LETTER

Imaging cycler microscopy

The article in PNAS by Gerdes et al. (1) adds to the evidence that spectral resolution limits of fluorescence microscopy can be overcome by reiterative cycles of tagging, imaging, and bleaching of fluorophores attached to ligands for target biomolecules. In fact, the robotic imaging cycler microscopes are based on this principle and have been used for decades (2–5). The current ready-to-use robotic Toponome Imaging System (TIS) for biomarker discovery and cell research can comap at least 100 molecules in individual cells and tissue sections and, because of its high functional resolution (<40 nm) (5), provides insights into their organization, including their supramolecular clusters.* Now Gerdes et al. offer a modified protocol using H_2O_2/NaOH (pH~12.0) to quench the fluorescence of CyDye-tagged ligands (1). However, as their own data show, the NaOH treatment decreases signal intensity for a significant percent of proteins/epitopes probed. This result contradicts the claim that their multiplex microscopy method (MxIF) enables "quantitative, single-cell, and subcellular characterization of multiple analytes" (1). Specifically, of the 72 immunostained antibody/antigen pairs probed, 59 (82%) were tested in up to 10 dye-inactivation cycles (figure S5 *A*–*C* and Dataset S1 in ref. 1). In eight (13.5%) of the pairs, the dye-inactivation decreased the fluorescence signal. The decrease was judged moderate after one and five cycles, with some residual staining remaining after 10. In the case of ribosomal protein S6, the fluorescence was largely decreased already after the first and fifth cycles, and virtually eliminated after the 10th. The effect of the order of antibody application on fluorescence intensity was tested for only 11 proteins/epitopes and the outcome was apparently judged only visually (figure S11 in ref. 1). Protein/epitope integrity after 100 cycles is shown for only four proteins using H_2O_2/NaOH for quenching (1). In contrast, the photobleaching used in TIS has been shown not to affect the fluorescence intensities for hundreds of proteins tested, and alterations of the cycle sequences (forward, inverted, randomly permuted runs) shown not to alter the specificity of the procedure or the overall molecular structure/antigenicity of the proteins/epitopes in the tissues (3). With TIS, the successive steps are carried out robotically, with the slides in fixed position under controlled conditions, which ensures high reproducibility. In contrast, in the MxIF method the successive steps were apparently carried out manually [slides removed from the microscope, cover slips removed, and so forth (1)]. Evidence of MxIF procedure's reproducibility under these conditions is not provided. Claims, such as that the method provides "unprecedented resolution" (see *Discussion* in ref. 1) need to be substantiated, especially in light of the ~40-nm functional resolution achieved by TIS (5). Without more detailed information it is not possible to evaluate the method properly, nor —most importantly—to test it in other laboratories. Taken together, the data reported by Gerdes et al. do not support the claim that the MxIF method "should be broadly applicable to problems in the fields of basic biological research, drug discovery and development, and clinical diagnostics" (see the *Abstract* in ref. 1), at least not in its present stage of development.

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1 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.
2 Schubert W (1997) Automated device and method for measuring and identifying molecules or fragments thereof. European patent EP 0810428 B1 [see also Schubert W. US patent 6,150,173 (2000); Japanese patent 3739528 (1998)].
3 Schubert W, et al. (2006) Analyzing proteome topology and function by automated multidimensional fluorescence microscopy. *Nat Biotechnol* 24(10):1270–1278.
4 Friedenberger M, Bode M, Krusche A, Schubert W (2007) Fluorescence detection of protein clusters in individual cells and tissue sections by using toponome imaging system: Sample preparation and measuring procedures. *Nat Protoc* 2(9):2285–2294.
5 Schubert W, Gieseler A, Krusche A, Serocka P, Hillert R (2012) Next-generation biomarkers based on 100-parameter functional super-resolution microscopy TIS. *New Biotechnol* 29(5):599–610.

Author contributions: A.D., M.R., A.K., R.H., A.G., and P.W. analyzed data; and W.S. wrote the paper.

The authors declare no conflict of interest. Walter Schubert is voluntary chief representative of the academic human toponome project and of its agency ToposNomos Ltd.

*Schubert W, Gieseler A, Hillert R, Krusche A (2013) Direct, spatial imaging of randomly large supermolecules by using parameter unlimited TIS imaging cycler microscopy. *International Microscopy Conference 2013, August 25–30, Regensburg, Germany.* Available at www.toposnomos.com/doku/MC2013.pdf. Accessed September 23, 2013.

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