Point/Counterpoint: A Critical Appraisal of the Lens Fluid Circulation Model

Introduction

Cataract is the leading cause of blindness in the world, accounting for approximately 42% of all blindness.[1] Surgical treatment of cataracts imposes a substantial economic burden on health systems. Since cataract is primarily a disease of old age, we are facing a looming cataract epidemic in which the demand for cataract surgery will place greater demands on the resources available for treatment. An alternative approach to surgery is the development of therapies designed to prevent or delay the onset of cataract. It is therefore not surprising that the ultimate goal of many international lens research groups is to determine the causes of lens cataract, with a view toward developing novel anticataract therapies. A major obstacle to achieving this laudable goal is our current understanding of how the normal lens maintains its transparency. It has been proposed that the lens operates an internal microcirculation system that contributes to lens transparency by delivering nutrients to, and removing metabolic wastes from, the deep fiber cells while maintaining steady state lens volume (the lens fluid circulation model [FCM]).[2–4] Key features of the model remain to be tested. Such scientific debate is a normal and healthy component of the research discovery process, but the lack of an accepted understanding of lens physiology is compromising progress toward the ultimate goal of developing targeted anticataract therapies.

The purpose of the two perspectives presented in Point/Counterpoint is to formalize this debate. Evidence for and against the FCM will be presented, with the goal of identifying areas of future experimentation that are needed to test its validity. A general overview of the model is provided, followed by a summary of the evidence supporting it by Richard Mathias, Paul Donaldson, and Linda Musil. In the Counterpoint, David Beebe and Roger Truscott present a critique of the model. These articles are followed by brief rebuttals that summarize the critical experiments needed to test the model.

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

It is important to acknowledge that our understanding of lens physiology has evolved from an initial view of the lens as inert tissue to one that recognizes it as a complex and dynamic organ. This evolution in understanding was initially driven by advances in histologic and electrophysiological recording techniques and then by our ability to determine the molecular identity and cellular localization of key transport proteins associated with the circulation system. Most recently, the ability to combine whole lens electrophysiological recording with transgenic animal models has enabled us to study the physiological roles that specific lens proteins play in the maintenance of lens transparency. It is highly likely that the application of new technologies to the lens will cause us to further modify our current understanding of lens structure and function, a summary of which is provided herein.

The Lens Internal Microcirculation: A Brief Overview

Lens transparency is the direct result of its specialized cellular architecture, which we have proposed is actively maintained by a unique lens physiology. The lens is an avascular tissue surrounded by a tough but porous collagenous capsule (Fig. 1A). Beneath the capsule, a single layer of cuboidal epithelial cells covers the anterior surface. Near the equator, these epithelial cells divide, and the daughter cells elongate and differentiate into the fiber cells that form the bulk of the lens. The fiber cells adopt a flattened hexagonal profile that facilitates their packing into an ordered array in which the spaces between the cells are smaller than the wavelength of light. During differentiation, the fiber cells lose their intracellular organelles and undergo significant changes in the expression of cytoplasmic and membrane proteins. An abundance of soluble cytoplasmic proteins, called crystallins, creates a high index of refraction. The concentration of crystallins is highest in the center of the lens, creating a radial gradient in refractive index that corrects inherent spherical aberration. Lens growth continues throughout the lifetime of an individual, with younger fiber cells being laid down on top of existing fiber cells, resulting in the progressive positioning of older cells deeper in the lens. Each mature fiber cell extends from the anterior to the posterior pole, where it forms a suture with other fiber cells. The lens maintains this precise cellular architecture and prevents light-scattering by controlling the volume of its constituent cells, preventing dilation of the normally narrow extracellular space, and maintaining the solubility of lens crystallins to stop their aggregation.

Although the energy necessary to drive these processes in the differentiating fiber cells of the outer cortex can be provided by aerobic metabolism, the remaining bulk of lens fiber cells lack mitochondria and therefore must use anaerobic glycolysis to satisfy their energy requirements. Because of its size, the lens cannot rely on passive diffusion alone to transport nutrients to deeper lying cells or to transport waste products back to the surface (discussed later). Furthermore, most fiber cells lack the usual potassium channels and Na/K pumps necessary to generate the negative membrane potential needed to control their steady state cell volume. Faced with these metabolic and physiological constraints, the lens requires a specialized transport system to deliver nutrients, remove waste products, and impose the negative membrane potential necessary to the maintain steady state volume of the fiber cells.

A common feature of all vertebrate lenses studied to date is the existence of a standing flow of ionic current that is directed inward at the poles and outward at the equator (Fig. 1A). Using a combination of electrical impedance measurements and the-

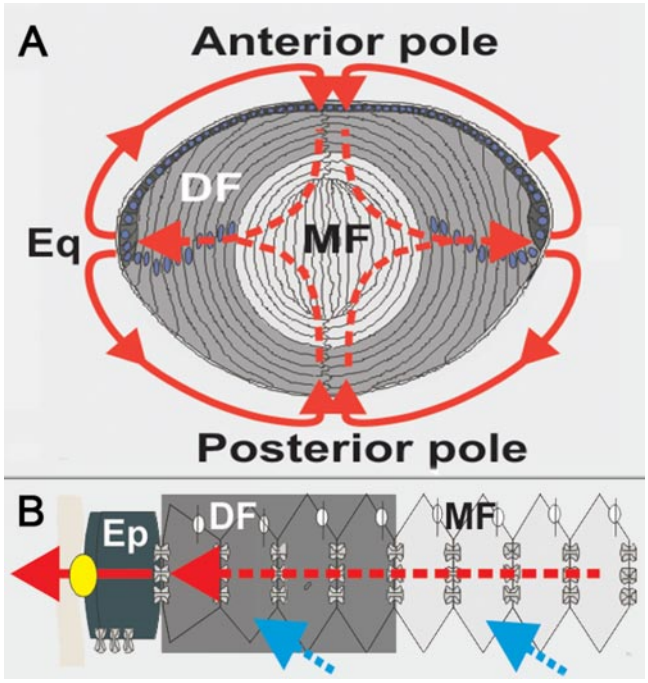

FIGURE 1. Lens structure and function. (**A**) Differentiating (DF) and mature (MF) fiber cells with measured external (*solid arrows*) and hypothesized internal (*dotted arrows*) current flows. (**B**) Equatorial (Eq) cross section showing ion uptake from extracellular space (*blue*) and cell-to-cell efflux (*red*) via gap junctions and Na pump (*yellow*). Ep, epithelial cells.

oretical modeling, Mathias et al.[4] have proposed that these currents measured at the lens surface represent the external portion of a circulating ionic current that drives a unique internal microcirculatory system that maintains fiber cell homeostasis and therefore lens transparency. Briefly, the working model is that the current, which is carried primarily by Na^+, enters at all locations around the lens along the extracellular clefts between fiber cells. It eventually crosses the fiber cell membranes, then flows from cell to cell toward the lens surface via an intracellular pathway mediated by gap junction channels. Because the gap junction coupling conductance in the outer shell of differentiating fibers (Fig. 1A) is concentrated at the equator,[5,6] the intracellular current is directed to the equatorial epithelial cells where the highest densities of Na/K pumps are located to actively transport Na^+ out of the lens.[7–9] Thus at the equator, the intracellular current that is leaving the lens is highly concentrated, causing the net current to be outward. At the poles, there is very little intracellular current. The net current is therefore predominantly inward, along the extracellular spaces (Fig. 1B).

The driving force for these fluxes is hypothesized to be the difference in the electromotive potential of surface cells and inner fiber cells. Data from ion substitution experiments performed on whole lenses[4] and more recently on isolated fiber cells,[10] suggest that the surface cells, including epithelial cells and newly differentiating fiber cells, contain Na/K pumps and K^+ channels, which together generate a negative electromotive potential. Fiber cells deeper in the lens lack functional Na/K pumps and K^+ channels, and their permeability is dominated by nonselective cation and Cl^- conductances, with molecular identities that remain to be determined. In these inner cells, a negative membrane potential is maintained by virtue of their connection to surface cells via gap junctions. This electrical connection, together with the different membrane properties of the surface and inner cells causes the standing current to flow. In this model, the circulating current creates a net flux of solute that in turn generates fluid flow. The extracellular flow of water convects nutrients toward the deeper lying fiber cells, whereas the intracellular flow removes wastes and creates a well stirred intracellular compartment. Furthermore, the active removal of Na^+ at the lens equatorial surface serves to maintain a favorable transmembrane Na^+ gradient that is used by secondary active transporters expressed by mature fiber cells to accumulate nutrients delivered to the lens core by the circulation system. Thus, transport by surface cells is not only responsible for the delivery of nutrients to inner fiber cells and their subsequent uptake, but also imposes the negative membrane potential necessary to maintain the steady state volume of the fiber cells.

Modeling Lens Function Predicts the Existence of a Circulating Fluid Flux

Although we acknowledge that the circulation model is not universally accepted, it is important to distinguish between the circulating ionic currents that have been experimentally measured[7,11,12] at the surface in lenses from different species and our model, in which a circulating current carried primarily by Na^+ generates a circulation of fluid inside the lens.[4] The data on the existence of the circulating ionic currents are firm and well supported by data on the distinct spatial localizations of gap junction conductance,[5] Na/K pump currents,[8] and the preferential influx of Na^+ at the anterior and posterior poles.[7,11,12] In contrast, circulating fluid flows in the lens have been more difficult to measure directly and are at present only predicted to occur from indirect measurements and models of the measured electrical properties of the lens.

The initial model of the electrical properties of the lens predicted the distribution of induced voltages when a current was injected into a central cell of a spherical syncytial tissue.[13] The model was based on the structure of the lens and was used to determine the membrane conductances of fiber and surface cells and to determine the effective resistances of intracellular and extracellular pathways.[4] Our impedance data suggest that most of the Na^+ leak conductance of the lens is associated with fiber cell membranes,[14] whereas others have localized Na,K-ATPase activity to the epithelium at the lens surface.[4] This spatial segregation of Na^+ influx (inner cells) from Na^+ efflux (surface cells) suggests that there is a circulation of Na^+. However, in our initial attempts to use the model to calculate the magnitude of this circulating Na^+ current, we naively neglected water flow. In the absence of water flow, the model predicted that voltage and ion gradients would develop in the intracellular and extracellular spaces that actually opposed the circulation. This result was contrary to experimental measurement of both surface current flows[12] and Na,K-ATPase activity, which suggests that a large Na^+ leak into the lens must exist to account for the large pump current. Even more bothersome was the prediction that large transmembrane osmotic gradients would develop, making it impossible to neglect water flow unless membrane water permeability was zero. Because it is clearly not zero,[15] it is now apparent that water flow cannot be neglected in modeling lens current flows.

The subsequent inclusion of water flow[16] dramatically altered the model and produced a series of specific predictions about the distributions of intracellular ions, voltages, and hydrostatic pressures that could be experimentally tested. Predictions of an intracellular voltage gradient from peripheral to central fiber cells of ~10 mV have now been confirmed by microelectrode measurements in lenses from several species.[16–18] Similarly, a prediction of a surface-to-central-fiber-cell gradient in the intracellular Na^+ concentration of ~10 mM was recently confirmed by measurements of the intracellular Na^+

concentrations in different regions of the mouse lens.[19] Earlier models also did not include the increase in gap junction coupling from the poles to the equator that was measured experimentally.[5] Higher coupling in peripheral equatorial fiber cells is proposed to guide intracellular outwardly directed current flow to the equator where Na,K-ATPase activity is concentrated.[8,9] Inclusion of this axial variation in gap junctional coupling into the model accurately predicted surface current inflow and outflow of a magnitude similar to those recorded by vibrating probe measurements.[11,12] Last, the model predicted that there would be a large intracellular hydrostatic pressure gradient (several hundred millimeters of mercury) to drive the intracellular flow of fluid from the central cells to the surface cells. We have recently measured this intracellular hydrostatic pressure in mouse lenses (Mathias RT, et al. *IOVS* 2010;51: ARVO E-Abstract 3459) and found that it varied from ~300 mm Hg in the central cells to 0 mm Hg in the surface cells.

The Lens Circulation System: Unresolved Questions

Although these many successes of the model calculations have served to reinforce our conviction that water fluxes convect nutrients into the lens faster than can be achieved by passive diffusion, it is fair to say that for many *seeing is believing*, and water flow in the lens has yet to be measured experimentally. Furthermore, some have questioned the need for a specialized transport system to enhance the delivery of nutrients to the "metabolically inert" fiber cells of the lens core. Finally, the model is evolving as new data are accumulated. These issues are now discussed, to identify areas for future experimentation.

Visualizing Circulating Ion and Fluid Fluxes within the Lens. Although fluid flow patterns such as those represented in Figure 1 are difficult to measure within the lens, findings in some recent studies are consistent with their existence. Fischbarg et al.[20] were able to detect translens (anterior to posterior) fluid movement when the lens was placed in a Ussing chamber that forced the pattern of current flow to be translens (anterior to posterior). Although interpreted differently by Fischbarg et al., this observation is consistent with our prediction that fluid follows the path of Na^+ flux such that when the Na^+ flux is forced to be translens, the pattern of fluid flow should follow. In this regard, magnetic resonance imaging (MRI) techniques offer the potential to noninvasively probe the structure and function of the lens. We have obtained preliminary data from MRI-based diffusion tensor imaging that show that the technique can be used to map the directionality of water movement in the bovine lens.[21] As would be predicted, the direction of water movement appears to follow the directionality of the Na^+ flux. Future work is needed to determine whether it can be altered after perturbations designed to disrupt the Na^+ flux that drives the circulation system.

Nutrient Delivery to Mature Fiber Cells. Because the lens is a large (with respect to diffusion distances), avascular organ,[23,24] it is our basic contention that some form of delivery system other than passive diffusion is necessary to support ionic and metabolic homeostasis of central fiber cells. This requirement is based on Einstein's law of diffusion, which simply states that the average time for diffusion to occur is proportional to the distance squared. This law means that diffusion is quite rapid over short distances, but is extremely slow over the longer distances found in organs. For example, glucose diffuses 10 μm deep into a lens in ~1 second but requires 11 days to diffuse 1 cm. In comparison, a convective fluid flow with a velocity of 1 $\mu m/s^2$, the rate calculated for fluid entry into the lens,[4] moves glucose up to 1 cm in less than 3 hours. Obviously, convection becomes essential when diffusion distances are significantly larger than cellular dimensions, the lenses of all mammalian species are sufficiently large that the diffusion of metabolites such as glucose to the central fiber cells would take many hours at least, whether it be via the extracellular space or an intracellular route mediated by gap junction channels.

Therefore, if the physical constraints imposed on a simple diffusion-based delivery system are accepted, then the issue shifts to whether the metabolic requirements of mature fiber cells are sufficiently high to warrant a faster delivery system. The metabolic requirements of the lens core are obviously 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. In the core, the major known metabolic requirement is to maintain the reduced levels of glutathione (GSH) to prevent protein cross-linking, crystallin aggregation, and light-scattering.[24] The replenishment of GSH from glutathione disulfide (GSSG) is mediated by the enzyme glutathione reductase and requires NADPH as a reducing equivalent, which is in turn produced via the hexokinase shunt pathway.[25] Hence, it is apparent that the core of the lens needs energy, but it is not clear whether the necessary reducing environment in the core of the lens is maintained by the local metabolism of glucose to maintain NADPH levels, or the importation of reducing equivalents from the cortex. We do know that low levels of glutathione reductase activity persist in the lens nucleus, albeit at lower levels than in the cortex,[26] and that mature fiber cells express transporters that are potentially capable of mediating the uptake of nutrients convected to them via the circulation system.[27] Furthermore, in preliminary experiments, we have shown that the Na^+-dependent glucose transporter SGLT2 is expressed in the nucleus and can accumulate SGLT-specific glucose analogues.[28] Thus, the production of reducing equivalents via the anaerobic metabolism of glucose convected into the nucleus by the circulation and the expression of glutathione reductase in the nucleus suggest that the recycling of GSH can occur locally.

In summary, the lens is a complex and dynamic tissue that requires an understanding of how regional differences in lens biochemistry, physiology, and cell biology contribute to the maintenance of lens transparency. It is our contention that the lens internal circulation system is central to this integrative lens biology that controls lens transparency. However, the circulation system remains a model that must be rigorously tested and refined if we are to accumulate the necessary functional insights to combat the looming cataract epidemic.

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Counterpoint: The Lens Fluid Circulation Model—A Critical Appraisal

In a study first reported more than 25 years ago, the investigators detected electrical currents outside the lens that depended on the activity of lens ion transporters.[1] Based on these ionic currents and measures of lens electrical impedance, a model was developed that postulated the existence of fluid flow through the lens fiber cell cytoplasm (hereafter referred to as the fluid circulation model [FCM]).[2,3] Although the FCM is more than 20 years old, direct evidence to support it is still lacking. Our analysis suggests that the FCM, as previously described, has conceptual shortcomings and does not appear to be consistent with published data. Contrary to the postulates of the FCM, it is unlikely that fluid circulation through the fiber cell cytoplasm is needed to maintain the metabolism of fiber cells that have degraded their organelles. We suggest that, if water did flow through the cytoplasm of fiber cells from the lens center to its periphery in the manner outlined in the FCM, it would be harmful to lens transparency.

We accept the initial observation that ion currents, generated by active transport, flow around and through the lens. However, we argue that these ion movements do not create a microcirculatory system in which water flows from the lens center to its periphery.

Measuring Fluid Movement in the Lens

Water has been shown to enter across the anterior epithelium and flow out of the lens across the posterior of the fiber mass.[4] The authors of this study suggested that water moves through the extracellular space between the fiber cells. When fluorescein was placed on the anterior surface of the rabbit lens in situ, the dye moved through the lens and appeared at the posterior surface 5 to 10 minutes later.[5] Candia[6] reported in a review article that, by isolating the lens equator from the anterior and posterior surfaces, fluid was observed to move into the lens across the epithelium and out of the equatorial and posterior surfaces. Although these measurements are informative, they do not reveal the pathway taken by water through the lens or whether the water traverses the fiber cell cytoplasm, as predicted by the FCM.

When a fluorescent dye small enough to pass through gap junctions was microinjected into a fiber cell of an intact lens, the dye diffused along the length of the injected cell and radially to fiber cells both deeper and more peripheral in the fiber mass.[7] If the injected cell was marked, the peak of fluorescence of the dye remained centered on the injected cell (Bassnett S., personal communication, July 2009).[8] The latter observation is not consistent with the FCM, which predicts that the dye would be carried into more peripheral fiber cells by the flow of water toward the lens equator (Fig. 1). One might imagine the fate of dye dropped into a still pool or a slowly flowing river. In both cases, the dye would diffuse radially from its initial position. However, dye placed in the river would move downstream with the flow, whereas dye placed in the pool would spread uniformly from its origin. In

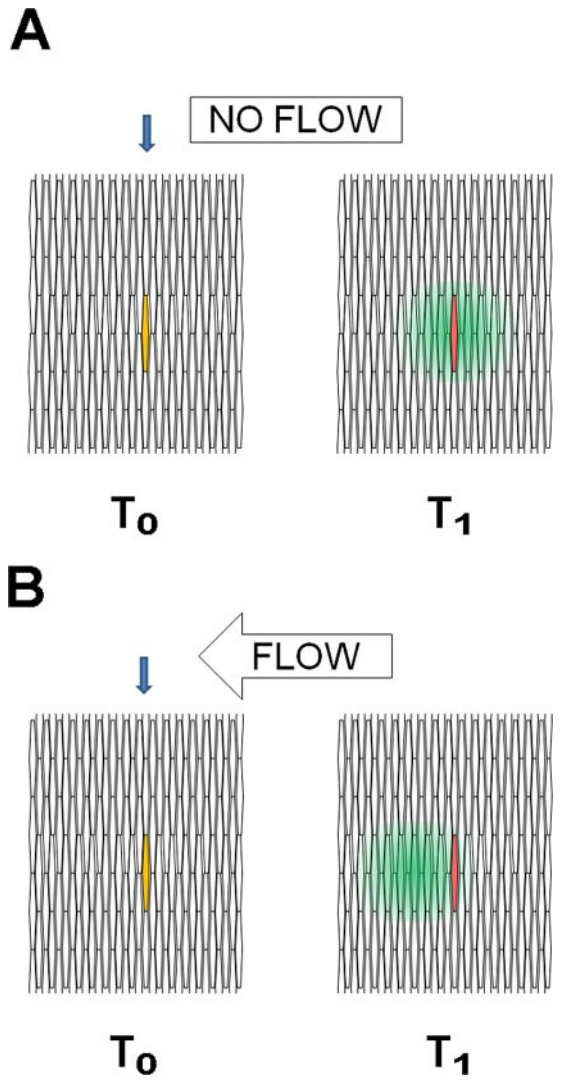

FIGURE 1. A cross-sectional view of fiber cells in which a single fiber cell is co-injected with a low-molecular-weight fluorescent dye (*green*) that can pass through gap junctions and a larger fluorescently labeled molecule (*red*) that cannot pass through gap junctions. The distribution of fluorescence is shown with increasing time after injection. At the time of injection (T_0), the injected cell (*blue arrow*) appears *yellow*, because of the co-localization of the green and red dyes. (**A**) The result if there is no fluid flow through the lens. With increasing time (T_1), the low-molecular-weight dye diffuses out on either side of the cell, with the peak of green fluorescence centered over the injected cell (*red*). (**B**) The results expected if there were flow through the lens. With increasing time after injection, the peak of green fluorescence of the low-molecular-weight dye would move "downstream," away from the red fluorescence in the injected cell. The available experimental data support the scheme outlined in (**A**).

the experiments reported to date, the lens behaved like a still pool.

These dual dye-injection studies were performed on lenses isolated from chicken embryos. It is not known when during its development the ion currents are first detectable around the lens. It is possible that no centrifugal flow was observed in these studies because no ion currents were present to drive flow. Thus, one way to explicitly test the predictions of the FCM would be to repeat these studies on adult lenses and monitor the extralenticular ion currents and dye movement at the same time. Such studies would test whether water flows through the cytoplasm of the lens fiber cells, from the center of the lens to its periphery, as predicted by the FCM.

The predictions of the FCM were also not supported by direct measurement of the movement of water molecules in the lens. Nuclear magnetic resonance (NMR) imaging was used to monitor water movement within intact human lenses in real time.[9] The pattern of flow predicted by the FCM (i.e., that water would enter initially at the poles and be seen last at the equator) was not observed. Instead, the water moved toward the nucleus uniformly from the lens surface and at a rate consistent with passive diffusion, with no evidence of flow. In a subsequent investigation, diffusion tensor NMR microimaging was used to study the movement of water within the fiber cell cytoplasm.[10] In this study, the water diffused freely within the lens fiber cell, but diffusion was constrained significantly by cell membranes. Of note, quiver plots, which reveal the "preferred" direction of diffusion, demonstrated that access to the center of the lens was largely via movement of water along the equatorial plane, not from the poles, as predicted by the FCM.

Potential Problems with the FCM

The FCM assumes that ions (Na^+ and Cl^-) and small metabolites such as glucose diffuse from the fluids surrounding the lens into the extracellular space surrounding mature fiber cells, deep in the lens. The gradients that result from the movement of ions across fiber cell membranes are postulated to provide the motive force for fluid flow.[3] Glucose is assumed to provide a substrate for metabolism in the lens core. However, studies by proponents of the FCM showed that a barrier to extracellular diffusion, located at the interface between the differentiating and mature fiber cells, inhibits small molecules such as glucose from diffusing into the extracellular space of the lens core.[11] This obstruction was not discussed in papers describing the FCM. Its existence seems contrary to the model.

Lens fiber cell membranes have a high concentration of gap junctions and aquaporin water channels. The FCM suggests that water channels contribute to the flow of water through the lens, with ions and some of the water passing through gap junctions.[3] However, unidirectional movement of water through water channels would create osmotic differences across the lateral membranes of the fiber cells. As water flows through these channels, intracellular solutes would be left behind. The resulting gradients would create an osmotic force that would oppose the flow of water at the surface of every fiber cell (Fig. 2). This effect would preclude unidirectional flow through aquaporin channels as a means of moving the water from the center to the periphery of the lens. 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. Although the osmotic braking force generated at gap junctions would be less than at water channels, it would still be additive for every fiber cell along the radius of the lens. Neither of these impediments to unidirectional flow was discussed in the published version of the FCM.[3]

Mature Fiber Cells Have No Need for Metabolic Activity, and Published Studies Show that Little Metabolism Occurs There

The FCM assumes that centrifugal fluid flow is essential for providing nutrients such as glucose to the fiber cells that lack organelles and for removing the waste products of metabolism (lactate, in the case of anaerobic glycolysis). Therefore, a basic assumption of the FCM is that mature fiber cells have active metabolism.

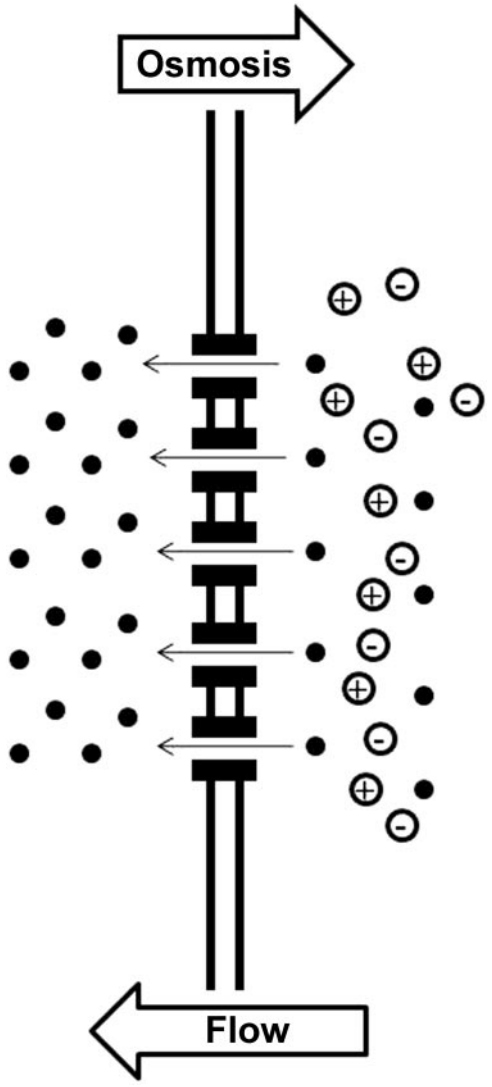

FIGURE 2. The consequence of unidirectional flow through aquaporin water channels. Water molecules (●) pass through the channels, leaving ions (○) behind. This movement creates lower water concentration on the upstream side of the membrane, generating osmotic pressure opposite the direction of flow. Without a means of selectively moving ions across lens membranes, unidirectional flow through aquaporin channels is not possible.

We know of no metabolic pathway that has been demonstrated in mature nuclear fiber cells (Fig. 3). Typical cells expend most of their metabolic energy maintaining their transmembrane potential and synthesizing proteins. Neither of these activities occurs in mature fiber cells. Even if some low level of metabolic activity were found in the cells, substrates for these reactions could diffuse through gap junctions from metabolically active superficial cells, which have organelles.

Consistent with this view, results of studies suggest that little enzyme activity survives in fiber cells after they have finished elongating and degraded their organelles. In all species examined, enzyme activity is concentrated in the outer cortical zone of the lens. In the young rat, the cortex contains 75% of the total phospholipase A2 activity.[13] Incorporation of tritiated water into cholesterol and fatty acids, as well as leucine into aquaporin-0, occurred in the outer 10% of the lens, with peak incorporation in the outer 3% to 6%, corresponding to the fiber cells that contain organelles.[14] Similarly, immunochemically detectable transglutaminase was localized to epithelial cells and a thin zone of the peripheral cortex in human lenses.[15]

Glucose transporters are readily detectable in the membranes of mature fiber cells,[16,17] which raises the question of whether these transporters are functional and whether glycolysis persists deep in the lens. Glycolysis requires the concerted activity of 10 enzymes. Loss of the activity of any one would block the pathway. No glucose-6-phosphate dehydrogenase (G6PD) activity was detected in the center of lenses in rats older than 6 months, but inactive G6PD molecules were detected.[18,19] The presence of inactivated enzymes in the nuclei of lenses may well be a general phenomenon, as the presence of superoxide dismutase, aldolase, and glyceraldehyde-3-phosphate dehydrogenase has also been documented.[18–21] Active lactate dehydrogenase (LDH), the enzyme that converts pyruvate to lactate in the final step of anaerobic glycolysis, was readily detected by histochemical assay in the nucleated superficial fiber cells of bovine and human lenses, but not in the fiber cells that were 50 to 150 μm beneath the capsule and lacked nuclei.[22] The sharp boundary between cells with LDH activity and those lacking it suggests that the activity of the enzyme was lost during or soon after fiber cell denucleation. In the absence of mitochondria, LDH activity is necessary to produce the NAD^+ that is essential for upstream steps in glycolysis. Without the NAD^+ produced by LDH, glycolysis would come to an abrupt halt. Given these observations and the fact that macromolecules present in the nuclei of adult human lenses have been "cooking" at body temperature for decades with no means to replace them, it is unlikely that glycolysis or any other major metabolic pathway functions in human nuclear fiber cells (Fig. 3). With little metabolic activity in the nucleus, there is no requirement for fluid flow to deliver substrates and remove the products of metabolism. If the FCM is necessary for metabolism in the lens nucleus, metabolic activity should be demonstrable there.

Is There a Need for Coupling between the Cortex and Nucleus?

It is apparent from the previous section that the lens nucleus, particularly in adult humans, is quiescent, with little or no enzymatic activity. A corollary is that, for nearly all purposes, there is no need for appreciable communication between the metabolically active cortex and the lens nucleus. There is, however, at least one exception.

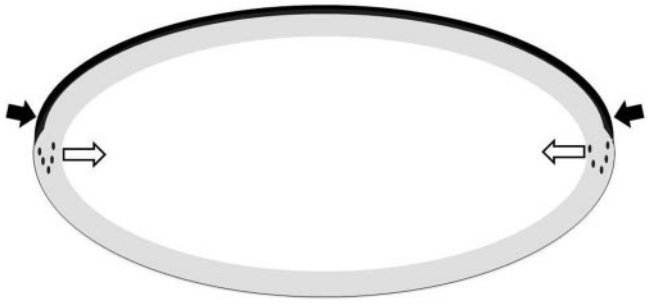

FIGURE 3. The zone of active metabolism in the adult human lens. The *dark band* at the *top* represents the lens epithelium. The *gray oval* at the *periphery* of the fiber mass includes all the lens fiber cells with nuclei and other membrane-bound organelles. The epithelium and the outer fiber cells are responsible for nearly all the metabolism in the lens. The available data suggest that the lens center has little or no metabolic activity. Based on experimental data, the *filled arrows* indicate the major route of entry of metabolites at the germinative zone, adjacent to the lens equator.[12] The *open arrows* depict the direction of diffusion of critical metabolites such as glutathione, from the metabolically active superficial fiber cells where they are produced, to the deeper fiber cells.

Glutathione (GSH) is essential for maintenance of lens transparency.[23–25] Like the enzymes just described, the enzymes and cofactors necessary for the reduction of GSH (glutathione reductase [GR], NADPH, and enzymes of the hexose monophosphate shunt) are located in the lens periphery. The lens cortex contains more than 20 mM GSH.[26,27] GR, the enzyme that is necessary for the reduction of oxidized glutathione, has been measured in the cortex and nucleus. Precise dissection methods have revealed a steep gradient of GR, with highest specific activity in the outermost cortical fibers, decreasing to no detectable activity in the inner parts of older lenses.[28] GSH is 80% to 90% lower in the lens nucleus than in the cortex,[29] and age exacerbates this difference (for example, see Ref. 30). Treatment of rabbit lenses with hyperbaric oxygen (HBO) for 4 hours decreased GSH levels by less than 10% in the superficial cortex, but by 70% in the nucleus.[31] In guinea pigs given prolonged HBO treatments, the level of GSH in the nucleus became nearly 10 times lower than that in the cortex, and GSH- and cysteine-protein–mixed disulfides in the nucleus increased dramatically.[25,30] It has been suggested that the decline in GSH concentration that occurs in the nucleus of the human lens with increasing age is caused by a barrier to diffusion from the periphery to the center of the lens.[32] The decline in GSH and the resulting increase in GSSG leads to oxidation of cysteine and methionine residues in proteins, a hallmark of age-related nuclear cataracts.[33]

When monkey lenses were incubated with ^{35}S cysteine for various times and the movement of label within the lens followed by autoradiography, the amino acid entered primarily at the equator (Fig. 3).[12] The movement of cysteine within the lens was then followed over time. The major pathway appeared to involve diffusion along the length of the fiber cells and orthogonal movement across the fibers in the equatorial plane, in accordance with the distribution of connexons (Fig. 3). These experiments showed that metabolites are transported into the cytoplasm of lens cells close to the equator and then move inward toward the nucleus along the equatorial plane. This direction is opposite that of the water and solute flow predicted by the FCM.

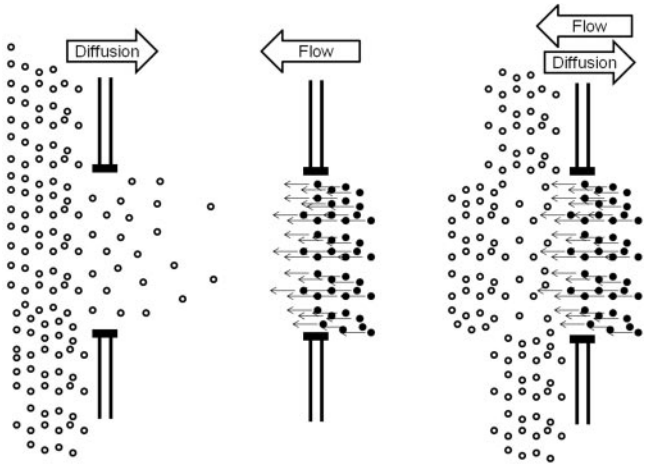

Figure 4. The diffusion of a metabolite such as glutathione and the flow of water in the opposite direction through a gap junction channel. *Left*: metabolite molecules moving through a gap junction by diffusion down a concentration gradient. Each molecule moves randomly. Net movement is due to the concentration difference across the junction. *Middle*: the flow of water through a gap junction, as proposed by the FCM. The net movement of all water molecules is in the same direction, resulting in bulk flow of solvent. *Right*: these movements are combined. It is evident that, if the magnitude of flow and diffusion were similar, the vectorial flow of water would oppose the movement of the metabolite, retarding or preventing its movement across the gap junction.

The experiments cited herein have shown that the capacity to reduce GSH is minimal in the lens nucleus. GSH is synthesized and reduced in the cortex, and reduced glutathione diffuses from the periphery of the lens to its center to maintain a reducing environment there. Therefore, to maintain lens transparency it is necessary to preserve a pathway for diffusion between the cortex and nucleus.

Fluid Flow from Center to Periphery Would Harm the Lens

The proponents of the FCM estimate that the magnitude of water flow and diffusion in the lens are similar.[3] They also point out that, because flow is directional and diffusion is random, flow would be more efficient at moving metabolites and waste products through the lens. However, if water flowed from the center to the periphery of the lens through gap junction channels, it would counter the diffusion of GSH through these same channels in the opposite direction (Fig. 4). Therefore, directional flow from the lens center to its periphery would effectively restrict reduced glutathione from reaching the lens nucleus. As described earlier, maintaining sufficient reduced glutathione in the nucleus is essential in the maintenance of transparency. Therefore, the mechanism of the FCM, as it is presently described, would be harmful to the lens.

Conclusions

Based on the arguments herein, we suggest that the theory behind the FCM is unlikely to be correct and that such movement is not needed by the lens. The model also appears to be incompatible with maintenance of the reducing environment that is necessary for the transparency of the lens nucleus. Before the FCM can be accepted, it must be demonstrated, not just hypothesized, that an internal circulatory system moves fluid through the cytoplasm of adult lens fiber cells. Its proponents should also show that a major metabolic pathway—for example, glycolysis—functions in mature fiber cells.

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Reply to: The Lens Fluid Circulation Model—A Critical Appraisal

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 $\sim 1.7 \times 10^{-3}$ μm/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 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 μm, 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 μm 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 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,[1] 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

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, Na^+ concentration gradients, and Ca^{2+} concentration gradients[2] and our more recent studies of hydrostatic pressure gradients (Mathias RT, et al. *IOVS* 2010;51: ARVO E-Abstract 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.[4] 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.[5] 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 Lim[6] 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 ^{35}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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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 extralenticular 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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