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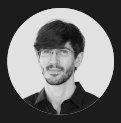
High-throughput measurement of cell confluency with a multi-camera array microscope

AUTHORS



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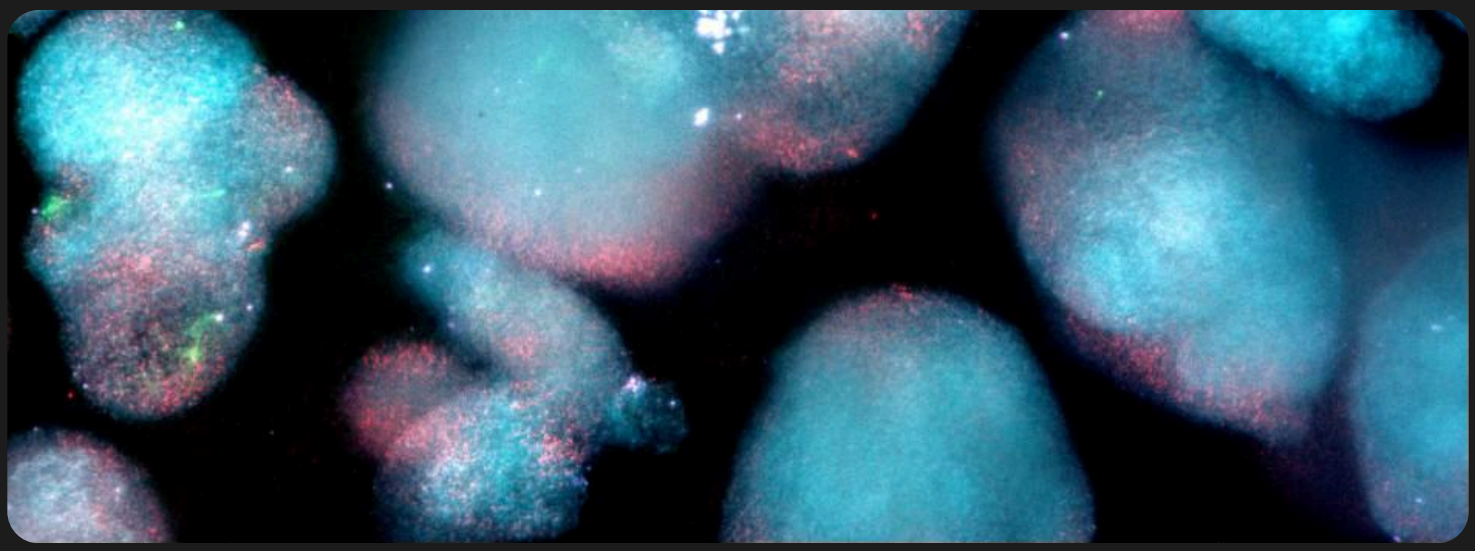
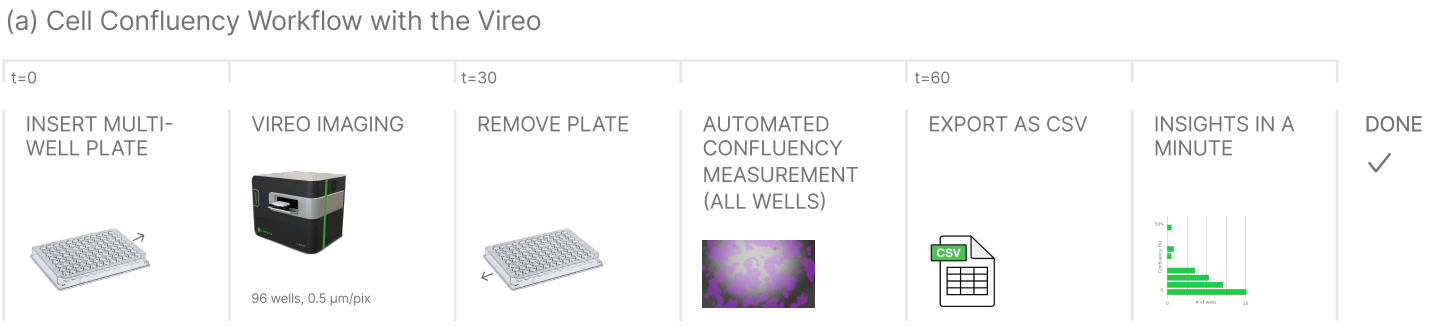
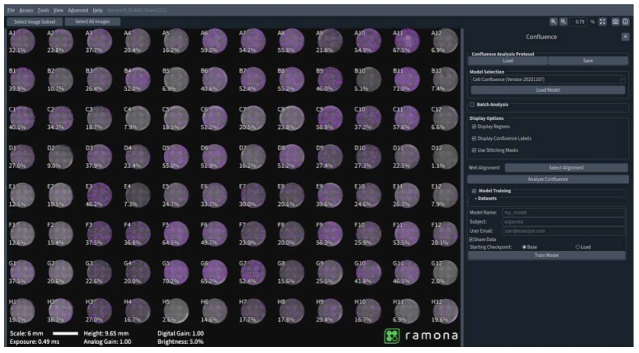
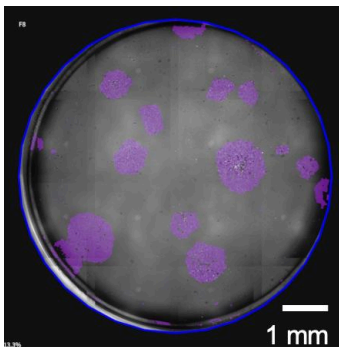


Abstract

In this work, we demonstrate how Ramona's Vireo™ microscope can quickly and accurately measure cell confluency across a variety of multi-well plate formats. The Vireo™ is an excellent system for general high-throughput microscopic image and video measurement of cells, organoids and small model organisms. Leveraging Ramona's unique Multi-Camera Array Microscope (MCAM™) technology, the Vireo™ scans and analyzes multi-well plates at faster speeds than any other imaging system on the market - accomplishing tasks that typically require 15+ minutes in tens of seconds. Here, we showcase the Vireo™ with rapid (< 1 minute) measurement of cell culture confluency, where it scans and statistically reports the area of each well within a multi-well plate that is covered by cells - a critically important measurement in a variety of workflows. After providing an overview of the Vireo™, we show how the system can be configured to automatically scan entire 6, 12, 24, 96 or 384 well plates and how customized machine learning software can rapidly segment, analyze, and report confluence statistics in a simple and openly interpretable manner.







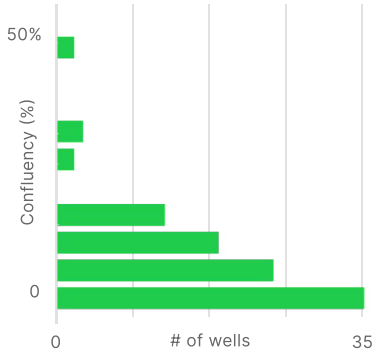


Figure 1: Overview of Vireo™ workflow for cell confluency measurement. (a) Within 1 minute, the Vireo™ images and analyzes all wells within any standard multi-well plate to produce accurate statistical reports of cell confluency. (b) Overview of an analyzed 96-well plate from Ramona's software. (c) Example well with cellular areas (pink) and well boundary (blue outline) automatically identified. (d) Plot of confluency results for 96 wells in (b).

Introduction

Cell confluency can be used in a variety of workflows, including to establish optimal growth conditions, to monitor when cells are in an ideal growth phase for a particular experiment, for quality control purposes, for metabolic and physiologic students, and for research consistency and reproducibility. Often, researchers utilize multi-well plates (6, 12, 24, 96 or 384 well plates) to culture and monitor different populations of cells, where cell confluency is generally measured across each of the wells individually. Likewise, researchers can either measure cell confluency at a single time-point, or can repeatedly measure cell confluency over time.

The Vireo™ microscope by Ramona Optics offers researchers the ability to rapidly obtain measurements of cell confluency across multiple wells of a multi-well plate and at different moments in time with a few different clicks of a button. The Vireo™ is the fastest such measurement system on the market - it uses an array of 24 individual compact microscopes to image whole plates in parallel with minimal lateral scanning for a simple, quick, and accurate means to quantitatively assess cell cultures. The Vireo's 24 microscopes are each outfitted with their own compact objective lens and 13 megapixel (MP) digital image sensor (312 MP total). Users may select between multiple bright-field and 4-channel fluorescence imaging modalities with the click of a button. For cell confluency, bright-field capture is used to enable unique applications in live-cell screening. The Vireo also includes a stage-top incubator with temperature, humidity and CO2/O2 control for such live-cell imaging studies. Additional details are available in Ref. [1-4], with more specifications at: **https://www.ramonaoptics.com/products/vireo.**

Alternative high-throughput microscopes that can measure cell confluency generally rely on autofocus to bring each well area into sharp contrast. Autofocus naturally fails in thicker specimen areas and can often lock on to incorrect z-planes to return blurry image data. In contrast, the Vireo automatically captures full z-stacks at each and every scan location, thus allowing the system to never miss an important well area. The Vireo™ uses focus identification software to carefully find the best focal plane in post-processing, thus ensuring that all data used for cell confluency measurement is of the highest quality. This helps avoid erroneous confluency measurements that are commonly encountered with alternative single-lens scanning microscopes.

In the following, we detail how the Vireo™ can provide key measurements of cell confluency by providing a step-by-step guide for how the microscope hardware and software can be configured to measure confluency and by showing example results. Additional resources are available at **docs.ramonaoptics.com.**

Measuring cell confluency with the Vireo, step by step

The first step in obtaining cell confluency measurements with the Vireo™ is populating a plate with a cell culture of interest. The Vireo™ microscope is configured to accept any format of SBS well plate. In the following workflow, we focus our attention on the popular 96 well plate format, but note that other multi-well plate formats (with 6, 24, or 384 wells, for example) are fully compatible with slight changes to the instructions outlined below

Here, we showcase cell confluency measurement with HCT116 human colorectal cancer cells prepared in 96 well plates but note that the workflow can be accurately applied to a wide variety of alternative cell types and well plate formats **(see below)**.

After inserting the well plate of interest into the Vireo™ and opening the MCAM™ Image Capture software, users next have the option to measure cell confluency at one of two differently supported imaging magnifications: 4X and 10X. It is currently recommended to use 4X imaging magnification. Depending on the conditions of the cells of interest, which can be established through visual assessment during live imaging, 10X magnification may be required, which is also an option. Below we list ideal imaging and cell confluency measurement workflows for both 4X and 10X capture. We note here that black-walled multi-well plates help minimize contrast loss at image edges, so are encouraged. In addition, the use of 200 µL of media is also preferred, as it leads to less image vignetting for bright-field image capture.

(a) 96-well plate image overview (4X)

(b) Select Wells

(c) Confluent Cell Area Overlay

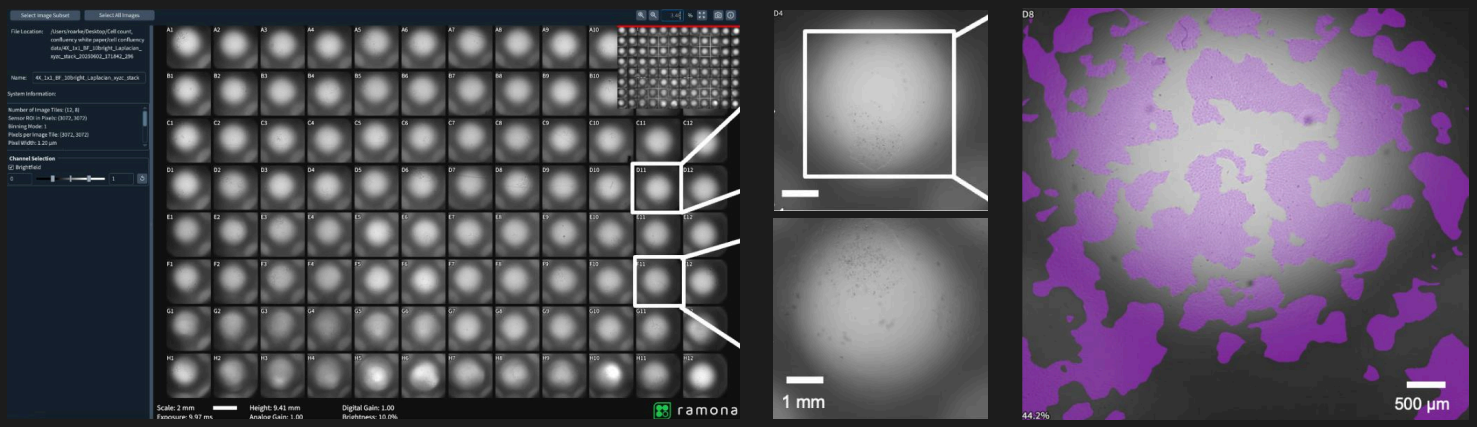


Figure 2: Ramona's MCAM Image Viewer displaying a 96 well-plate captured via XYZ stack at 4X resolution with (middle) example raw images and (right) example output segmentation mask overlay from Confluency Assay software.

Cell confluency workflow at 4X magnification

4X Vireo image capture scans the entire 96 well plate in 2×2 steps, resolving each full well in each unit microscope's full field-of-view. It is useful to use 10 z-slices at 100 µm step size to acquire a z-stack of every well to ensure that thicker specimen areas across the entire well plate are faithfully resolved. With the above settings, it currently takes 30 seconds to capture a z-stack across an entire 96 well plate with a Vireo outfitted with 24 4X objective lenses. We note that a time-lapse acquisition may additionally be used to capture and measure cell confluency as a function of time via the appropriate Acquire Time Lapse setting. Following image capture, users simply select "Compute Confluence" to obtain automated whole-plate analysis via a custom-developed machine learning segmentation model. This creates both a graphical overlay of confluent areas (Figure 2) for visual review, as well as an associated statistical report of confluency area, reported as a percentage of well area, for all wells (i.e., a 96-entry spreadsheet along with associated metadata).

STEP	FUNCTION
01	**Load** your **96-well plate** onto the MCAM.
02	**Acquire** and XYZ stack at **4x magnification** with **brightfield illumination** and **Laplacian projection.**
03	**Open the acquired dataset** in the MCAM™ Viewer software.
04	**Navigate to Assay > Confluence.** A panel will open on the right.
05	**Click “Compute Confluence”** to run the analysis.
06	Analysis results **are displayed** on the screen **and saved** in a folder named “confluence_results” in the parent folder of the analyzed dataset. Two files are generated, “analysis_metadata.nc” file containing the analysis results and a csv file summarizing the quantification of confluence on a per well basis.

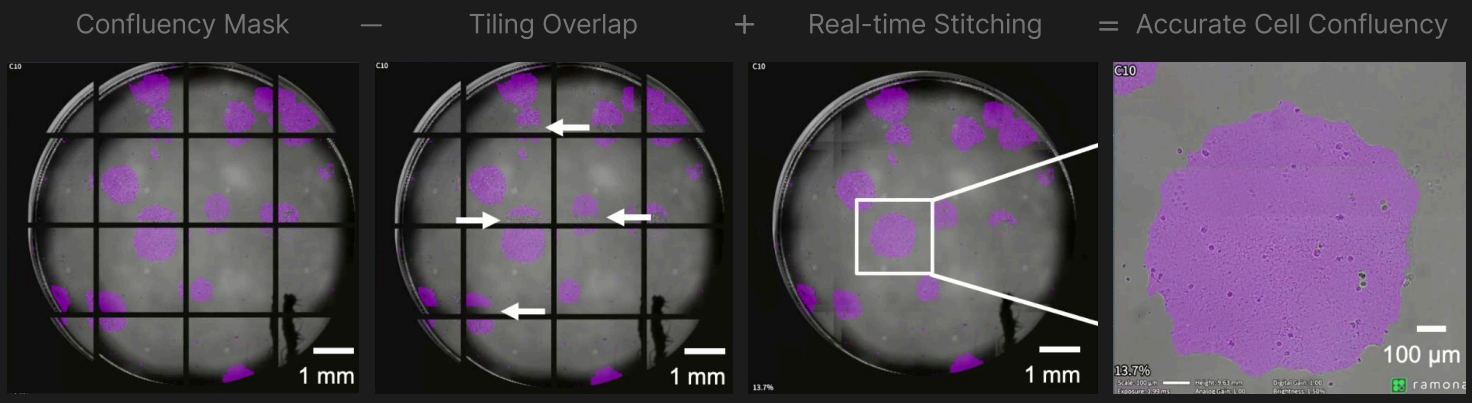


Figure 3: Visual overview of cell confluency calculation at 10X magnification with a 96-well plate. Following imaging wells with a 4X4 lateral scan (16 tiles per well), cell confluency is automatically calculated and displayed for each tile. A “well alignment” file is used to account for tile overlap to ensure accurate cell confluency is computed when multiple tiles are imaged per well.

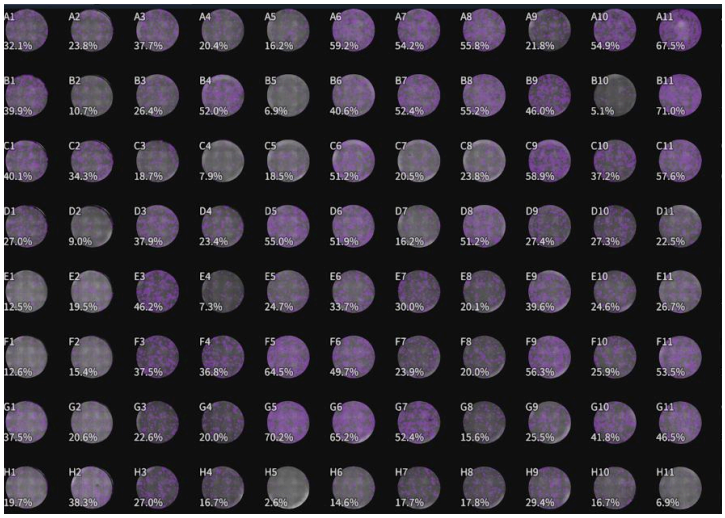
Cell confluency workflow at 10X magnification

As noted above, cell confluency data can be captured at 10X magnification, where additional time is required to scan across the well plate at higher resolution. 10X image capture scans 96 wells in 4×4 steps (a 3×3 configuration can also be selected for select 96 well plates). It is useful to use 20 z-slices at 20-30 µm step size for most applications. The total time required is 2 minutes for a 24-camera 10X Vireo system.

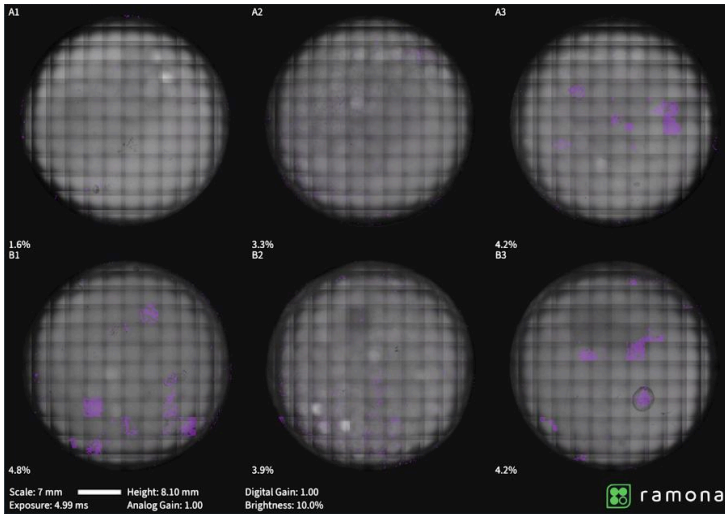
STEP	FUNCTION
01	**Load** your **96-well plate** onto the MCAM.
02	**Acquire** an XYZ stack at **10x magnification** with a **4×4 well plate scan** with **brightfield illumination** and **Laplacian projection.** Please see the workflow guide for “Full Wellplate Scan” for more information on acquisition.
03	**Open the acquired dataset** in the MCAM™ Viewer software.
04	Navigate to **Assay > Confluence.** A panel will open on the right.
05	**Select a well alignment file** specific to the well plate in use. Please see “Create a Well Alignment File” for more information.
06	**Click “Compute Confluence”** to run the analysis.
07	**Analysis results are displayed** on the screen **and saved** in a folder named “confluence_results” in the parent folder of the analyzed dataset. Two files are generated, “analysis_metadata.nc” file containing the analysis results and a csv file summarizing the quantification of confluence on a per well basis.
08	**Interpretation:** Regions outside of the well alignment radius are excluded. Regions in one half of overlapping images are excluded. The bottom and right overlap margins are excluded selectively. The percent confluent is displayed in the bottom left corner of the screen.

Following image capture, users can once again select “Compute Confluence” to automatically process the entire well plate. Due to the need to capture multiple images within each well area, a “well alignment” file is generally required for accurate confluence area calculation at 10X magnification on a per well basis. New well alignment files can be easily created via the “Create a Well Alignment File” button within the Ramona GUI. As diagrammed in Figure 3, well alignment files allow the Vireo to accurately take into account overlapping image areas created by Tiling Overlap during cell confluency calculation. Ramona’s software allows users to generate and view accurate confluency masks for each captured FOV at 10X. Often, image FOVs can partially overlap due to specific capture settings (i.e. to facilitate accurate image stitching). Via the “well alignment” file, Ramona’s software will automatically account for parameters such as FOV overlap to report highly accurate confluency area calculations in a flexible manner.

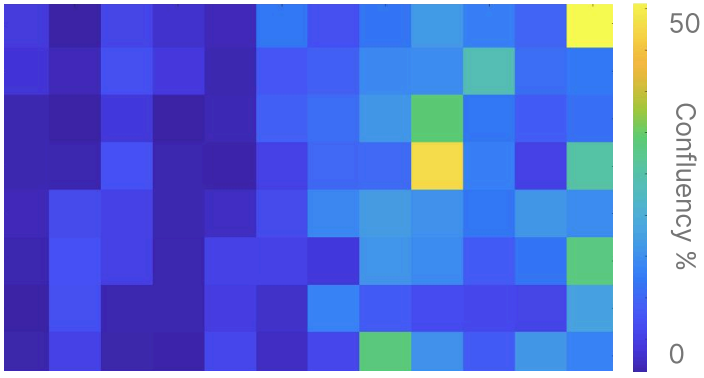
(a) 96 well plate, high confluency



(b) 6 well plate, low confluency



(c) 96 well plate, confluency map



(c) 96 well plate, confluency map

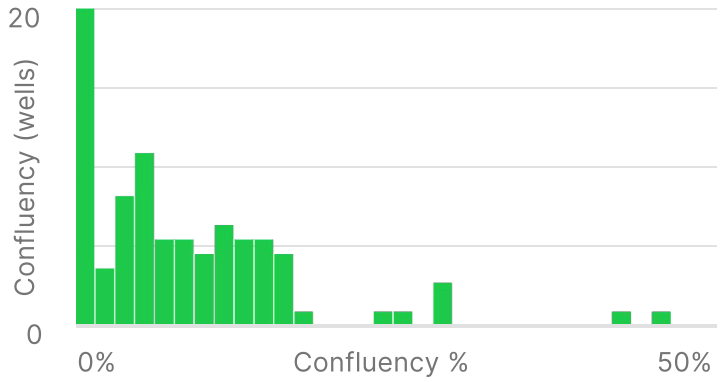


Figure 4: (a-b) Example whole-plate scans of a 96 and 6 well plate. (c-d) Plots of confluence results from (a) viewed as a well-plate map and as a histogram offer direct experimental insight, all available within 1 minute of plate insertion.

Alternative plate formats and results

The above workflows naturally extend to other well plate formats beyond 96 well plates (e.g., 6, 24 and 384), as well as a variety of confluency conditions. Figure 4 displays results from both a 96 well-plate with high cellular confluency, as well as a 6-well plate with very low cell confluency. Both plates were 3D-scanned by the Vireo at 10X resolution within the same approximate amount of time, and both were accurately processed to provide meaningful insights. When multiple image captures per well are required (e.g., as for the 6-well plate), a similar workflow as outlined for the 10X magnification case may be followed to ensure well alignment is taken into account for accurate area calculation. As highlighted in Figure 4(c-d), automated cell confluency results are output in CSV format for simple and easy statistical assessment and direct visualization with any preferred visualization software, all within just a few minutes of starting the imaging experiment. For more information about the Vireo and its exciting capabilities, please visit **https://www.ramonaoptics.com/products/vireo** to learn more.

References

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[2] M. Harfouche et al., “Imaging across multiple spatial scales with the multi-camera array microscope,” Optica 10(4), 471-480 (2023).

[3] K. Kim et al., “Rapid 3D imaging at cellular resolution for digital cytopathology with a multi-camera array scanner (MCAS),” NPJ Imaging 2, 39 (2024).

[4] K. Kim et al., “Parallelized brightfield and fluorescence imaging of organoids using a scalable multi-camera platform,” BioRxiv (2025). Available at: https://doi.org/10.1101/2025.10.07.681020