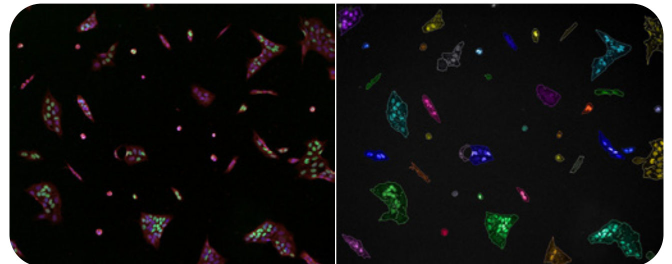
CASE STUDY

A workflow to characterize and benchmark human induced pluripotent stem cells using the Operetta high content analysis system

Human induced pluripotent stem cells (iPSCs) offer tremendous opportunities for disease modelling and discovery of novel therapeutics. To advance iPSC technology, the Human Induced Pluripotent Stem Cell Initiative (HipSci) offers the scientific community access to a large panel of cell lines with thorough characterization and data analysis tools[1]. Bringing together clinical genetics with genomics, proteomics and cell biology, HipSci has generated a UK national iPSC resource, with over 500 iPS cell lines from healthy volunteers and several hundred lines from individuals with genetic disease, to discover how genomic variation impacts on cellular phenotype[2]. One of the key partners in HipSci, the Centre of Stem Cells and Regenerative Medicine directed by Prof. Fiona Watt, is now implementing a 'Stem Cell Hotel' to engage with external scientists from academia and biotechnology companies. Within this framework, the HipSci Cell Phenotyping group led by Dr. Davide Danovi, is developing 'cell observatory' assays to study cell responses to chemical, physical or biological stimuli. Assay and image analysis pipelines have been used in particular to characterize iPSCs from multiple donors.

Human iPSCs exhibit considerable phenotypic variation within the same cell line and between different donors. In order to characterize and quantify this heterogeneity, the Cell Phenotyping group has developed a simple, highly reproducible high content assay to phenotypically quantify cell behavior. Analyzed features include cell proliferation, cellular and nuclear morphology and intercellular adhesion. Collating this data with genomics and proteomics will ultimately help to understand gene function and contributors of variance in iPSCs. This case study details the phenotypic screen used to characterize human iPSCs on diverse extracellular matrix substrates that was described earlier[3]. Furthermore, it describes how to distinguish single cells from cells in clumps, which allows the capture of specific phenotypes emerging upon cell-to-cell contact. The first set of images analyzed using this method have been released and are freely available[4].



Experimental overview

To establish a scalable workflow for the characterization of a large panel of iPSCs, the group first aimed to identify an effective, robust and inexpensive substrate promoting cell attachment and spreading. A sufficient number of single cells (vs. cells in clumps) was also considered important for substrate selection. In total, 74 extracellular matrix (ECM) conditions, including single ECM proteins and mixtures of different ECM proteins, were tested. Human iPSCs from a single control line in undifferentiated culture conditions were seeded on the different ECM coatings. After 24 hours, cells were labelled with DAPI, CellMask Deep Red and EdU (30 min pulse) to label the nucleus, cytoplasm and replicating cells, respectively.

For research use only. Not for use in diagnostic procedures.

Phenotypic analysis

Phenotypic analysis of the images acquired on the Operetta® system was performed in Harmony® high content analysis software (Figure 2). Briefly, nuclei were detected on the DAPI channel and the nucleus area, roundness, width to length ratio and DAPI median intensity were calculated. Next, the median intensity in the EdU channel was determined. Filters were set to remove feeder cells and artefacts by deselecting very small or large nuclei, or very weak or intense nuclei. Using this valid nuclei population, the cytoplasm was detected on the CellMask Deep Red channel and border objects that were not completely imaged were removed. Finally, cell morphology parameters such as cell area, roundness and width to length ratio were calculated and the intercellular adhesion was determined by the *cluster by distance* method in the *modify population* building block. By setting the distance to 0, all cells touching each other were defined as "Clump". Finally, the number of cells per clump was calculated. For cells not touching each other, i.e. "Singles", this was equal to 1. This strategy allows the efficient capture of phenotypes emerging upon cell-cell contact. Image analysis results were then transferred to the TIBCO Spotfire® based software High Content Profiler™ for secondary analysis.

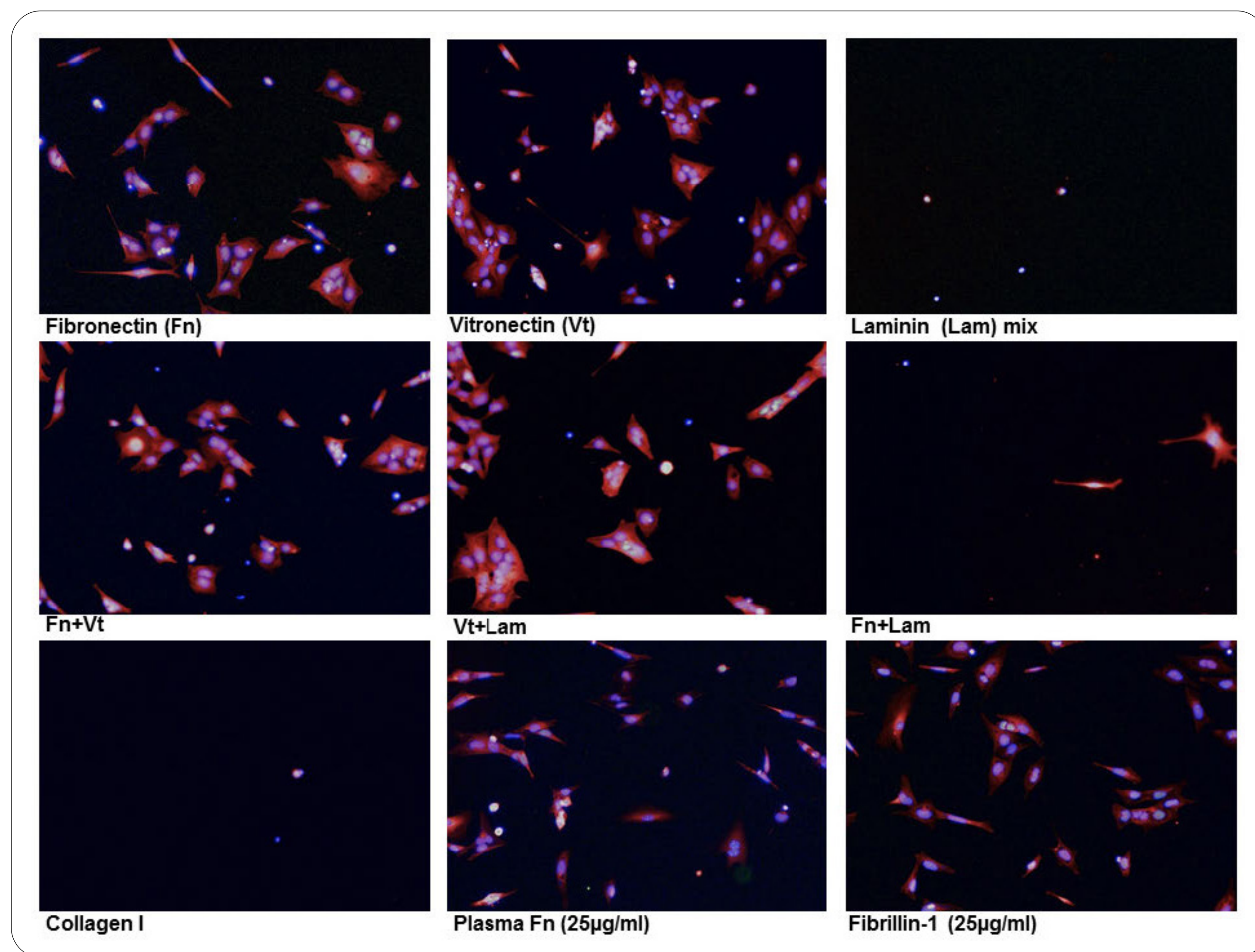


Figure 1: Phenotypic variation of iPSCs within the same cell line induced by different ECM conditions. Human iPSC morphology was analyzed 24 hours post seeding on 74 different ECM conditions (only selected conditions are shown here) using the Operetta system with 10 x magnification. Individual cell attachment (total number of cells) as well as different amounts of cells in clumps vs single cells can be observed on the different ECMs (Scalebar = 100 µM).

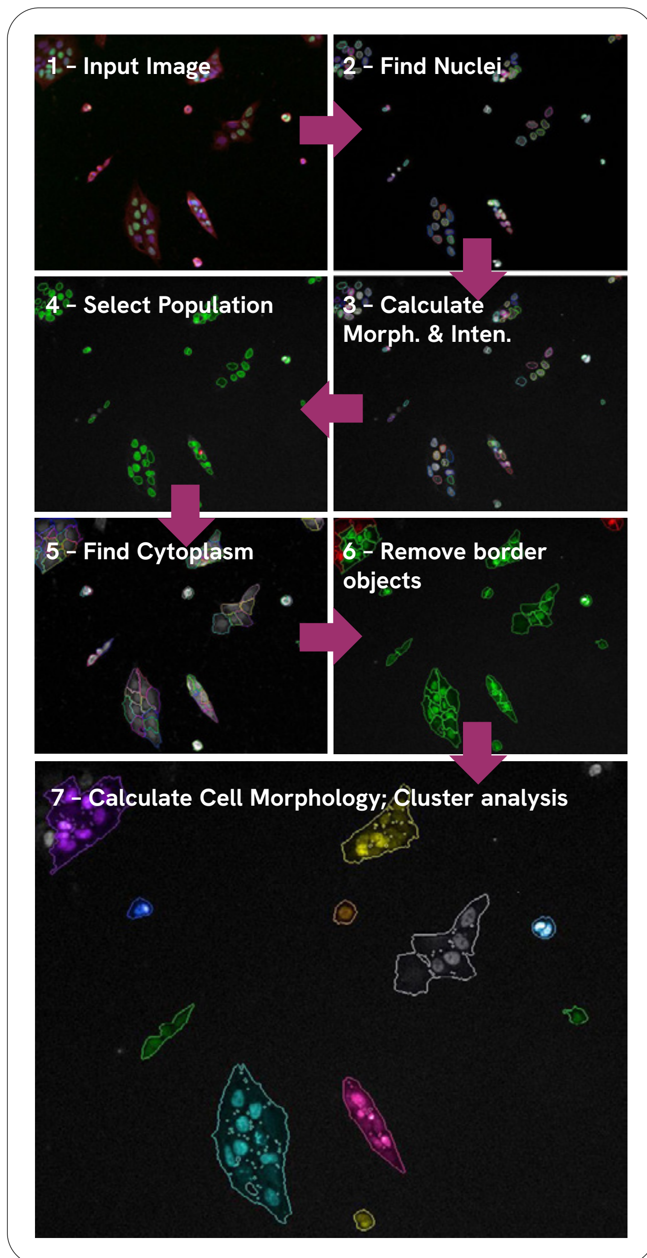


Figure 2: Image analysis strategy for phenotypic analysis of human iPSCs using Harmony software. Channels used: DAPI [Nuclei], Alexa488 EdU [replicating cells], CellMask Deep red 647 [cytoplasm]. Quantified phenotypic features include single cell variations (e.g. cell or nuclear roundness, area, width to length ratio) and aggregation features (single cells vs. cells per clump).

The ECM screen was performed in two different plates named “Orla” and “Manchester”, which were seeded as represented in the plate layout generated in High Content Profiler (Figure 3a). Plotting the XY coordinates (based on centroid position of each cell) within each well, leads to a visualization of the cellular distribution (Figure 3b), which can be indicative for coating, seeding or washing artefacts within a screen.

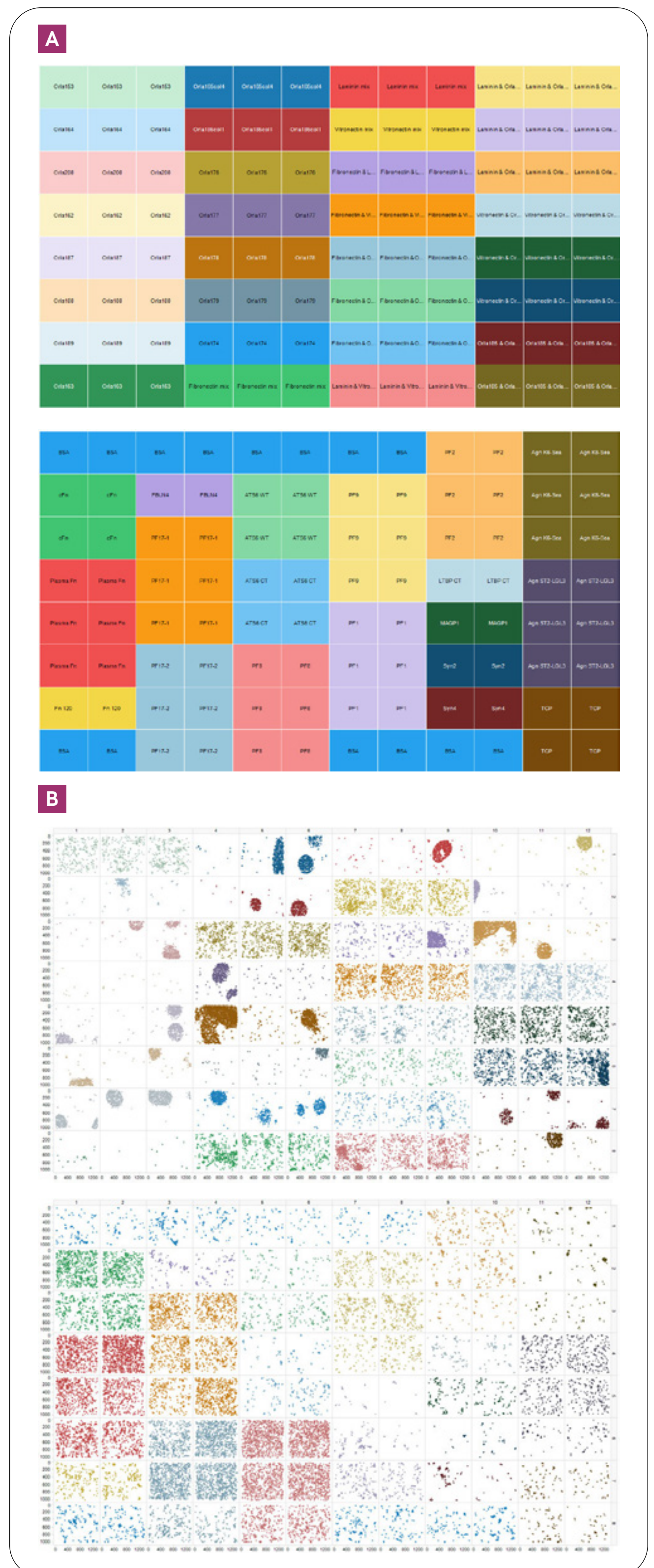


Figure 3: a) Plate Layouts, color coded, for “Orla” (top) and “Manchester” (bottom) as generated in High Content Profiler. b) Single cell plate layouts, color coded, showing centroid position of all cells, irrespective of individual cell morphology. Single cell plate layouts show differences in cell numbers and aggregations.

To create meaningful visualizations of the phenotypic screening data, nine ECM coatings with a broad range of phenotypes and attachment behaviors were chosen (Figures 4-7). Overall, Plasma Fn and PF8 promoted the highest level of cell attachment and a reasonable number of single cells vs. cells in clumps (Figure 4). Cells on PF8 and Plasma Fn also showed a relatively large phenotype (Figure 5) and formed a tight phenotypic cluster within principle component analysis (Figure 7). Based on these results and practical reasons, such as cost and robustness, the authors decided to use plasma fibronectin in further experiments. A more detailed description of cellular phenotypes on Plasma Fn and a comparison of single cell vs cell clump phenotypes has been published[4].

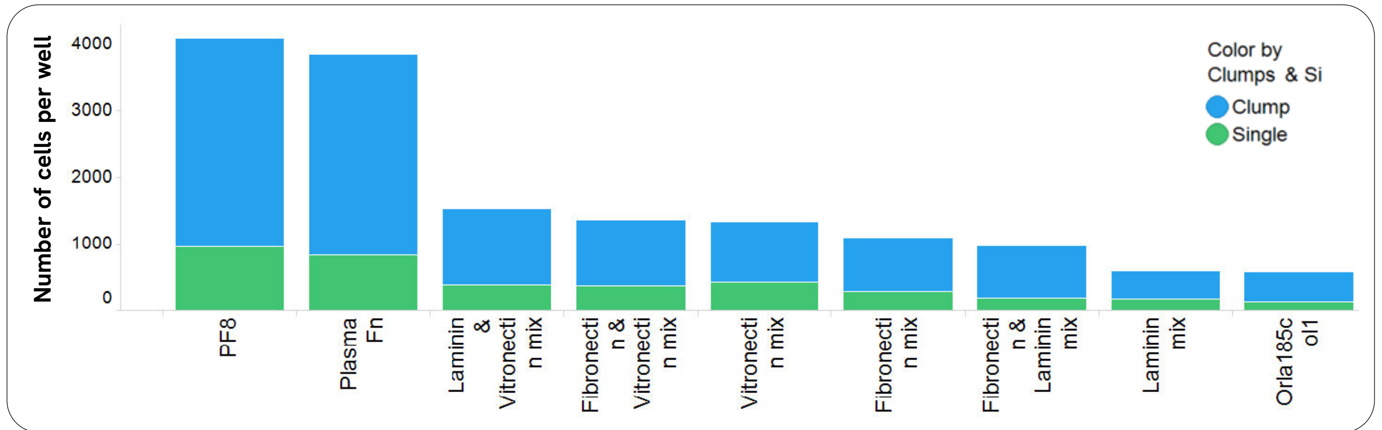
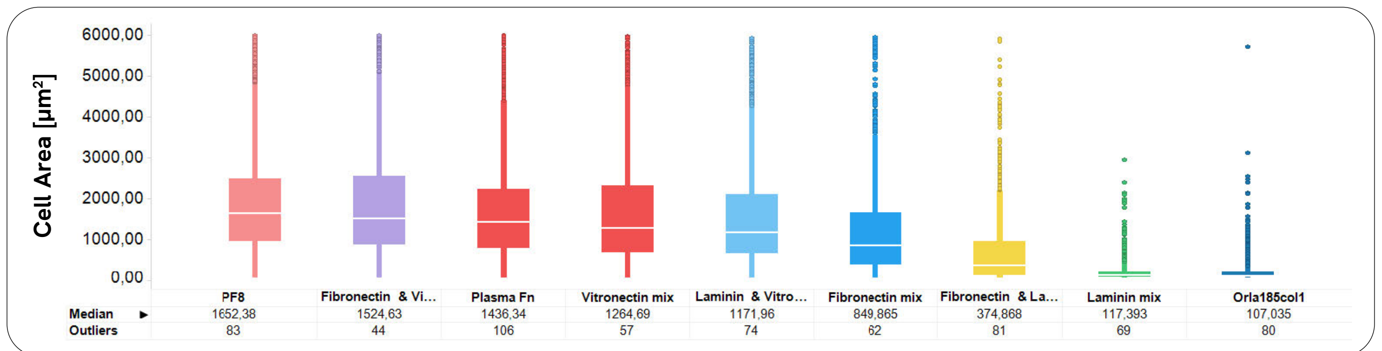
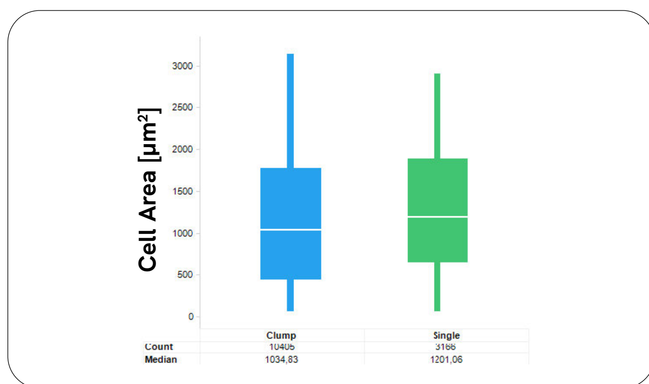


Figure 4: Different ECM coatings promote different levels of cell attachment. A bar chart was generated with TIBCO Spotfire® and sorted by value. After 24 hours, more cells were detected on PF8 and Plasma Fn due to favorable adherence, survival and proliferation. Laminin mix and Orla185col1 led to the fewest number of cells.



	PF8	Fibronectin & Vi...	Plasma Fn	Vitronectin mix	Laminin & Vitro...	Fibronectin mix	Fibronectin & La...	Laminin mix	Orla185col1
Median ▸	1652,38	1524,63	1436,34	1264,69	1171,96	849,865	374,868	117,393	107,035
Outliers	83	44	106	57	74	62	81	69	80

Figure 5: Phenotypic differences induced by ECM coatings. The median cell area in µm² was plotted against the nine different ECM coatings using a Box plot and sorted by value. Cells are significantly larger (>10 x) on PF8 compared to Laminin and Orla185col1.



	Clump	Single
Count	10405	3166
Median	1034,83	1201,06

Figure 6: Comparison of cell area for single cells and cells in clumps. Across the nine ECM coatings, single cells are slightly larger than cells growing in clumps. However, the majority of cells are in clumps (data table).

Distribution of wells after PCA

Data table:
hc_Aggregated cells

Marker by
(Row Number)

Color by
protein coatings

- Fibronectin & Laminin...
- Fibronectin & Vitrone...
- Fibronectin mix
- Laminin & Vitronectin...
- Laminin mix
- Orla185col1
- PF8
- Plasma Fn
- Vitronectin mix

hc_Comp.2

hc_Comp.1

hc_Comp.3

Figure 7: Principle component analysis (PCA) to visualize phenotypic variation across the nine ECM coatings. Using all calculated phenotypic features, a PCA was performed using the High Content Profiler plugin of TIBCO Spotfire®. The nine ECMs lead to three more or less distinct phenotypic clusters.

Conclusion

This case study illustrates how the Operetta high content analysis system is enabling stem cell research by generating phenotypic signatures of iPSCs. While the experiments described here focus on a single iPSC control cell line, the methods developed have been the foundation for up-scaling to characterize a much larger panel of human iPSCs. One of the main initiatives of the HipSci consortium is to understand how genomic variation impacts cellular phenotypes. Besides genomic and proteomic analysis, high-content analysis is a key technology for this task, as it not only provides image-based phenotyping but also the required throughput to automatically analyze hundreds of cell lines. Secondary analysis tools such as TIBCO Spotfire® based High Content Profiler allow a better understanding of multiparametric datasets such as phenotypic profiles, as they provide tools for interactive data visualization, analysis and phenotype classification.

Dr. Davide Danovi

"We have used the Operetta and Operetta CLS high content devices extensively to quantitate cell behavior. It is important to combine an offer of intuitive off-the shelf solutions whilst allowing further analysis tools to be developed by users."

References

1. www.hipsci.org.

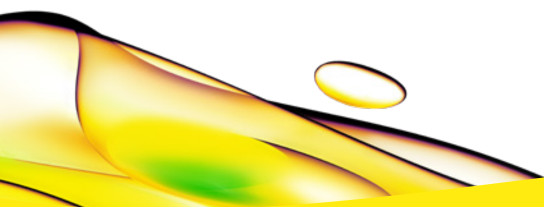
2. Kilpinen, H., Goncalves, A., Leha, A., Afzal, V., Alasoo, K., Ashford, S., et al. (2017). Common genetic variation drives molecular heterogeneity in human iPSCs. Nature, 546(7658), 370–375. doi:10.1038/nature22403.

3. Leha, A., Moens, N., Meleckyte, R., Culley, O. J., Gervasio, M. K., Kerz, M., et al. (2016). A high-content platform to characterize human induced pluripotent stem cell lines. Methods (San Diego, Calif.), 96, 85–96. doi:10.1016/j.ymeth.2015.11.012.

4. https://idr.openmicroscopy.org/about/.

Acknowledgement

Many thanks to Ruta Meleckyte and Mia Gervasio for providing the data described in this case study. Thanks also to Dr. Andreas Leha, Dr. Nathalie Moens, Prof. Cay Kielty, Dr. Stuart Cain and the other authors of the original study. Thanks also to Marie Mattock, Zuming Tang, James Hutt and Dr. Karin Boettcher (Revvity) for help with image analysis pipeline set-up, data analysis, generating the TIBCO Spotfire® visualization and writing of the manuscript.



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Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com

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