LETTER

Reply to Schubert et al.: Regarding critique of highly multiplexed technologies

We are surprised by the concerns raised by Schubert et al. (1) regarding the quantitative nature and methodological approaches presented in our recent PNAS report (2). I will attempt to clarify some of confusion and misstatements, as several errors and misrepresentation of our data are stated in the critique. The first incorrect assertion in the letter regards the use of NaOH to quench fluorescence of the Cy dyes. As clearly stated in the *Methods* of our report (2), all experiments presented in the paper used the basic peroxide method for consistency. Although certain NaOH techniques are included in patent applications for the MultiOmyx platform by our group, they were not included in this article.

Regarding criticism at the platform for not being "quantitative," our reference to quantitation referred to relative quantitation following single-cell segmentation. We recognize the difficulty in developing truly quantitative immunohistochemical methods. Because all antibodies have different affinities and labeling properties, providing absolute quantification would require an enormous amount of standards. As the system we presented was optimized for using routine formalin-fixed, paraffin-embedded tissue sections, quantification is further complicated because of thin sectioning of the samples and changes in protein conformations. Perhaps the biggest factor that inhibits absolute quantification is the sample itself; upstream sample handling and preanalytical variability introduce a larger factor of error than all experimental steps together. Similarly, the use of the term "unprecedented resolution" is clearly in reference to "resolution" of cell phenotypes and not to the optical resolution of a traditional wide-field fluorescent microscope.

As presented, we do identify some epitopes that are affected by the dye inactivation. Testing for antigen sensitivity is a critical component of our characterization process. A priori knowledge of antigen sensitivity allows the antigen to be detected in the earliest stages of the procedure. Proteins with substantial effects are evaluated in the first rounds. Moreover, because the quantitation is relative and all samples are stained at the same time, we are presuming that each epitope will behave more or less the same regarding dye-inactivation sensitivity. Also embodied within the critique is the claim that "manual" methods introduce variation that prohibits quantification from the data. Our experiences in developing this platform have indicated that advantages conveyed in automation would be largely reflected in labor reduction, not the quality of staining produced or data collected. However, care was taken for quality control and reproducibility of our staining protocol and image acquisition, and we would consider the manual process presented not to be a limitation to meaningful analysis.

Finally, we have shown through additional publications (3, 4) additional applicability of the technology. General Electric strives for the highest level of quality and rigor and has validated the system both internally and with luminaries in the field.

Readers interested in more details regarding the MultiOmyx platform are encouraged to contact GE Healthcare at multiomyx.info@ge.com.

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1 Schubert W, et al. (2014) Imaging cycler microscopy. *Proc Natl Acad Sci USA* 111:E215.

2 Gerdes MJ, et al. (2013) Highly multiplexed single-cell analysis of formalin-fixed, paraffin-embedded cancer tissue. *Proc Natl Acad Sci USA* 110(29):11982–11987.

3 Nelson DA, et al. (2013) Quantitative single cell analysis of cell population dynamics during submandibular salivary gland development and differentiation. *Biol Open* 2(5):439–447.

4 Clarke G, et al. (2013) A novel, automated technology for multiplex biomarker imaging and application to breast cancer. *Histopathology,* 10.1111/his.12240.

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Conflict of interest statement: M.J.G. is an employee of the General Electric Corp.

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