion the scan^R system has applications for the identification of relevant transport genes, using RNAi and the localisation of encoded protein during intracellular transportation via multi-colour labelling. The Olympus scan^R is a microscope-based screening platform for efficient acquisition of high quality imagery and importantly, the automated collection and analysis of bio-informatic data. The modular nature and open platform design of the scan^R allows the user to customise the apparatus for individual experimental design.

ACKNOWLEDGEMENTS

We thank the Pepperkok laboratory and the Mitocheck project team for providing data and comments on the text.

References

1. Liebel U, Starkuviene V, Erfle H, Simpson JC, Poustka A, Wiemann S, Pepperkok R. A microscope-based screening platform for large scale functional protein analysis in intact cells. FEBS Letters 554 (2003) 394-398.
2. Starkuviene, V., Liebel , U., Simpson, J.C., Erfle, H., Poustka, A., Wiemann, S., and Rainer Pepperkok. High Content Screening Microscopy Identifies Novel Proteins with a Putative Role in Secretory Membrane Traffic. Genome Res. (2004) 14 1948-1956.
3. Erfle, H., Simpson, J.C., Bastiaens, P.I.H. and Pepperkok, R. siRNA cell arrays for high content screening microscopy. Biotechniques (2004) 37 454-462.

Figures

1 scan^R screening system with plate-loading robot

2 Image screen shots following data acquisition using scan^R, demonstrating the detection and separation of labels. The blue DAPI-stained nuclei are circled in cyan; the detected CFP tagged VSVG in the Golgi are circled in red and the detected Cy-5 VSVG antibodies at the cell surface are circled in blue

3 Gating, classification and data evaluation by multiple selection of data sub-populations according to specific criteria

4 Burner Stability. Comparison of the burner light output normalized to the average intensity (100%). Red: MT20; yellow: standard illuminator with mercury burner

Information

Further information on the Olympus scan^R system and the screening assays described is available at:

www.olympus-europa.com/microscopy
www.embl-heidelberg.de/ExternalInfo/pepperko/index.html
www.mitocheck.org

Specifications are subject to change without any obligation on the part of the manufacturer.

OLYMPUS

OLYMPUS LIFE AND MATERIAL SCIENCE EUROPA GMBH
Postfach 10 49 08, 20034 Hamburg, Germany
Wendenstrasse 14–18, 20097 Hamburg, Germany
Phone: +49 40 237730, Fax: +49 40 23773647
E-mail: microscopy@olympus-europa.com