Membrane molecules mobile even after chemical fixation

To the Editor: Fixation of cells and tissues is the critical first step for histochemical or cytochemical investigations[1,2]. Recent efforts to visualize lipid rafts by immunofluorescence and immunoelectron microscopy[3], particularly in nonstimulated cells, have yielded varied results, raising a question about the efficacy of chemical cross-linking fixation protocols for blocking the lateral diffusion of membrane molecules and thus their antibody-induced clustering[3,4]. In a literature search we did not find any systematic investigation of this issue. Here we investigated whether the lateral diffusion of both raft-associated and non–raft-associated molecules is blocked upon chemical cross-linking and treatment with cold methanol in human T24 cells. We used single fluorescent molecule tracking, which allowed us to directly observe the variation in molecular immobilization after fixation (**Fig. 1a**, **Supplementary Figs. 1** and **2**, **Supplementary Videos 1** and **2**, and **Supplementary Methods**).

With the frequently used fixation conditions of 4% paraformaldehyde at 25 °C for 30 min, contrary to general expectation glycosylphosphatidylinositol (GPI)-anchored proteins were difficult to immobilize and at least ~40% of H-ras, ~70% of transferrin receptor (TfR) and ~2% of Cbp diffused laterally (**Fig. 1b** and **Supplementary Figs. 3–5**). Including 0.1% glutaraldehyde in addition to 4% paraformaldehyde did not immobilize Halo-GPI (51% immobilized) but immobilized ≥90% of monomeric (m)GFP-GPI, Lyn, H-ras, Cbp and TfR (we observed ~70% immobilization of GPI-anchored proteins, on average, depending strongly on the protein; **Fig. 1b** and **Supplementary Figs. 3–5**). Increasing glutaraldehyde concentration to 0.2% or methanol (100%) fixation resulted in immobilization of >80% GPI-anchored proteins but not of phospholipids or cholesterol.

We then investigated antibody-induced clustering under different fixation conditions by measuring the fluorescence intensities of individual spots (**Supplementary Fig. 6**). Whereas treatment with 4% paraformaldehyde with or without 0.1% glutaraldehyde did not itself induce clustering, paraformaldehyde treatment did not block antibody-induced clustering of Halo-GPI and Halo–H-ras. Including 0.1% glutaraldehyde in addition to 4% paraformaldehyde suppressed clustering but did not entirely inhibit it. These results indicate that, in interpreting immunolocalization data, antibody-induced clustering

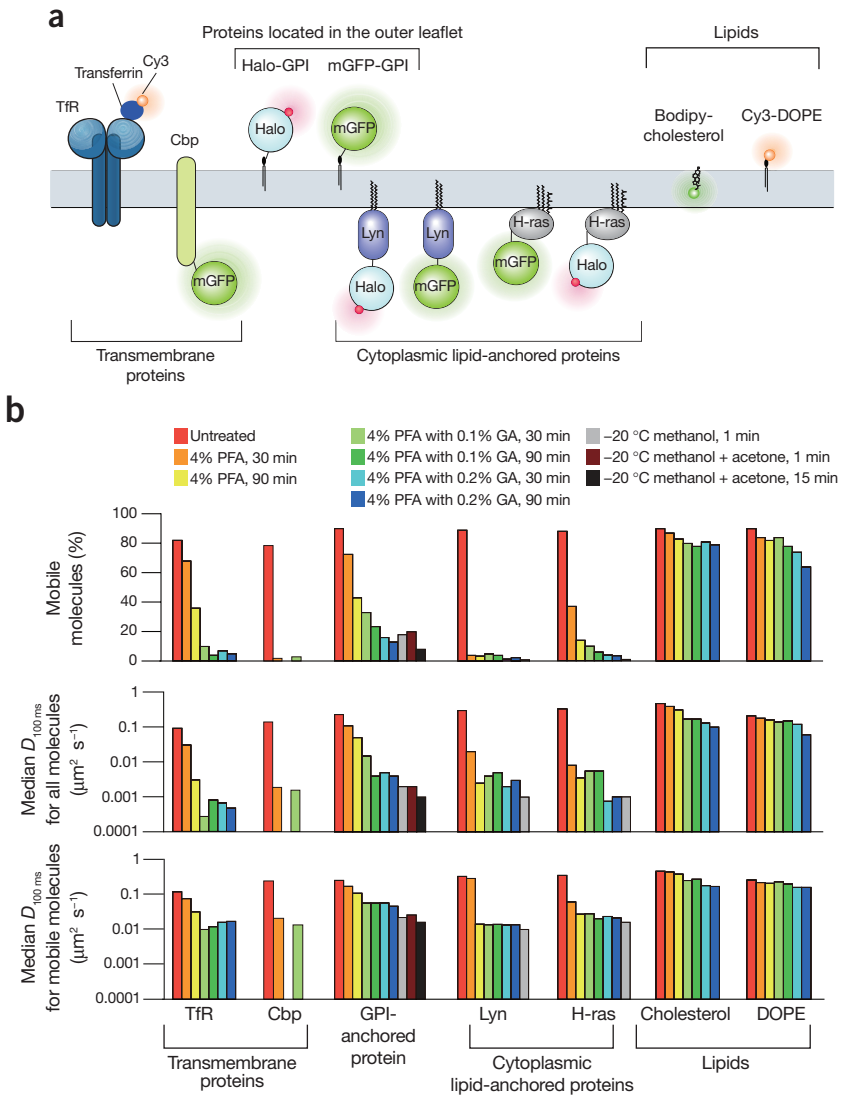

Figure 1 | Single-molecule mobility under different fixation conditions. (**a**) Schematic of the molecules observed in this work. The Halo protein was covalently labeled with a Halo-tag ligand conjugated to tetramethylrhodamine (TMR), which was added to the cell culture medium. DOPE, L-α-dioleoylphosphatidylethanolamine. (**b**) Plots of mobile fractions (minimal estimate) and the median effective diffusion coefficient in a 100-ms scale ($D_{100\ ms}$) for all molecules and for mobile molecules after fixation of T24 cells in the indicated conditions. Paraformaldehyde (PFA) or PFA and glutaraldehyde (GA) treatment was at 25 °C. For Lyn, H-ras and GPI, the averages of the median values for mGFP and Halo conjugates are shown.

even after chemical fixation must always be considered, especially for GPI-anchored proteins and lipids.

These results show that subtle differences in fixation conditions could lead to large variations in the extent of immobilization of molecules, and thereby in the extent of antibody-induced clustering[5,6], which might result in apparently contradictory localization data. Our advice is to fix specimens with 4% paraformaldehyde with 0.2% glutaraldehyde at 25 °C for 30 min or longer, or with cold methanol, and to consider the possibility that up to 20% of the molecules of interest could still be clustered by antibodies after fixation. For fluorescence microscopy, it would be better to observe live cells using fluorescent protein–conjugated molecules or monovalent fluorescent probes (organic dye–labeled fragment antigen-binding (Fab) fragments). Furthermore, these probes can be used without secondary antibodies even with chemical fixation.

Note: Supplementary information is available on the Nature Methods website.

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Federal policy and the use of pluripotent stem cells

To the Editor: In March 2009, US President Barack H. Obama ended eight years of restrictions on federal funding for human embryonic stem cell (hESC) research[1]. Obama's policy expanded funding for research and directed the US National Institutes of Health (NIH) to develop a process for certifying the ethical derivation of any line submitted to an NIH registry, including a recertification of lines approved during the George W. Bush administration (NIH Human Embryonic Stem Cell Registry Under Former President Bush, 9 August 2001–9 March 2009). What little relief the policy provided to researchers was dashed in August 2010 when a US district court temporarily barred the use of federal funds for any type of hESC research[2].

How does protracted controversy in an uncertain policy environment impact scientists working with hESCs? We began to answer this question by using information we gathered at the June 2010 International Society for Stem Cell Research (ISSCR) meeting in San Francisco. Our results, taken from 120 short surveys of researchers and an analysis of 381 research posters using hESC and human induced pluripotent stem cell (iPSC) lines, show how policies may impact the trajectory of a scientific field. Information taken from posters included the cell line name, funding sources, location of first and last authors, and whether the research used embryonic or induced pluripotent cells. Our surveys asked four basic questions: (i) "why did you choose these cell lines?"; (ii) "how did you get them?"; (iii) "why did you use iPSC lines?"; and (iv) "how important were federal and state policies in your thinking about which cell lines to use?".

It appears that iPSCs do not yet offer a solution for a field seeking an alternative to lines derived from human embryos: 53% of the projects described on posters (203 projects) used hESCs alone and 90 of 178 iPSC projects also used hESC lines as controls or comparisons. The apparent reliance of much iPSC research on hESC lines suggests that policy decisions about hESC research will also shape the future of iPSC science[3].

We also examined the type of hESC lines used, their frequency of appearance on the ISSCR posters, and their status under the Bush and Obama administrations. Forty-three different hESC lines had been used in two or more projects, and an additional 81 lines had been used in just one project each. Of the 43 lines, most (33 lines, 76.6%) were not listed on the Obama registry (NIH Human Embryonic Stem Cell Registry) (**Fig. 1**). Most studies used one or more of seven lines approved during the Bush era (281 studies, 78.83%). Four of these seven lines are currently ineligible for funding under the Obama policy. The remaining three—H1, H7 and H9—appeared in 185 experiments, which is over 63% of the total. In prior work examining materials transfer agreements and publication data, we found that the use of these three lines underpin the bulk of hESC research[4]. The growing diversity of human stem cell materials we observed here is good news, but the relative lack of use of new lines approved under the Obama policy presages trouble.

Much of the diversity of cell lines reported at the meeting results from research conducted outside the United States. More than half of the research had US-based first and last authors (164 projects, 55.97%). The remainder either had both first and last authors affiliated with non-US organizations or represented collaborations between a US author and researchers abroad (129 projects, 44.03%). We take the latter two cases to represent instances in which at least one of the major contributors works in a setting where American stem cell policies do not apply.

Consider the H1, H7 and H9 lines. More than 57% of US-based research (94 projects) used one or more of the three lines, compared to just over 32% of non-US and collaborative projects (42 projects), a difference that is highly significant ($Z = 4.218$, $P < 0.0001$). We believe this difference represents the legacy of the Bush years, followed by uncertainty created in implementing the Obama policy. The large numbers of experiments using the H1, H7 and H9 lines may reflect researchers' certitude about Bush-era lines being grandfathered into the new, more permissive regime. But anticipation of approval does not necessarily lead American