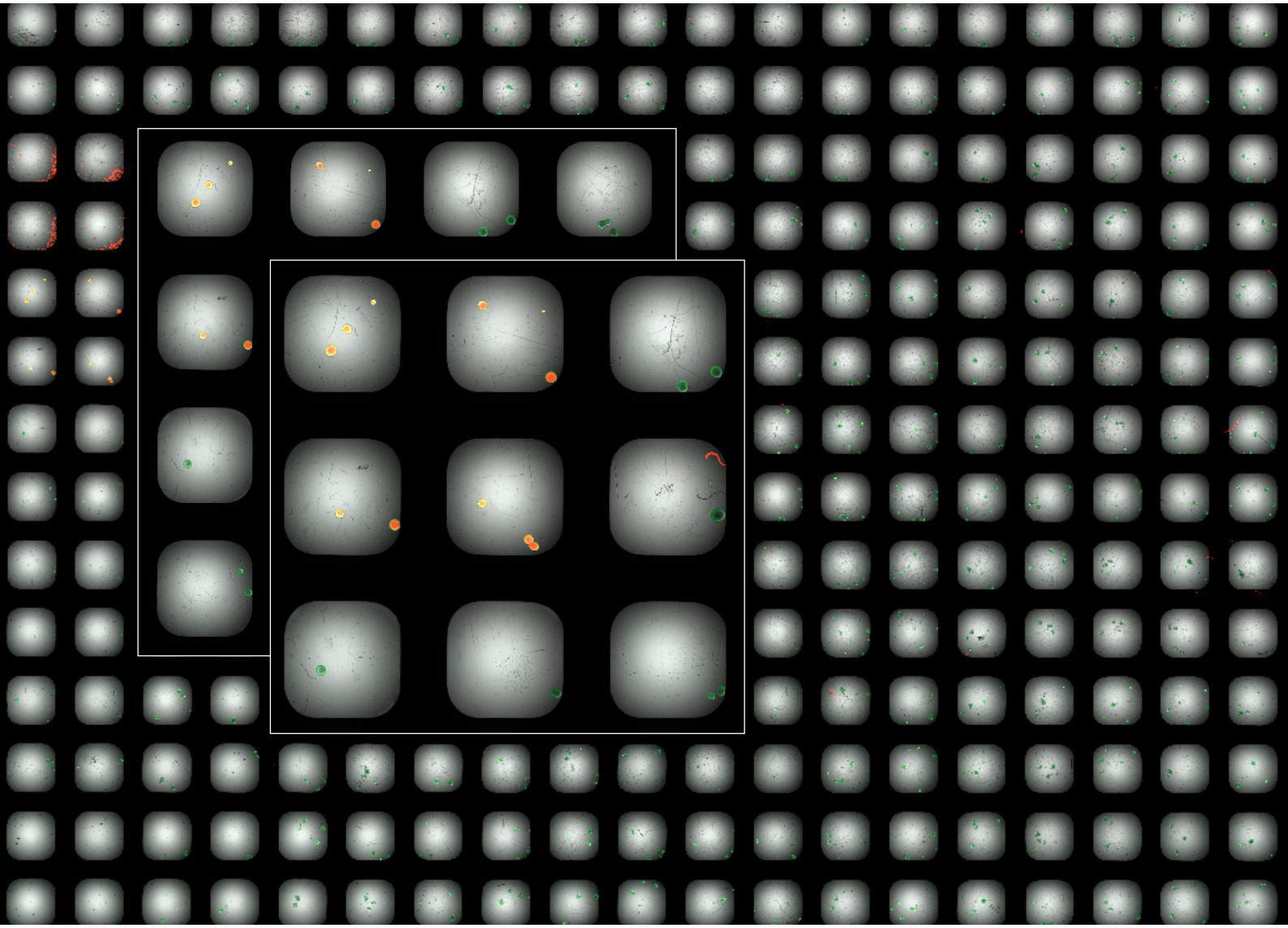

ZEISS Celldiscoverer 7

Analysis of three-dimensional cell culture using fast and sensitive widefield microscopy

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Seeing beyond

ZEISS Celldiscoverer 7

Analysis of three-dimensional cell culture using fast and sensitive widefield microscopy

Authors: René Buschow, Thorsten Mielke, Jesse Veenvliet, Marie-Laure Yaspo
Max-Planck-Institute for Molecular Genetics, Berlin, Germany

Jennifer Bischoff
Greiner Bio-One GmbH, Frickenhausen, Germany

Volker Doering, Thorsten Kues
Carl Zeiss Microscopy GmbH, Jena, Germany

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Introduction

The use of three-dimensional cell culture models emerged as fundamental tool over the last years throughout various life science disciplines. Among those are growing fields such as tumor and translational biology as well as developmental and basic cell biology.

Experimental challenges like tracing of individual events, marker-based signal quantification, large throughputs in time-series or end-point measurements have been widely solved in conventional 2D cultures. Introduction of the next spatial dimension adds an additional degree of complexity and novel tasks regarding acquisition and analysis of diverse samples. Further, identification and quantification of cellular and compartmental events within those complex three-dimensional structures must be conducted in minimal harming condition to keep the underlying biology intact (source 1).

3D culture systems often require special supports with thick well bottoms, non-planar u-bottom shapes or very high skirt heights which are challenging for most optical devices as well as automation routines and analysis algorithms. Here we present new strategies for the investigation of 3D cell culture models exploiting ZEISS Celldiscoverer 7. A special interest will lie on the post-processing of the acquired images enabling to use the instrument as real High-Content-Imaging platform (source 2).

This application note presents three experimental workflows based on ZEISS Celldiscoverer 7 imaging platform in combination with Perkin Elmer Columbus, arivis Vision4D as well as ZEN image analysis and post processing functions, integrated into the software package ZEISS ZEN (blue edition). The involved software packages are able to conduct quantitative analysis in an automated yet flexible way, making them an ideal tool for a complex and diverse academic research environment.

Example I: Compound Response

Two-dimensional cell models have been used for a long time as gold standard to gain insights into cellular responsiveness, signal transduction mechanisms, sensitivity and/or specificity towards compounds (source 3, 4). Within this example, we introduce an approach for the analysis of label-free 3D cell cultures over time using 384 multiwell plates. Our approach enables the characterization of up to 70 and more compound-conditions plus controls in quadruplicates. The Greiner Bio-One 384-Well Bioprinting Kit (GBO #781976) including NanoShuttle™ (source 5) and the magnetic 384-well spheroid drive was used for the preparation of organoids. The huge advantage of this system is that, due to magnetic bioprinting, the cell aggregation is independent of geometric formats. This way, the system provides optimal physical properties for sensitive high quality imaging.

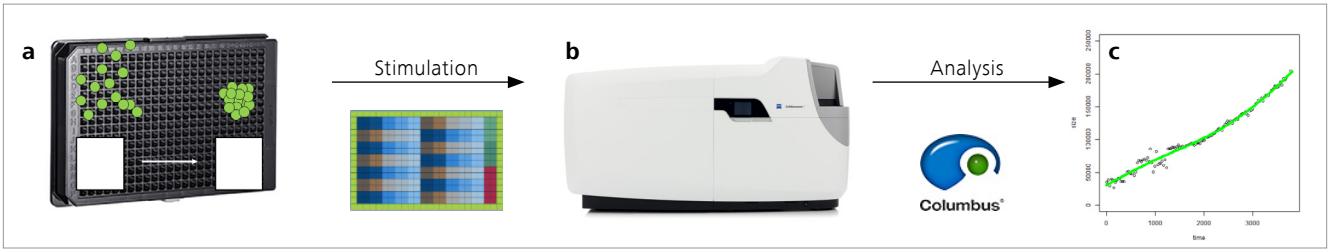

Figure 1 Experimental pipeline for 3D cell culture in 384-Well formats a) *Within the presented strategy, we use the Greiner Bio-one 384-Well Bioprinting Kit. Herein, 250 cells/well were incubated with Nanoshuttle™ (1 µl / 10,000 cells) in a 384-Well Microplate with Cell-Repellent surface (GBO #781976-SIN) and placed on the bioprinting magnet drive. The spheroids are formed within 2 hours. On the next day, spheroids are stimulated with the given compound panel in quadruplicates (scheme between a) and b)) **b)** ZEISS Celldiscoverer 7 allows automated image acquisition under stable environmental conditions (temperature, CO_2, humidity) for 72 h and more. Image data is automatically transferred into Perkin Elmer Columbus storage and analysis environment. **c)** Analysis results (total area - Y - plotted over time - X -) from Perkin Elmer Columbus is visualized using third party software (ImageJ/R).*

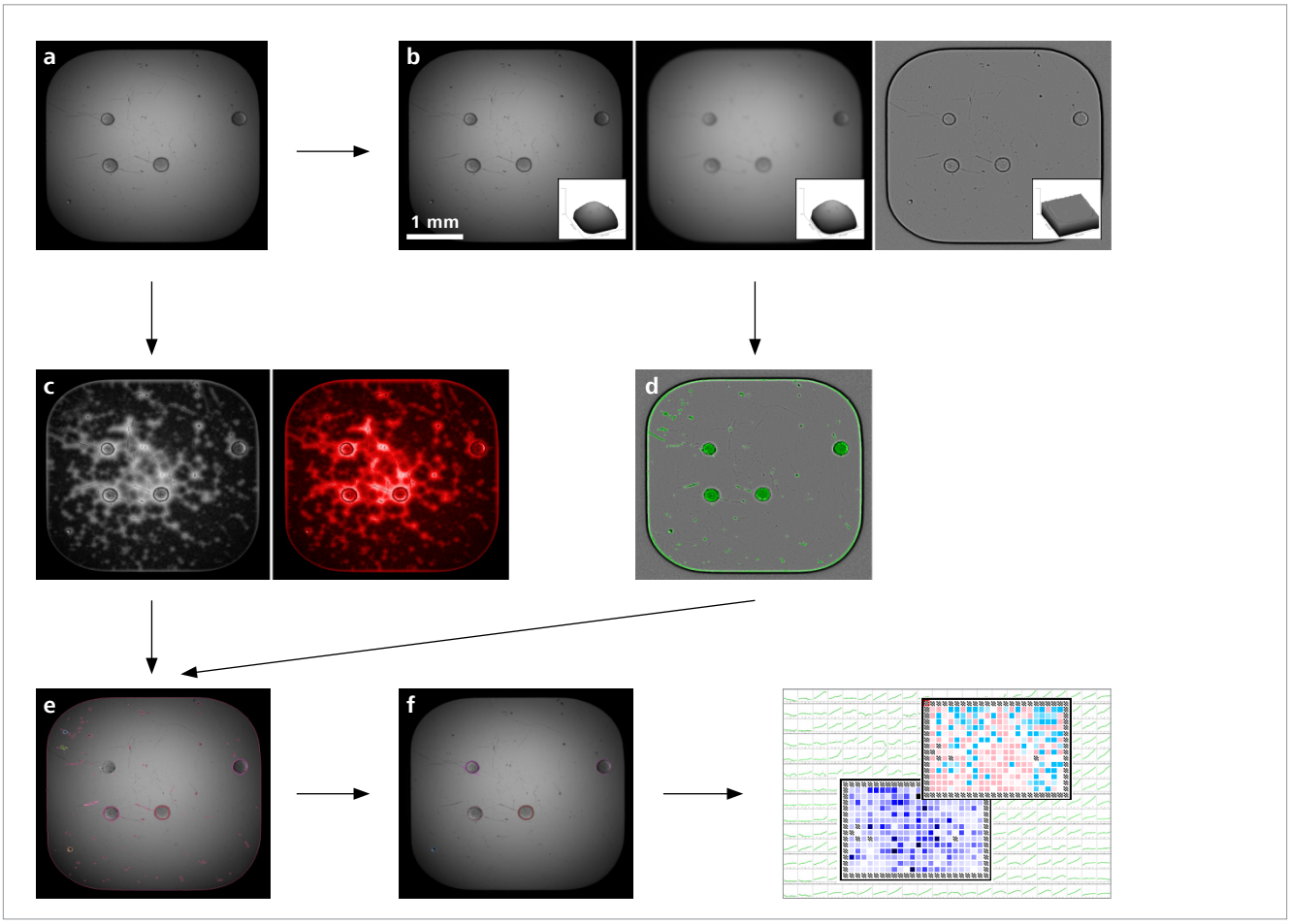

Figure 2 Perkin Elmer Columbus Analysis Strategy *The illustrated routine is performed on more than 40,000 images in a step wise manner.*
a) *Load raw image input.* ***b)*** *Custom-developed correction of image artefacts (liquid meniscus and well-geometry), shown are single steps with the matching surface plots.* ***c)*** *Identification of primary region of interest, (i.e. total well bottom surface).* ***d)*** *Identification of secondary regions of interest (all objects within the primary object).* ***e)*** *Overlay of raw images with secondary regions of interest and parametrization (geometry, intensity, texture of raw images a)).* ***f)*** *Filtering of true positive objects based on pre-defined geometrical parameters.* ***g)*** *Data visualization and further analysis using third party software (ImageJ and R).*

The resulting data sets can be used to make predictions for substance efficiency as well as to provide important information about biological functionalities in basic or translational research.

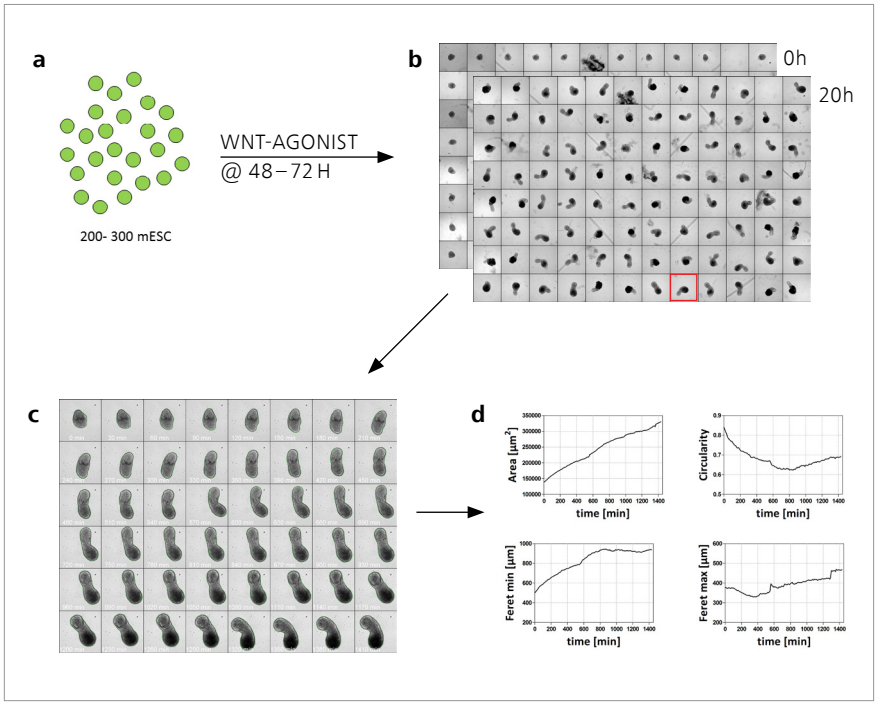

Figure 3 Monitoring of embryo-like-structures a) *Cell suspensions of 200 – 300 murine embryonic stem cells were cultured in a 96-Well ULA plate and stimulated with a WNT-agonist.* ***b)*** *The subsequent differentiation process is monitored fully automated using the ZEISS Celldiscoverer 7.* ***c)*** *Acquired image data is transferred to an analysis routine integrated into ZEISS ZEN blue. Geometrical parameters like perimeter, convexity, area and ellipticity are analyzed automatically.* ***d)*** *The comparison of phenotypes provides information on how the development is influenced by the different treatments.*

Example II: Monitoring of embryo-like-structures

The second example is a developmental biology application. The goal here was monitoring of the self-organization of embryonic stem cell aggregates. To this end, we used the protocol as published by Beccari & colleagues (source 6). We transferred their differentiation approach into ULA 96-well formats, integrated an automated image acquisition using ZEISS Celldiscoverer 7 and analyzed data in ZEISS ZEN (blue edition). Within these three-dimensional structures we investigated the morphometric changes.

We used a 5× objective with a numerical aperture of 0.35 offering large field of view and working distance.

The combination of multiwell plate formats with embryonic stem cells and modern imaging is able to provide deep insights into the self-organization of developing (stem-) cells systems. Moreover, the working strategy presented here has the potential to reduce, replace and refine animal experiments in developmental biology and probably future routine toxicity tests.

Example III: Elucidation of complex structure composition

Unravelling the composition of three-dimensional cell aggregates using light microscopy approaches comprise diverse challenges. First, penetration and capturing depth of the excitation and emission light in widefield microscopy is limited, depending on light-scattering properties of the sample. Second, the heterogeneity of 3D cell systems or conditions requires a significant throughput. Moreover, working in multiwell plate formats using confocal or multiphoton approaches producing superior optical sections, is time consuming and often phototoxic. The ZEISS Celldiscoverer 7 widefield system allows the acquisition of 96 individual Z-stacks (~20 planes) in 4 channels in less than 30 min. Further, to overcome limitations in the optical sectioning in Z-stacks, the raw data can be deconvolved to significantly increase contrast, signal-to-noise ratio and resolution. The Deconvolution module of ZEISS ZEN (blue edition) imaging software provides different algorithms including depth variant point-spread-functions for deep imaging. In combination with high-end autocorr objectives it delivers excellent image quality from thick samples, thereby offering maximum information density. The deconvolved, high resolution data generated in this way can be used for further processing and analysis such as evaluation of the cellular composition of spheroids/organoids with arivis Vision4D.

The arivis Vision4D software package allows a streamlined, batchable convertion of multicolor Z-Stacks acquired on ZEISS Celldiscoverer 7. Within a customer build analysis pipeline, nuclei are identified using the so called "blob finder"-function to segment 3 dimensional objects. In a next step, the identified objects are filtered by customer-chosen volumes to analyse only nuclei with proper dimensions. From these positive objects we interpolated fluorescence intensities in all channels (here specific cell markers in red and green).

To identify cell populations, a customer-based thresholding allocated objects/cells as marker positive or negative, and allows sorting of the objects into subgroups (compare figure 4d). The combination of arivis Vision4D and ZEISS Celldiscoverer 7 allows for a real quantification of cellular composition in 3 dimensional cell systems in a reasonable time.

Conclusion / Summary:

The interaction between ZEISS Celldiscoverer 7 and downstream platforms as Perkin Elmer Columbus, arivis Vision4D, ZEISS ZEN (blue edition) and fully customizable pipelining allows for a streamlined and efficient image processing and analysis. This, in combination with a high degree of experimental automation, qualifies ZEISS Celldiscoverer 7 as a real High-Content-Imaging-platform in academic research.

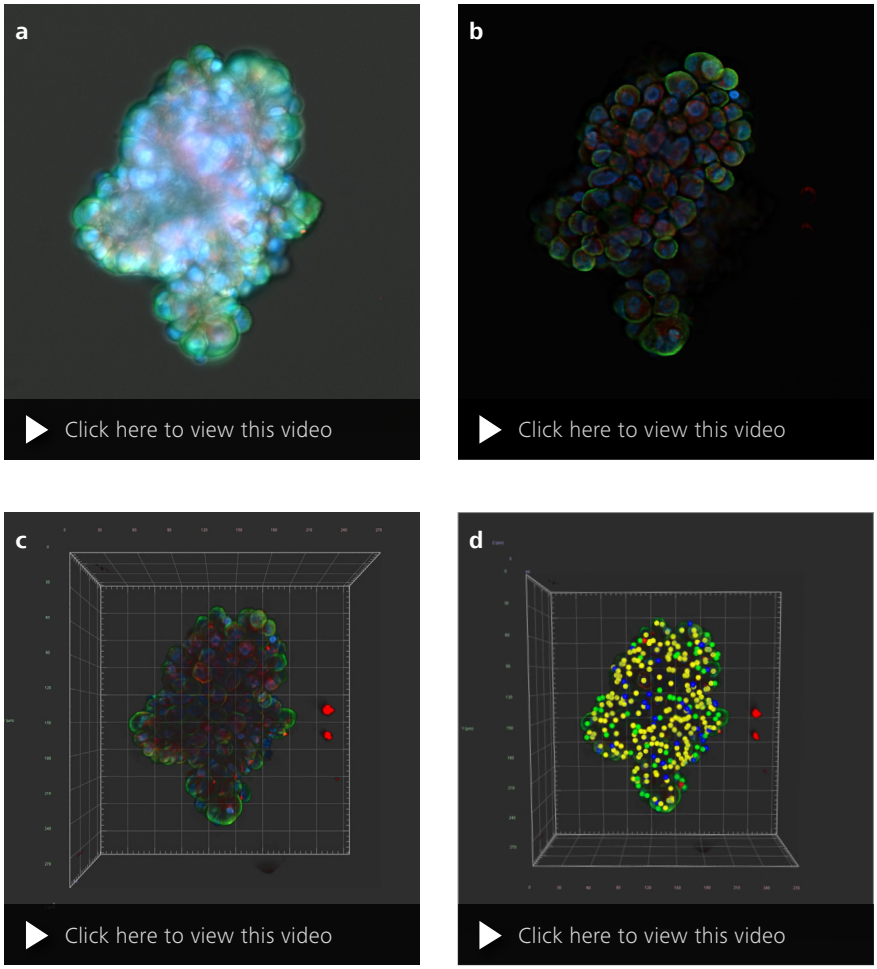

Figure 4 Elucidation of cell composition in 3D. *The primary tumor cells depicted here formed aggregates in 96-Well ULA plates and were triple stained with a nuclear counterstaining (blue) and two sub-population dyes (red and green) and imaged in a 96-Well SCREENSTAR plate (GBO #655866).* ***a)*** *Shows a top view of the raw Z-stack acquired with ZEISS Celldiscoverer 7 using a 5×/0.35 objective with 2× post-magnification for a total magnification of 10×.* ***b)*** *The same data after using the constrained iterative algorithm with the Deconvolution module for ZEISS ZEN (blue edition).* ***c)*** *The results of the calculated projection rendered in 3D.* ***d)*** *The results of the image analysis with the arivis Vision4D software, yellow circles show cells that are both red and green, green and red cells that belong to only one subpopulation, blue no subpopulation. Scale bar indicates 100 µm.*

Literature (PMID)
[1] 21042962
[2] 28520521
[3] 30002710
[4] 24831787
[5] 28524079
[6] 30283134

Carl Zeiss Microscopy GmbH
07745 Jena, Germany
microscopy@zeiss.com
zeiss.com/celldiscoverer