The Hidden Variable in High Content Screening: A quantitative assessment of microplate quality with a focus on speed, flatness and signal

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SUMMARY

How do plates impact high content screening (HCS)?

- **We quantify functional differences in HCS plates:**
  - Optical properties (signal intensity, signal to background)
  - Number of cells observed
  - Focus: focal evenness, out of focus percentage
  - Overall imaging speed
- **Using 4 channel staining on epithelial cells, measurements are done where HCS happens: the level of the cell, at submicron resolution**
- **Material and manufacturers are compared, finding significant differences**

INTRODUCTION

High throughput high resolution imaging at scale needs quality plates

HCS requires detailed subcellular imaging of cellular function and phenotype enabled by cutting edge imaging on purpose-built 96- 384- and 1536- well plates. Advances in optics, autofocus, and the development of large format CMOS cameras has led to fast imaging of large areas at higher resolution: more cells at greater detail. For instance, a 384-well plate can be imaged in 5 channels with single 1.2mm2 field of view at 0.27um/pixel digital resolution in 60 seconds on the Araceli Endeavor® Ultra.

Pitfalls in plates adversely affect imaging and assay quality

- Plate bottom unevenness and manufacturer error lead to slow imaging times and focus failures
- Impurities and lack of optical clarity result in diminished signal and higher background, or signal overlap between wells
- Plate bottom unevenness within wells leads to loss of focus within a FOV, necessitating multiple z planes or resulting in unusable images

Microwell plates details

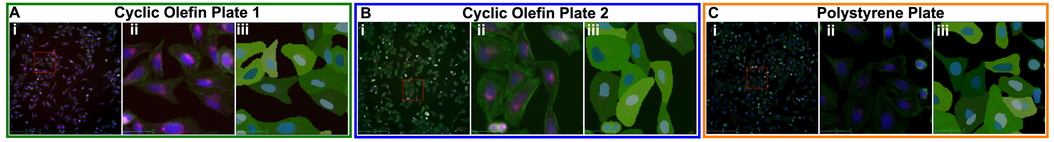
- All plates here designed for high content imaging
- 3 different manufacturers, anonymized
- #1.5 coverslip bottom (150-190 micron) thickness, optically clear, tissue culture treated
- Material differences in plate bottoms compared:
  - Two Cyclic Olefin plates, with different formulations, from separate manufacturers
  - One Polystyrene plate purpose-made for HCS
  - No glass plates tested, as they are cost prohibitive for high throughput

METHODS

3 plate types were assessed in 3 cohorts of staining (1 plate type/cohort), fixed, stained, and imaged within the same day. Stained cells were used to assess plate quality, looking at quantitative measures of staining and focus.
Cell culture and staining: human epithelial cells (U2OS) plated at 1600 cells/well, incubated overnight then stained with 0.2mM MitoTracker DeepRed (Invitrogen M22426), fixed with 10% NBF for 15 min at RT, permeabilized/ 10min RT with 0.1% Triton X100/PBS, and stained with 10ug/mL Hoechst 33342, 1:1000 phallodin-488 (Cayman 20549), and 1:200 WGA-555 (Biotium29076)/60min RT, with 3x washes in PBS between steps
Imaging: done with the Araceli Endeavor® with submicron resolution, 1.18 x 1.18mm field of view (FOV) with either 1, 4, or 9 FOV/well
Analysis: Analysis and segmentation from Araceli Clairvoyance™ with DNN-based segmentation on a per object basis based on either nuclear or actin stain to define foreground. Whole FOV focus measured with Clairvoyance's QC tool, any well <0.85 manually assessed for focus. Best focus within FOV measured in custom applet finding highest frequency component distinguishable from noise via parallel 1D fast Fourier transform run on 5x5 tiles/FOV through z stack, tiles with low content discarded. Graphing/statistics from GraphPad Prism. All measures normalized within experimental cohort.

Minor differences in cell count, significant channel-specific differences in signal

Figure 1: representative 4400x4400 FOVs (i) for all 3 plate types, with 4 channels of staining, as well as zoom in (ii, red box), and segmentation (iii).



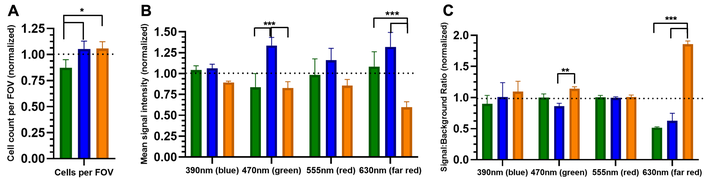
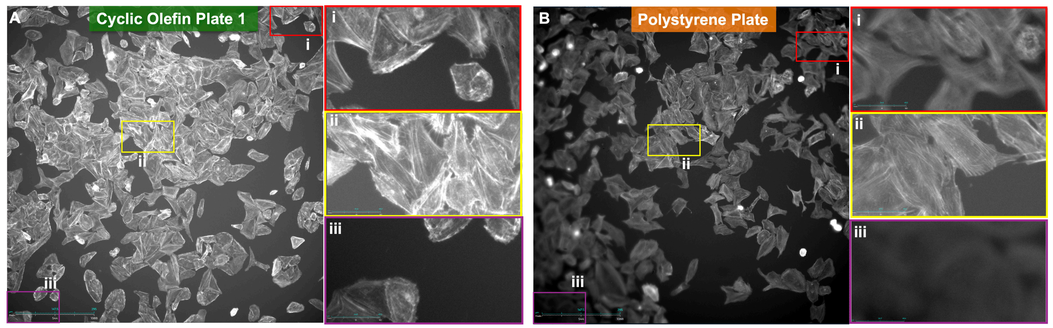


Figure 2: Quantification of cell number (**A**, nuclei count), with signal intensity (**B**) and signal to background ratio (**C**) measured in each channel. Cell and nuclear segmentation (Fig 1) is used to determine signal. Exposures and display identical. *** P<0.0005, * P<0.05

Major differences found in within FOV flatness

Figure 3: Qualitative comparison of cyclic olefin #1 (A) polystyrene (B) plates with actin staining (phallodin-488): exposure and display parameters are identical between images. Colored boxes indicate ROIs. Note the difference in focus across the 1.2 x 1.2 mm FOV and in background intensity.



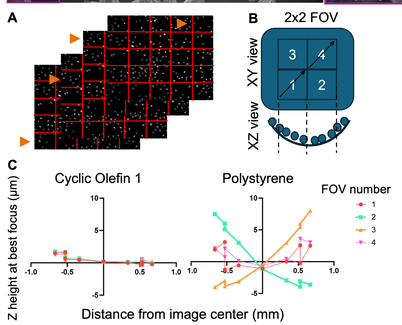
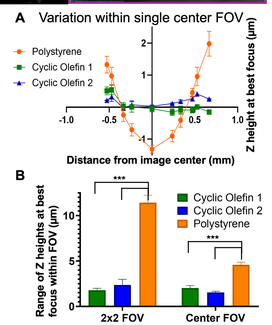


Figure 4 (left): Focus measured between -6 and 12 µm in 5x5 subdivisions per FOV (**A**) with example images from polystyrene plate at z positions -4, 0, 4, 8 µm: orange carrot marks best focal position for that sub-FOV. Best focus position for each subdivision plotted (**C**) for right-to-left diagonal for FOV sites 1-4 (schematic in **B**) within a well, indicating an extreme curvature in polystyrene but not cyclic olefin plates affecting focus. N>150 wells/data point.

Figure 5 (right): A) Z height at best focus is measured across 25 subdivisions within a single FOV in the center of the well, across all plates and experiments. **B**) plots the range of best focus z heights within an FOV for both 2x2 and single FOV/well data. P<0.0005



Moderate imaging time differences, with no differences in out of focus well counts

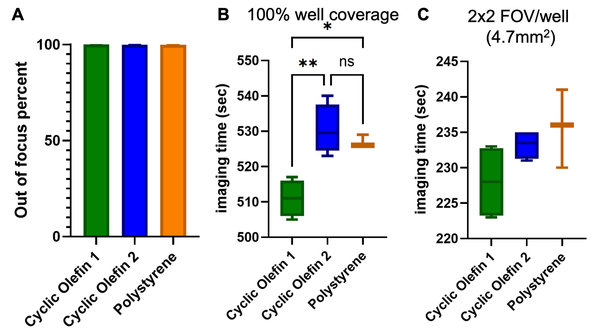


Figure 6: A) No differences seen in whole-FOV focal failures, with no more than 2 (2x2) and 6 (whole well) out of focus FOVs found per 384-well run. Some timing differences seen, with Cyclic Olefin plate 1 consistently faster, though this was only significant for 100% well coverage runs (**B**) , not maximized coverage (**C**), perhaps due to speed in finding focus. **P<0.005, * P<0.05 Tukey's multiple comparisons test

CONCLUSIONS

- **Polystyrene plates are not well-suited for high resolution imaging with a large FOV**
- >10µm variation in focus across a FOV results in focal loss at edges, resulting in unusable data
- Cyclic olefin plates have <2µm variation in focus
- Polystyrene plates have less overall signal, but an improved signal:background ratio
- Minor differences in cell count not attributed to material differences
- Endeavor's laser autofocus mitigates plate bottom unevenness, resulting in no difference in out of focus well count between materials
- Minor, but significant difference in acquisition speed

REFERENCES

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