Blebs, chemical fixation, and the optical stretcher

John M. Maloney, Department of Materials Science and Engineering, Massachusetts Institute of Technology

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The optical stretcher—a tool that deforms single cells serially by photonic pressure—allows observation and mechanical characterization of fully suspended cells, absent chemomechanical influences from a contacting probe or substratum. Such capabilities are useful for examining dynamic mechanical events such as blebbing and chemical fixation. Here, we briefly summarize our observations of these mechanisms in the course of studying the viscoelastic nature of cells.

Blebbing first came to our attention as a challenge when analyzing phase-contrast images of single cells to quantify deformation during optical stretching (OS, Fig. 1). In OS, single cells are sent through a hollow capillary to be serially examined and deformed through photonic interaction with dual counterpropagating laser beams.[1]

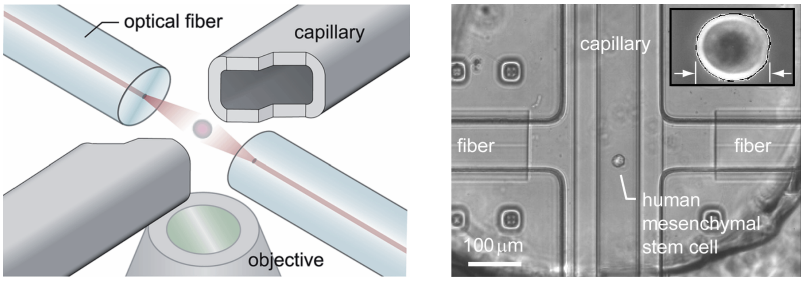

Figure 1: Schematic of optical stretcher, in which dual optical fibers emit infrared laser beams that hold and deform single suspended cells within a glass capillary by photonic pressure. Inset, quantification of cell deformation by elongation of laser-axis diameter (Maloney (2010)).

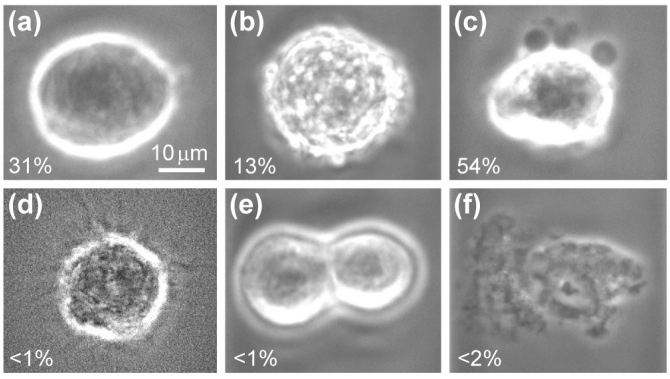

Figure 2: Suspended human mesenchymal stem cells exhibit surface morphology variety including (a) smooth cells and cells with (b) folds/ruffles, (c) blebs, and (d) filopodia; also occasionally observed are (e) cells undergoing cytokinesis and (f) dead cells, along with clumps of cells. Smooth, ruffled, and blebbing cells are most frequently observed (frequencies shown), and smooth and ruffled cells and cells with filopodia are most amenable to automated edge detection during analysis (Maloney (2010)).

We observed considerable variety in the morphology of suspended human mesenchymal stem cells (hMSCs) (Fig. 2) that had been detached from tissue culture polystyrene by trypsinization immediately preceding a several-hour OS experiment. These cells exhibited

[1] Guck (2001); Lincoln (2007); Lautenschläger (2009); Maloney (2010); Maloney (2013); Maloney (2014).

folds, blebs, and/or filopodia and were sometimes undergoing cytokinesis when detached. Dead cells were identified by the lack of a characteristic white ring revealed by phase contrast microscopy of an intact plasma membrane. Clumps of multiple cells were also observed. Single-cell appearance was divided approximately evenly between smooth and folded cells and blebbing cells, with the other morphologies observed only occasionally. Because the image analysis process relies on reliably locating the edge of the cell, blebbing makes it difficult to accurately track cell deformation during laser-induced stretching and subsequent recovery.

Cellular blebs are fluid-filled protrusions from cells that arise from detachment of the plasma membrane from the cortex; indeed, they were first termed "blisters."[2] Consensus from 1970s and 80s studies of cell morphology,[3] as characterized by optical microscopy and SEM, was that blebs are transient and are most prominent for several tens of minutes after detachment from a substratum, and that they represent excess surface membrane that cannot be immediately resorbed.

[2] Zollinger (1948).

[3] Erickson (1976); Vogel (1978); Furcht (1978); Harrison (1979); Garnett (1980); Kinn (1981); Moskalewski (1981); Rovensky (1984).

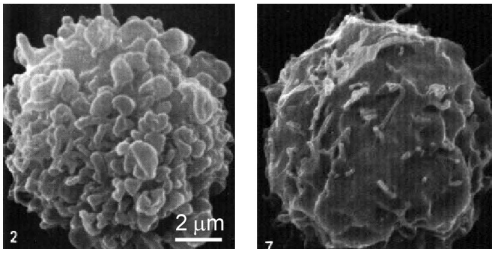

Figure 3: Resorption and reduction of blebs on cells, 6 and 40 minutes after substratum detachment (Kinn (1981)).

We experimented with serum starvation, the addition of sucrose, and different detachment initiators (EDTA, trypsin, Accutase) but could not eliminate blebbing. We concluded, in agreement with the previous morphological studies, that blebbing is more strongly linked to detachment than chemical exposure.

Earlier findings of bleb conversion to microvilli over tens of minutes (Fig. 3) suggested an explanation for our observation of a decrease in creep compliance over the same time scale (Fig. 4). We found significant stiffening over the first hour of stretching (approximately 30–90 minutes after initiating trypsinization & EDTA).

Through experiments decoupling temperature and pH changes from substratum detachment, we found that it was detachment that initiated the stiffening transition (Maloney (2010)). A look at cells over time showed that, indeed, the percentage of cells with blebs decreased from approximately 90% during the first 15 minutes to approximately 50% during the second hour. Coincidentally, in the same journal issue as our report was a dedicated study by Norman

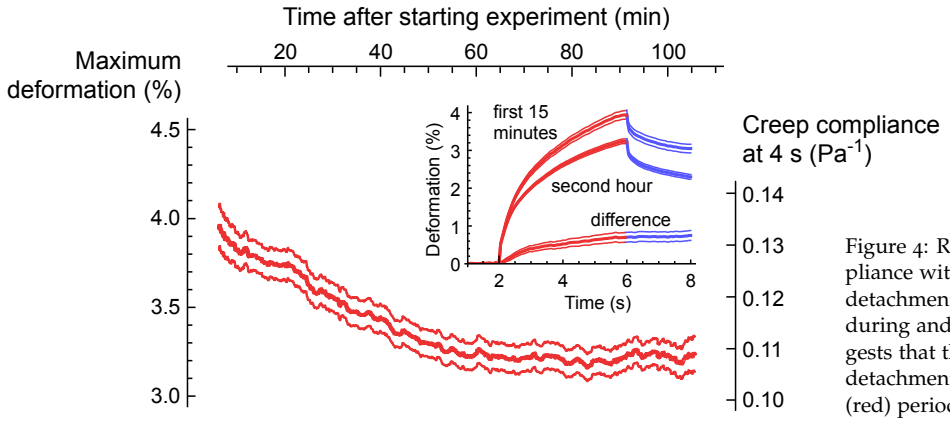

Figure 4: Reduction in creep compliance with time after substratum detachment; inset of cell deformation during and after 4 s laser pressure, suggests that the dependence on time after detachment is coupled to the stretching (red) period rather than the recovery (blue) period (adapted from Maloney (2010)).

et al. of blebbing after substratum detachment;[4] their findings, which included quantification of decreasing bleb density over tens of minutes, agreed with ours. We now allow adherent cells to remodel after trypsinization by incubation at 37°C in a centrifuge tube (or any container that resists cell adhesion) for at least 60 minutes before loading the cells into the OS. This incubation time reduces blebs considerably and thus aids image analysis and improves the quality of our cell deformation data.

[4] Norman (2010).

More insight was gained from several 1990s and 2000s studies that concluded that blebs are an adherent cell's healthy response to contact.[5] Fluid protrusion into hemispherical regions of detached membrane (pressurized by cortical actomyosin contraction) may aid cell spreading upon surface attachment. Such blebbing occurs on a time scale of seconds and consists of a cycle in which near-immediate fluid flow into the bleb is followed by new cortex deposition and contraction inside the bleb, withdrawing the protuberance. We have observed such behavior; dynamic blebbing of an hMSC is shown in Fig. 5 in the form of frames from a video available online as part of the supplementary information of Maloney (2010).

[5] Bereiter-Hahn (1990); Cunningham (1995); Charras (2006, 2008a,b); Fackler (2008).

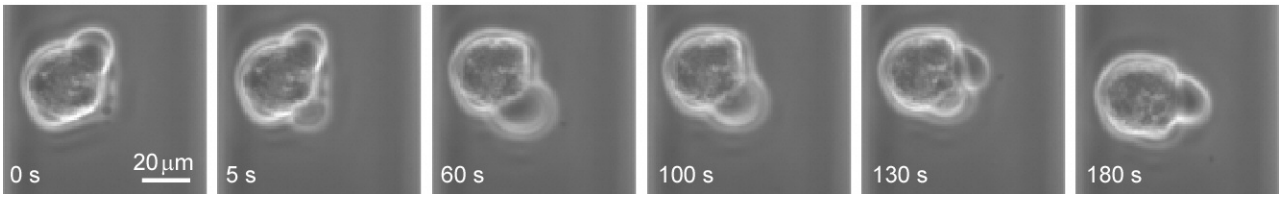

Figure 5: Dynamic blebbing on an hMSC over several minutes; note the transition around 100–130 s as new cortical material polymerizes just inside an existing bleb, rendering it less transparent and withdrawing it just as another bleb springs forth (Maloney 2010). (Direct video link: `http://www.sciencedirect.com/science/article/pii/S0006349510010489#mmc1`.)

This type of blebbing persists even when cells are allowed to remodel after detachment; it may be triggered by contact with the

tubing or capillary or by other cells. For certain OS studies, we now use the nonadherent CH27 lymphoma cell as a model cell type (Maloney (2013)). These cells do not require an incubation period, do not respond to substratum contact by blebbing, and exhibit a smooth surface morphology essentially 100% of the time.

Relative to blebbing, fewer studies have looked at fixation and the influence of fixative concentration and application time on cellular mechanics.[6] Cytoskeletal crosslinking is—in theory—a simple approach to remove the cells' viscoelastic character and stop metabolic processes. In practice, we find a dose-dependent continuum of viscoelastic response.

We studied fixation by applying glutaraldehyde at different concentrations, generally for 10 minutes, to a suspension of recently detached CH27 lymphoma cells and then performing OS (Maloney (2013)). The fixative was removed (by dilution, centrifuging, and resuspension) before stretching the cells. For these experiments, we applied a 1 Hz sinusoidal laser profile and looked at the phase lag and amplitude of the resulting sinusoidal deformation. From these values, we can calculate the mean cell fluidity[7] and stiffness (specifically, the complex modulus) (Fig. 6).

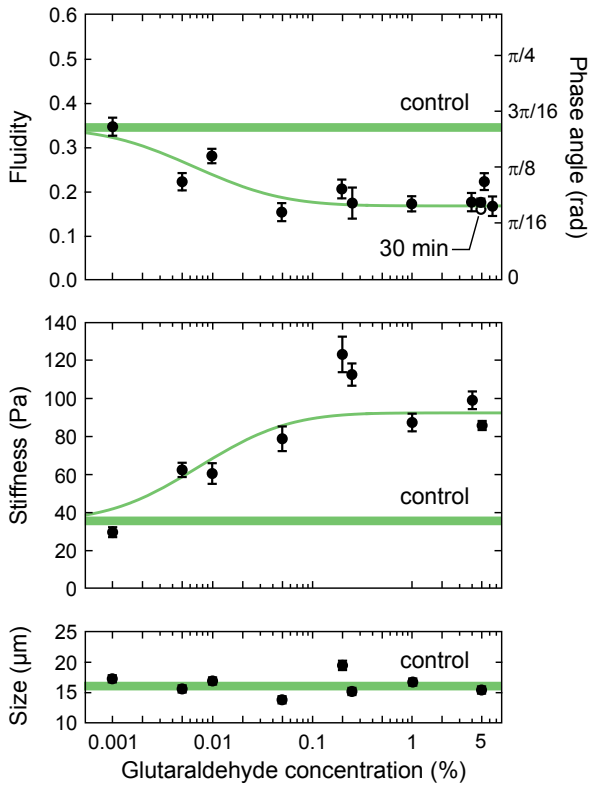

Figure 6: Alteration of whole-cell viscoelastic parameters (but not cell size) from exposure to glutaraldehyde, generally for 10 minutes (error bars represent standard error) (adapted from Maloney (2013)).

[6] Hoh (1994); Hutter (2005); Burns (2006).

[7] Fluidity is a measure of hysteresivity or viscous character that we have explored in detail in suspended cells (Maloney (2013), Maloney (2014)). Fluidity values of 0 and 1 would correspond to phase angles of 0 and $\pi/2$ and thus to perfectly elastic or viscous behavior, respectively. Biological cells are somewhere in between; they exhibit a fluidity between 0 and 1 and thus behave neither as an ideal solid or liquid but rather as a mix of both—a *complex fluid*.

It is clear that exposure to 0.001–0.1% glutaraldehyde changes the mechanical behavior of the cells in a dose-dependent manner. The fluidity (i.e., the phase lag) is reduced, indicating a transition towards elastic behavior, and the stiffness is increased. (The cell size, however, as characterized by the diameter along the laser axis, is unchanged by fixation.) An increase in fixative time from 10 to 30 minutes did not detectably alter the fluidity for 5% glutaraldehyde application (Fig. 6); this finding agrees with the conclusion of Hoh et al. and Hutter et al. that fixation is completed in a time scale of ~ 1 minute.

IN SUMMARY, the optical stretcher enables the researcher to examine and mechanically probe individual cells—useful capabilities for studying phenomena such as blebbing and chemical fixation, both of which feature coupled morphological and mechanical transitions observable at the single-cell level. In our concluding study (Maloney (2014))[8], we applied a panel of pertubative conditions, including osmotic pressure, temperature, pH, and multiple potent cytoskeletal-affecting drugs, to more fully understand the cell as a chemomechanical material.

[8] All of our OS studies are available at `http://john.maloney.org/picspubs.htm`.

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