Using IN Cell Analyzer 1000 to Characterize Flatness of Plate and Well Bottoms

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Introduction

The quality of data from high content screening depends on four important groups of factors: sample biology and preparation, sample plate type, image acquisition system and image analysis. Perhaps because of the scarcity of characterization tools, up to now little work has been done to describe how plate quality parameters such as plate- and well-bottom warp and tilt affect the final data [1,2]. IN Cell Analyzers 3000 and 1000 both offer tools to characterize overall plate bottom flatness. We are now able to utilize IN Cell Analyzer 1000 to similarly characterize well bottoms. In this work we report results for a range of plate types and discuss factors that affect the measurements.

Methods & Materials

Ten polystyrene clear-bottom 96-well microtiter plates were plated with a U-2 OS stable cell line utilizing GFP. Additional cellular labelling was done using Hoechst 33342, Texas Red®-X phalloidin and MitoTracker® Deep Red 633. The survey plates were donated from five manufacturers, here referred to as A - E. All plates were imaged with IN Cell Analyzer 1000, using a 20X objective. The instrument uses a laser based focus solution to detect the liquid-plastic interface of the plate well bottom. In this way the location (Z) of the interface for each focus attempt is recorded. In our protocols each well was acquired at 14 horizontal or 16 vertical adjacent fields bisecting the well centre, (Fig 1).

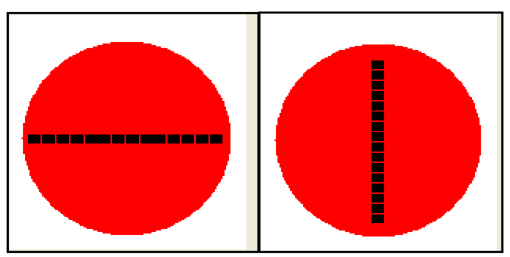


*Figure 1: **Acquisition of mapping points.** Focus positions were recorded at intervals of 449.6 μm horizontal (X) and 335.9 μm vertical (Y) directions.*

From the Z position of the liquid-plastic interface we can calculate various plate quality descriptors for plate- and well-bottom tilt and warp. Repeatability tests showed Z determinations have a precision of ± 0.2 μm.

Plate Bottom Tilt & Warp

Figure 2 shows bottom profiles of plates from two manufacturers. Manufacturer A shows greater warp than B. However, this work argues that features that most influence image quality are well-bottom tilt and warp. Focus is determined at specific well locations thus overall plate warp has less impact on image quality. However within the field bottom Z must remain near the objective depth of focus, typically a few μm (1.4 μm for the 20X objective used in this work).

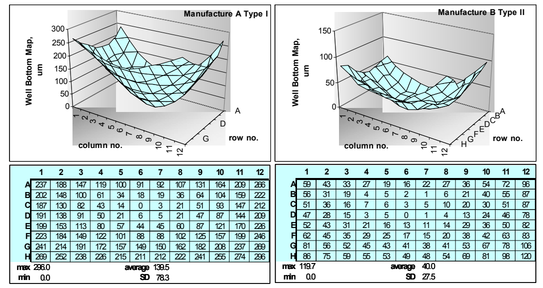


*Figure 2: **Examples of Plate Bottom Warp.** Bottom Z values determined from each well's center. These two illustrations are typical representations of each manufacturer's plates. Manufacturer A demonstrates a more severe bottom warp, nearly 2.5 fold that of the comparatively flat manufacturer B plate. However, what matters most to image quality is the degree of well bottom warp and tilt.*

Definition of Well Bottom Tilt and Warp

Fig. 3 shows how we define well bottom tilt and warp. Data are shown for bottom Z values taken horizontally, for wells G1-G3, two different plate types (both manufacturer A).

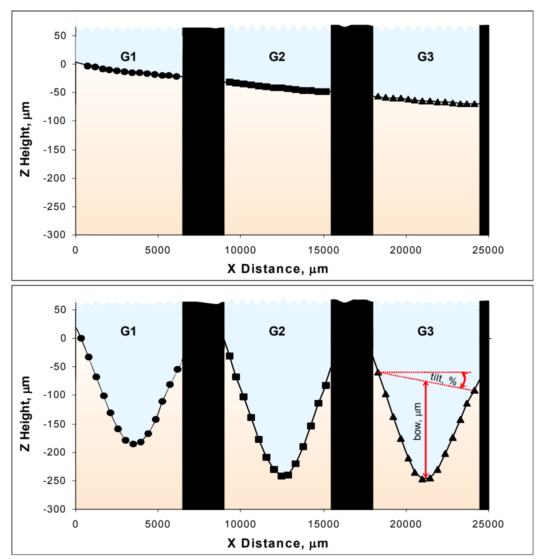


*Figure 3: **Definition of well bottom tilt and warp.** Actual bottom Z data for wells G1-G3 of two different plate types. Well bottom tilt is defined as the slope of the line connecting the two edges of the well relative to the horizontal, expressed as %. Well bottom warp is approximately measured by the length of the bow between the lowest point and the top of the bowl, as shown. Note that the figure is highly exaggerated in the Z direction to emphasize details. Actual well bottom tilts are of the order of 0.5 to 1% (~ 0.3°). Similar tilts and warps characterize the Y-direction profiles. For brevity this work focuses on the X-direction features only.*

It should be mentioned that the lower figure represents the most extreme case of well bottom warp we have observed.

Relationships of Plate- and Well-Bottom Tilts

As Fig. 2 shows, most plate bottoms have bowl-like profiles. We have already defined the meaning of well-bottom tilts. The plate-bottom tilt may be defined similarly. But because imaging takes place within a well, the kind of tilt that matters more is the local slope of the plate bottom at each well. These can be calculated from the local tangents to the plate-bottom profiles (cf. Fig. 2A).

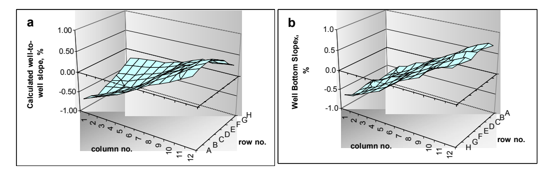


*Figure 4: **Comparison of Plate- and Well-Bottom Tilts.** Well-bottom tilts (b), as defined in Fig. 3, correspond closely to plate bottom tilts (a), defined as local well-to-well slopes of plate bottom profiles. All data in the X-direction.*

Interestingly, all 10 plates we have studied show a pattern similar to that seen in Fig. 4. We conjecture that well bottom tilts appear as natural extension of the overall plate warping that takes place in plate manufacturing.

Depth of Focus vs. Variance of Well Depth

Fig. 5 shows bottom measurements of several wells: row G (columns 1-12) and an expanded view of well E8, with each data point representing one field of view (450 μm). Bows are all < 3 μm, while in other plate types (cf. Fig. 3), they can reach ~ 100 μm.

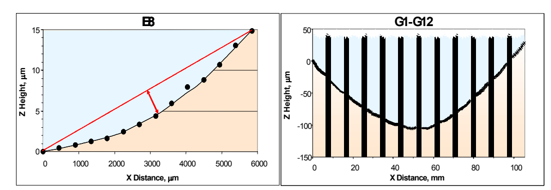


*Figure 5: **An example of moderate well warps.** Plate type Manufacturer A Type I. For each well, variance of well depth is defined as the average change of Z within each field of view.*

As far as image focus is concerned, we need a measure that tells us whether a particular well or plate would produce acceptable images. What is important is that Z variation within each field of view should remain less than the depths of focus of the objective (1.4 μm in this survey). We define the Z variation across a field as 'well depth variance', a quantity that depends both on the objective in use and plate quality. We therefore summarize all our data in Table 1, where the quantities for well bow and variance of well depth are averaged for all 96 wells of each plate.

Depth of Focus = 1.36 μm	Mean well bottom bow, μm	Variance of well depth, μm	# of wells > 1x depth of focus	# of wells > 3x depth of focus
Manufacturer A Type I	2.1	1.5	48	0
Manufacturer A Type II	32.5	5.1	96	54
Manufacturer A Type III	90.8	13.6	95	87
Manufacturer B Type I	1.6	0.7	9	0
Manufacturer B Type II	13.7	2.5	82	3
Manufacturer C Type I	11.9	2.1	91	0
Manufacturer C Type II	11.3	2.0	94	2
Manufacturer D Type I	9.5	2.5	86	6
Manufacturer D Type II	10.9	2.7	85	12
Manufacturer E Type I	2.7	0.6	2	0

*Table 1: **Summary plate quality data.** Well bottom bow is defined in Figs. 3 and 5. Variance of well depth is defined in Fig. 5. All values are averages over 96 wells. For each plate, the number of wells where variance of depth is greater than the depth of focus (1.4 μm) are indicated.*

Image Quality vs. Plate Quality

In Fig. 6 we show how plate quality affects image quality. The top figure corresponds to a better quality plate from manufacturer E /type I. Its mean bow is 2.7 μm and its variance of well depth is 0.6 μm (cf. Table 1). In contrast, the lower quality plate (manufacturer A /type III) has mean bow is 91 μm and its variance of well depth is 13.6 μm. Because the autofocus finds the best focal point at the middle of the field of view, the steep slope of the warp from manufacturer A /type III plate produces out-of-focus images around the periphery of the field of view.

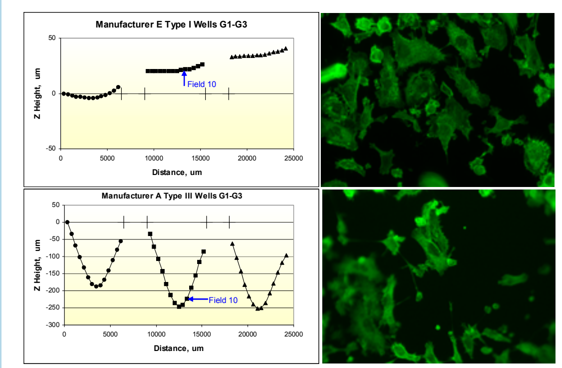
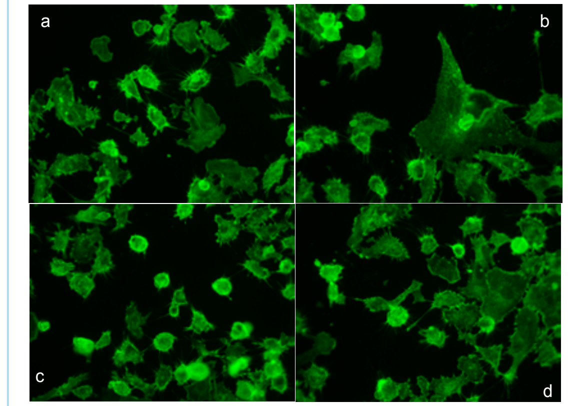


*Figure 6: **Impact of well bowing on image quality.** The images and graphs are representative of the same field and wells acquired from different manufacturers. Top: The data set acquired from Manufacture E Type I shows only minor warp and tilt across the field with bows measuring 7.8, 3.2 and 2.4 μm for well G01 – G03 respectively. This well is rewarded with a uniformly focused image. Bottom: Conversely this data set demonstrates severe warping, 161.4, 189.9 and 164.1 μm for wells G01 – G03, respectively, and is rewarded with a poorly focused image.*

Another way of demonstrating the role of variance of well depth on image quality is to image cells at field positions of increasing well depth variance. This is shown in Fig. 7 where cells were imaged at increasing well depth variance, from 1.6 to 5.6 μm (7a - 7d).



*Figures 7: **Blurring of images with increasing variance of well depth.** All images acquired from Manufacturer A Type II. Variance of well bottom depth within the field of view is: (a) 1.6 μm; (b) 3.2 μm; (c) 4.4 μm; (d) 5.6 μm. As the depth variance increases, images blur at the edges.*

We have demonstrated in other work that blurring due to imaging outside the depth of field of objective leads to image quality deterioration and consequent loss of data quality as measured by Z′ [3]. In future work we will investigate how plate bottom quality affects assay Z′.

Summary

- **IN Cell Analyzer 1000 was used to characterize the uniformity of well and plate bottoms for ten 96-well plate types from 5 different manufacturers.**
- **The IN Cell Analyzer 1000 autofocus system was used to measure the Z heights of the plastic-liquid interface. In each well 14 Z values were collected along the plate's long axis (X), and 16 Z values along the plate's short axis (Y). From these we learn:**
- **Plate and well bottoms can be characterized by tilt and warp, independently along X and Y.**
- **Features of the well are more important to imaging quality then those of the overall plate.**
- **Well-bottom bow (cf. Figs. 3, 5), and the variance of well-bottom depth within the field of view strongly correlate with image quality.**
- **Evidence is presented that best image quality is obtained when variance of well-bottom depth is less than the depth of field of objective in use (least blurring within the field of view).**

References

1. Rhem SM and Cowan CL, SBS Poster, Portland, OR (2003);
2. Rhem SM and Cowan CL, SBS Poster, Orlando, FL (2004);
3. Yekta A et al., SBS Poster # 2014, Geneva, CH (2005).

GE imagination at work

*To whom all correspondence should be addressed. **This poster was presented at the 11th Annual Conference of the Society for Biomolecular Screening, Geneva, Switzerland (11 - 15 September 2005)**.