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Underappreciated consequences of background and uneven illumination on microscopy-based measurements of single-cell variability

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Advances in high-throughput fluorescence microscopy (HTFM) and image informatics have allowed heterogeneous single-cell phenotypes to be characterized and related to putative biological functions. However, it is underappreciated that accurate estimates of single-cell phenotypic variability depend on image correction. Further, the methods used throughout the HTFM literature do not generally take advantage of shared image properties, instead correcting each image independently. In this brief note, we first recall the basic mathematical framework underlying image correction. We then show how the true distributions of commonly measured single-cell features are altered in uncorrected images. Finally, we report an observation that allows the use of a simple method for accurate image correction in HTFM.

The intensity of a pixel, I , can be modeled by $I = S(F + B)$, where: S is shading due to uneven illumination, F is the biologically relevant foreground fluorescence, and B is the background fluorescence¹⁻³ (see Supplemental §1 for full model description). Image correction is a data preprocessing step whose goal is to obtain F . (Subsequent normalization steps, not discussed here, may be applied to deal with systematic experimental errors, such as row or column biases in microtiter plates^{4,5}.)

How is apparent cellular variability (e.g. standard deviation or coefficient of variation) affected when S or B are not removed from images? In Figure 1a and Supplemental §2, we analyze such effects for the distributions of commonly used single-cell features, namely: Average, Total or Ratiometric biomarker intensities. For these simple features, background and shading can alter apparent phenotypic variability in surprising ways. For example, the effects of either on the Ratio feature are generally unpredictable, while background increases Total feature variability due to underlying differences in cell size. Such changes to single-cell feature distributions can be particularly important in screening, where variation is

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Author Contributions Statement

AC performed the experiments, analyzed the data, and prepared the figures. CW and SR provided critical interpretations of the data and revisions of the manuscript. AC, LW, and SA wrote the manuscript. SR, LW, and SA prepared the general mathematical formulations. All authors reviewed the final manuscript.

Additional Information

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commonly used to determine whether experimental conditions differ statistically from controls.

In practice, the challenge for correction is to estimate shading within an image, as subsequent background subtraction is relatively simple. How then should shading in large HTFM image datasets be estimated? In principle, every image from a microtiter plate could have its own unique shading pattern and background. Indeed, this is an (implicit) assumption of correction methods common to HTFM studies. However, we find that shading is not unique to every image, but is rather predictable based on image position within a well (Figures 1b, S1, S2). This observation suggests an image correction strategy for HTFM that only depends on within-well position. In short, images are corrected by within-well position using reference shading patterns estimated from the data (Figure 1b, Supplemental §3). This empirical approach using shared image properties across well positions is simpler than commonly used methods that estimate correction parameters for each individual image, yet more accurate than applying one set of correction parameters to all images. Accurate estimates of variability are important for large-scale studies of single-cell phenotypes, which are increasingly used to link functional significance to patterns of cellular heterogeneity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

a, Image background and shading can alter measured distributions of cellular phenotypes. The effects of image background (“B”, blue) or shading (“S”, red) on distributions of three measured cellular features were mathematically analyzed (assumptions in Supplemental Note §2). Insets show predicted directions of change ($\uparrow$, $\downarrow$, $=$, or $?$: increase, decrease, none, or indeterminate) of the mean (μ), standard deviation (σ), or coefficient of variation ($cv = \sigma/\mu$). For Hoechst- and Smad-stained cells ($n > 3700$), histograms illustrate these changes to single-cell feature distributions before (black) and after the addition of synthetic background (blue) or shading (red). x-axes are in arbitrary fluorescence units (left and middle) or are dimensionless (right). **b, Shading in microtiter plates is defined by within-well position.** Image grids from each well were normalized to the within-well median intensity and montaged across the entirety of a 384-well plate before (left and blue outlines) and after (right and red outlines) positional image correction (additional details in Supplemental Figure S2). Middle: zoomed in images of wells before or after image correction.