Calibration of quenching of CellTracker by AB9

CellTracker Orange CMTMR was prepared as a 10 mM stock in DMSO and brought into the aqueous environment by adding a 100-fold excess of 5% BSA in PBS. It was further diluted with PBS to bring its final concentration to 5 μM. Various amounts of Acid Blue 9 (AB9; ranging from 12.5 μg/mL to 800 μg/mL, which, in the molar units, corresponds to 15 μM to 1 mM, respectively) were added to CellTracker, resulting in a decrease in fluorescence. To quantify the intensity, a drop of the solution was placed on a coverslip and fluorescence images were collected on an Olympus Fluoview 500 confocal microscope using a ×20/0.7 UPlanApo objective and illumination from a HeNe Green laser at 543 nm. The intensity was collected from the plane immediately adjacent to the coverslip to minimize loss of light due to absorption. It follows from the calibration curve (Supplementary Figure S1) that the presence of 0.2 mg/mL of AB9 (4% or less of its typical extracellular concentration) should be easily detectable by this method.

Stability of fixed cells

T24 cells were grown in Lab-Tek chamber slides (Bioexpress, Kaysville, UT, USA), stained with CellTracker Orange CMRA (see the Materials and methods section of the main text), and fixed with 4% paraformaldehyde (PFA). Fluorescence images were collected on an Olympus IX70 microscope using a ×10/0.4 objective. After collecting the first set of images, solutions of AB9 in PFA were added to the wells, and more fluorescence images were collected every 20 min from randomly selected fields. The average fluorescence intensity per cell was analyzed as described by Model and Blank (1). As shown in Supplementary Figure S2, fluorescence was reduced by 30%–40% compared with that before fixation but remained stable in the presence of either 5 mg/mL or 100 mg/mL AB9 for at least 100 min; the extent of quenching by both solutions was approximately the same. These results, along with those presented in the Results and discussion section of the main text, indicate the lack of dye penetration into PFA-fixed cells. (As follows from Supplementary Figure S1, even if one assumes that a 40% drop in intensity was due to dye leakage through the membrane, the

Supplementary Material For:
Thickness profiling of formaldehyde-fixed cells by transmission-through-dye microscopy

Mariana Pelts, Sahil M. Pandya*, Christine J. Oh, and Michael A. Model
Department of Biological Sciences, Kent State University, Kent, OH, USA

Keywords: Cell morphology; Acid Blue 9; formaldehyde fixation; membrane integrity

*S.M.P.’s current address is Northeastern Ohio University Colleges of Medicine and Pharmacy, Rootstown, OH, USA
The intracellular concentration of the dye would have to be no more than a few percent of its external concentration to cause such an effect. According to Equation 3 of the main text, the error of thickness calculation resulting from the presence of this amount of dye in the cell would also be on the order of a few percent.)

Accuracy of thickness measurement

To compare transmission-through-dye (TTD) with confocal 3-D restoration, HeLa cells were stained with 10 μM WGA-Alexa 488 at room temperature for 1 h with slow agitation on a shaker. The cells on coverslips were fixed with 4% PFA for 15 min and put on slides in 7 mg/mL AB9 as described in the Materials and methods section of the main text. The same groups of cells were imaged in two ways: (i) TTD images were collected on a widefield IX70 Olympus microscope using a ×10/0.4 objective and (ii) fluorescence stacks were collected on a confocal Fluoview 500 microscope at a 0.4 μm vertical step using a water-immersion 40/1.2 UPlanApo objective and a 488 nm excitation from the argon laser. Confocal cross-sections and TTD profiles were computed approximately along the same line, then brought to the same scale, and superimposed using Photoshop CS4. (Although TTD images can be collected with a laser scanning microscope in transmission mode using an appropriate laser line, we usually obtain better images using widefield illumination because fluctuations may occur during scanning.) Since the refractive index of cells was assumed to be close to that of water within a few percent, no correction for the focal shift was applied (2–4).

The match between the confocal and TTD images shown in Supplementary Figure S3 is generally quite good except for the right side of the top cell. One reason for the mismatch between the two profiles is that the cell located under the top cell recedes from the coverslip. While the confocal image identifies the vertical profile, TTD only shows the combined thickness of all the layers. In addition, when an object has a refractive index substantially different from the refractive index of the medium, refraction on steep boundaries may create Becke lines similar to those formed by inclusions (see Figure 3 of the main text). To demonstrate this effect, we collected TTD images of spherical polystyrene particles with \( n = 1.59 \) (Spherotech, Lake Forest, IL, USA). To mimic the typical refractive index difference between water and cells (around 0.05), the beads were immersed in Cargille oil (Cargille Labs, Cedar Grove, NJ, USA) with \( n = 1.58 \) and \( n = 1.54 \). The lipophilic dye Sudan IV was dissolved in the oils to bring \( \alpha_{488} \) to 0.07–0.11 μm⁻¹ (the maximum absorption coefficient was limited by dye solubility). TTD images of beads were collected using a ×20/0.7 UPlanApo objective through a 520/10 bandpass filter. Supplementary Figure S3 shows the comparison between the experimental thickness profiles and the theoretical profile of a sphere. In the refractive index–matched medium (\( n = 1.58 \)), the spherical profile was restored with nearly perfect accuracy, while in oil with \( n = 1.54 \), significant distortions were present at the edges (Supplementary Figure S4).

Likewise, parts of cells with steep inclines may not be rendered correctly if the bright field contrast is comparable to the contrast due to absorption. By collecting control images at a wavelength where the dye does not absorb (as in Figure 3 of the main text), one can identify parts of cells where TTD images are contaminated by bright field contrast (Becke lines). As we show in the main text, by using higher dye concentrations, the contribution of Becke lines to the overall image contrast can be suppressed.

Acknowledgments

This work was supported by NIH grant number 1R15GM186816.

References