Because of space limitations, it is not possible to adequately address the individual points raised by our colleagues. Instead, we focus on a few reoccurring, fundamental concepts that are at the core of their arguments.

### Measuring Fluid Movement in the Lens
The intra- and extracellular fluid flow patterns, such as those represented in Figure 1A of our Point article are difficult to measure experimentally, because the extracellular space is very small and the intracellular water flow is thought to be very slow. From the model, we have predicted intracellular water flow velocity in the inner cortex to be \( \sim 1.7 \times 10^{-2} \) \( \text{mm/s} \). The flow velocity in the extracellular compartment (which is approximately 400 times smaller than the intracellular compartment) would have to be 400 times faster to preserve the overall steady state lens volume.

Because the relative fluid flows in the intra- and extracellular spaces are vastly different, the movement of a tracer molecule in the lens would depend on whether it is localized extracellularly or whether (like the fluorescent dye referred to in Fig. 1 of the Counterpoint article) it is injected intracellularly. We predict that an intracellularly injected tracer molecule about the size of a \( \text{Na}^+ \) ion, but which is uncharged, would be freely carried by convection through lens gap junction channels and would not be affected by voltage gradients within the lens. In this optimal circumstance, 1 hour after injection, the center of the diffusion pattern would be moved by the intracellular fluid flow only approximately 6 \( \mu \text{m} \), or approximately three cell widths. Because dyes are larger than ions and most are charged, and because the effective mobility of a dye in moving between lens fibers is greatly reduced over that in free solution, an estimate of 6 \( \mu \text{m} \) in 100 hours is a more realistic estimate of their flow in the intracellular compartment. For well-coupled cells like lens fibers, the movement of a gap-junction–permeable dye from its site of intracellular injection into the surrounding cells is detectable within seconds. Thus, the relatively rapid spread of an intracellularly injected fluorescent tracer dye due to diffusion would mask any small shift due to water flow or voltage gradients. If one artificially created a large diffusion gradient for any permeant molecule, that molecule would diffuse down its gradient at a rate much faster than transfer due to water flow or voltage gradients. If one artificially created a large diffusion gradient for any permeant molecule, that molecule would diffuse down its gradient at a rate much faster than transfer at steady state by the lens circulation.

A similar argument pertains to NMR imaging of water flow in which an isolated lens is immersed in heavy water, creating a large gradient for diffusion of heavy water into the lens. It is the small perturbations from equilibrium that create the lens circulation. It therefore follows that if one wants to study the lens circulation, large external perturbations must be avoided, and the steady state properties must be the focus. In modeling fluxes in

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the lens, we have invoked the circulation only in the steady state, when solutes and solvent are near equilibrium. Our measurements of standing voltage gradients, \( \text{Na}^+ \) concentration gradients, and \( \text{Ca}^{2+} \) concentration gradients\(^2\) and our more recent studies of hydrostatic pressure gradients (Mathias RT, et al. \textit{IOVS} 2010;51: ARVO EAbstract 3459) and MRI-based water tensor measurements\(^3\) were all conducted in the steady state lens.

**Water Flow through Aquaporins and Gap Junctions**

The surprising statement by our colleagues, “However, unidirectional movement of water through water channels would create osmotic differences across the lateral membranes of the fiber cells. …This effect would preclude unidirectional flow through aquaporin channels…” suggests that the transmembrane flow of water creates transmembrane osmotic gradients. This view negates the past 50 years of epithelial fluid transport research, which has established that the converse is true—that is, water follows salt transport.\(^3\) Fluid transport does not occur without establishment of an osmotic gradient by membrane transport of salt. Osmosis is a passive process, involving simple diffusion of water down its concentration gradient. Again, our colleagues have focused on fluid transport without considering the entire picture, which relates fluid movement to the ion fluxes that induce fluid flow.

Similarly, the statement, “Osmotic resistance would also be generated at gap junctions, since unidirectional flow of water and ions through the gap junctional channels would leave behind proteins and other solutes too large to pass through them,” and the depiction of this in their Figure 2 have two fundamental misconceptions. First, the lens has a steady state circulation of fluid and ions, so what leaves a cell is exactly equal to what has entered that cell, and nothing is “left behind.” The second misconception is that gap junctions create osmosis. To have osmosis, there must be a semipermeable membrane—that is, a membrane with water channels (e.g., the aquaporins) that exclude the movement of ions. Gap junction channels passively conduct both ions and water, and so they are not capable of osmosis.

**Formation of a Barrier to Extracellular Diffusion**

It has been shown that, at the transition between differentiating and mature fiber cells, the diffusion of dyes into the lens via the extracellular space becomes restricted.\(^3\) At first glance, this restriction seems contrary to the circulation model, but the barrier does not appear to exist for smaller molecules such as ions and (presumably) water. Our measurements of intracellular ion concentration gradients and voltage gradients show smooth increases from the surface to the center of the lens,\(^2\) indicating that the fluxes go all the way to the center of the lens and do not exhibit the step changes that would be expected of a barrier to ion movement. Based on histochemical mapping of amino acid distributions, Donaldson and Linn\(^4\) have proposed that this extracellular diffusion barrier restricts the radial diffusion of metabolites into the lens nucleus, causing them to enter the lens at the poles via the sutures. This finding suggests that this barrier to extracellular diffusion separates the lens into two metabolic compartments that obtain their nutrients via different pathways. Differentiating fiber cells in the outer cortex take up nutrients directly from the extracellular space, which is in free contact with the aqueous humor. Internalized mature fiber cells in the lens core have nutrients delivered to them via the sutures that form an entry pathway that transverses the extracellular diffusion barrier and allows low-molecular-weight molecules to be convected into the center of the lens. In both regions, gap junctions form an intracellular pathway for the removal of waste products from the lens.

**Metabolic Activity of Mature Fiber Cells**

It is obvious that the metabolic requirements of the lens core are much lower than those of the cortex, where the high levels of protein and lipid synthesis associated with fiber cell differentiation and elongation are fuelled by oxidative metabolism, but the lens nucleus is not totally metabolically inert. Indeed, Yorio et al.\(^7\) estimated that approximately 10% of total lens metabolic activity occurs in the mature fibers and detected glycolysis, even in the most central fibers. This finding is in keeping with those in several other earlier studies\(^8\)-\(^11\) in which the results demonstrated that the core of the lens “metabolizes glycolytically with monosaccharides principally used as a substrate and excretes lactic acid.”\(^12\) It appears necessary to re-evaluate the detection limits of the assays used to measure enzyme activity in the lens core and compare these to the actual level of activity needed to provide the energetic requirements of the metabolically sluggish (but not dead) mature fiber cells in the lens nucleus. Finally, even if all these arguments suggesting the existence of metabolic activity in mature fiber cells are disregarded, the statement that “a basic assumption of the [lens circulation] is that mature fiber cells have active metabolism” is not accurate. Providing the metabolic needs of mature fiber cells is simply one hypothesis on the physiological role of the circulation and has nothing to do with its existence or nonexistence.

**Coupling between the Cortex and Nucleus-Supplying of GSH to the Lens Nucleus**

A metabolic requirement of the core that both groups agree on is the need to maintain the reduced levels of glutathione (GSH) to prevent protein cross-linking.\(^13\) The replenishment of GSH from GSSG is mediated by the enzyme glutathione reductase and requires NADPH, which is in turn produced via the hexokinase shunt pathway.\(^14\) Our colleagues believe that GSH generated in the cortex diffuses into the lens nucleus via an intercellular pathway into the lens nucleus. This argument is based on a report that a 30-minute incubation of an isolated monkey lens in \(^55\)S-cysteine (not glutathione, per se) resulted in more label being detectable in the equatorial region than at either pole.\(^15\) Once again, the movement of such an externally added tracer into the lens is dictated mainly by its concentration gradient and would be essentially unaffected by the lens circulation. Moreover, it is not possible to deduce from this single measurement whether the amino acid in fact initially enters the lens at the equator or is taken up in other regions and is then redistributed to this most metabolically active region of the lens. Furthermore, in their critique of the model, our colleagues suggest that outwardly directed fluid flows generated by the circulation system would be deleterious to lens health by impeding the intracellular diffusion of GSH into the lens core. Contrary to this view, the circulation system would instead aid the diffusion of oxidized glutathione (GSSG) from the nucleus to the lens cortex, where our colleagues propose that glutathione reductase regenerates GSH from the GSSG that originated in the lens nucleus. Unfortunately, we do not even know whether GSH or GSSG is permeable to the lens gap junctions.

Our alternative view is that the circulation system convects glucose deep into the lens, allowing regeneration of GSH to occur locally in the nucleus. Although this view should be tested experimentally, the debate about how reduced levels of GSH are maintained in the lens nucleus is particularly pertinent to the initiation of age-related nuclear (ARN) cataract. In ARN cataract, the levels of GSH are abruptly reduced in the nucleus relative to the cortex, rendering the center of the lens susceptible to oxidative damage and protein aggregation.\(^16\) Since the levels of GSH and the activities of its associated enzymes have been shown to decline progressively as a function of age, it has been assumed that ARN cataract is the result of a failure of enzymatic activity.\(^17\) However, although the specific activities
of enzymes are reduced with increasing age, these reductions have been deemed to be insufficient to account for the decrease in GSH levels observed in the nucleus and do not explain the abrupt decline in GSH levels in ARN cataract.\(^{17}\)

To explain this observed decrease in GSH nuclear levels, Sweeney and Truscott\(^ {18}\) have proposed that, with advancing age, a barrier develops that restricts the gap junction–mediated diffusion of GSH from the cortex into the lens nucleus. However, if we assume that the regeneration of GSH can occur locally in the nucleus, then a failure to maintain an appropriate reducing environment in this region of the lens would also produce the abrupt decrease in nuclear GSH levels relative to the cortex. Thus, rather than the age-dependent formation of a barrier to the diffusion of GSH from the cortex to the nucleus, an alternative explanation for the decline in nuclear GSH levels in ARN cataract would be a failure of the circulation system to deliver sufficient glucose specifically to the nucleus, reducing glucose metabolism and the production of NADPH required for GSH regeneration. Regardless of the mechanism, it appears that ARN cataract is a transport problem, a realization that highlights the importance of having a clear understanding of overall lens physiology.

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**References**


**Reply to: A Critical Appraisal of the Lens Circulation Model—An Experimental Paradigm for Understanding the Maintenance of Lens Transparency?**

The authors of the Point article clearly describe the fluid circulation model (FCM) in its current form. The two articles present contrasting views of lens physiology. In the view of Donaldson et al., mature fiber cells in the adult lens are metabolically active, requiring a steady supply of substrates and removal of metabolic end products. If this view were correct, robust circulation of intracellular fluid might be needed. Our perspective, which is supported by several published studies not considered by proponents of the FCM, is that mature fiber cells have little or no metabolic activity. They depend on superficial fiber cells to maintain their ionic balance and to preserve a reducing environment in their cytoplasm. The movement of ions and reducing agents such as glutathione (GSH) to and from the lens core occurs by simple diffusion across the gap junctions of mature fiber cells, not by an internal circulatory system.

The data we present suggest that the FCM is unlikely to be valid. To test whether the FCM is an accurate view of lens physiology, it should be sufficient to perform only two experiments. One would be to repeat the study cited in Figure 1 of our Counterpoint article on adult lenses, while monitoring extracellular currents. Displacement of a gap-junction–permeable dye, relative to an impermeant marker, would directly measure fluid flow in the intact lens, if it exists. The second experiment should test whether homogenates of adult human nuclear fiber cells transform a substantial amount of labeled glucose into pyruvate and amino acid precursors into reduced glutathione. The first directly measures flow in the lens, and the second tests whether GSH can be synthesized and reduced in mature fiber cells.

Figure 4 of our Counterpoint article shows that unidirectional flow from the center to the periphery of the lens would impede the diffusion of small molecules such as GSH, in the opposite direction. Therefore, the FCM requires that sufficient metabolism persist in the nucleus to generate ATP from glucose and reduced glutathione from amino acids and NADPH. For this reason, flow and metabolism are inextricably linked. If flow cannot be demonstrated or GSH cannot be synthesized and reduced by local metabolism in the lens nucleus, the FCM hypothesis is untenable.

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