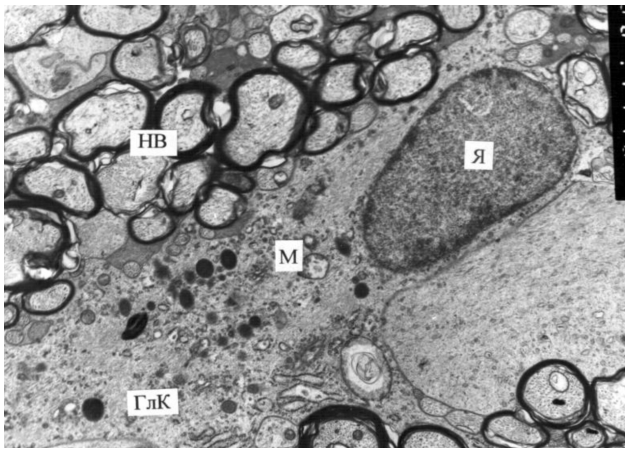

Рис. 3. Ультраструктура глиальных клеток зрительного нерва кролика через 1 месяц после моделирования контузии легкой степени. Нормальная ультраструктура глиальных клеток. Электронная микрофотография. Х 6 000. Условные обозначения: ГлК — глиальная клетка, Я — ядро, М — митохондрия, НВ — нервное волокно.

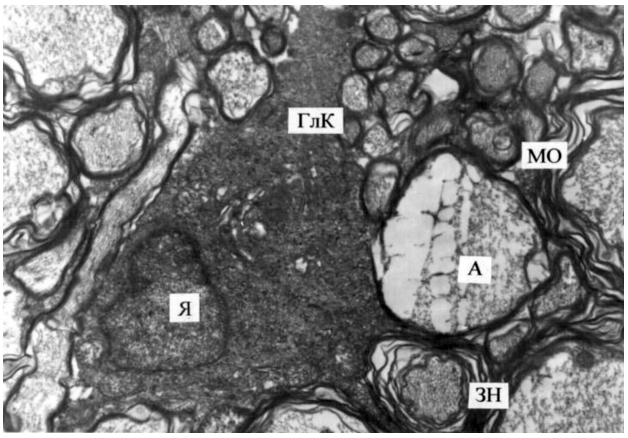

Рис. 5. Ультраструктура зрительного нерва кролика через 2 месяца после моделирования контузии легкой степени. Уплотненная структура глиальной клетки, фрагменты деструкции мембран миелиновой оболочки. Электронная микрофотография. Х 6 000. Условные обозначения: ГлК — глиальная клетка, Я — ядро, А — аксон, МО — миелиновая оболочка, ЗН –зрительный нерв.

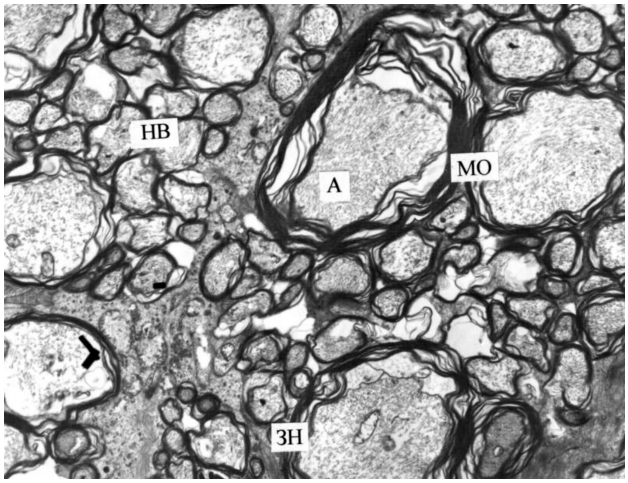

Рис. 4. Ультраструктура аксонов зрительного нерва кролика через 2 месяца после моделирования контузии легкой степени. Выраженные нарушения ультраструктуры аксональных структур и оболочек зрительного нерва. Электронная микрофотография. Х 4 000. Условные обозначения: А — аксон, НВ — нервное волокно, МО — миелиновая оболочка, ЗН — зрительный нерв.

нейрофиламентов, которые служат каналами для аксоплазматического транспорта веществ и способствуют прохождению нервного импульса по нервному волокну. Микрофиламенты являются также местом локализации мембранных протеинов [1, 9, 19, 20, 28].

Результаты проведенных нами исследований показали, что постконтузионные нарушения в структурах зрительного нерва первично касаются, в основном, тонких повреждений нейротрубочек и нейрофиламентов, а также митохондрий в аксонах нервных волокон. Ультраструктурные повреждения митохондрий приводят к снижению образования АТФ, энергии, которая необходима для аксоплазматического транспорта и других функций нервных волокон [5, 8, 15, 16, 21, 22]. Вследствие повреждения нейротрубочек и нейрофиламентов нарушается нормальное функционирование аксонов, что, возможно, в дальнейшем может привести к дегенерации нервных волокон, а также и нервных клеток [6, 13, 14]. В опубликованной нами ранее работе [3] в постконтузионном периоде на данной модели контузии в теле ганглиозных клеток отмечены признаки гидропических изменений ультраструктур, особенно элементов зернистой эндоплазматической сети с дегрануляцией их мембран, что свидетельствует о нарушении белкового синтеза и метаболических процессов во всей клетке. Кроме этого, в данном нашем исследовании в миелиновых оболочках нервных волокон также выявляются признаки отека и разволокнения их ламелл, что также усугубляет прохождение нервного импульса. При этом глиальные клетки, окружающие нервные волокна, более устойчивы. Однако при определенной степени контузии ультраструктуры глиальных клеток также могут очагово вовлекаться в патологический процесс, что еще больше усугубляет патологическое состояние нервных волокон, поскольку глиальные клетки не только способствуют проведению нервного импульса нервными волокнами, но и участвуют в их питании и регенерации [26, 27].

Заключение

Таким образом, в результате моделирования контузии легкой степени глаза у кроликов определены ранние и первичные повреждения ультра-

структуры нервных волокон зрительного нерва. Учитывая регенераторные способности аксонов, данные волокна могут восстановить свою структуру. Однако при более тяжелой степени контузии, повреждение изучаемых структур может привести к их глубокой патологии.

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Physical Characteristics of the Internal Limiting Membrane and their Significance in Ocular Surgery and Beyond

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Key words: Atomic force microscopy, brilliant blue G, chromaticity analysis, chromovitrectomy, indocyanine green, internal limiting membrane, trypan blue, vital dyes.

Introduction*: Efforts to render surgical removal of the internal limiting membrane safer and easier depend on an in-depth understanding of its physical characteristics.*
Purpose*: Studying the internal limiting membrane is particularly gratifying, as it represents the most easily accessible of all human basement membranes and many findings may contribute to an improved comprehension of human basement membranes in general.*
Data sources*: MEDLINE with no language restriction through March 2014.*
Data selection*: Overview of recent research results from the authors own study group as well as a context of the current literature.*
Conclusion*: New examination techniques such as chromaticity analysis and translational use of established techniques like atomic force microscopy allow new insights into mechanical properties of the internal limiting membrane. Chromaticity analysis permits objective measurements of the contrast behavior of the internal limiting membrane when exposed to vital dyes. Compared to various approved dyes, indocyanine green shows the best visible contrast, in accordance with its popularity despite its off-label status. Atomic force microscopy allows the examination of the native internal limiting membrane. It, thus, enabled the study group to provide an extensive topographic map including surface structure, thickness and stiffness, avoiding the fixation artifacts of previous descriptions. Surface structure, stiffness, rolling behavior and cell adhesion were found to be asymmetrical between the two opposite surfaces of the membrane and the same asymmetry could be reproduced for several other ocular basement membranes challenging the current model of basement membrane ultrastructure.*

Ключевые слова: атомно-силовая микроскопия, бриллиантовый синий G, анализ цветности, хромовитрэктомия, индоцианин зеленый, внутренняя пограничная мембрана, трепановый синий, витальные красители.

Введение. *Легкость и безопасность удаления внутренней пограничной мембраны (ВПМ) зависит от степени понимания ее физических характеристик.*
Цель. *Изучение внутренней пограничной мембраны, являющейся одной из наиболее доступных базальных мембран организма человека, будет способствовать расширению взглядов на строение базальных мембран в целом.*
Источники данных: *Медлайн без языковых ограничений в течение марта 2014*
Выбор данных: *Обзор последних результатов исследований авторов и мировой литературы.*
Заключение. *Применение новых методов обследования, таких как хроматический анализ и атомно–силовая микроскопия позволяют по-новому взглянуть на механические свойства внутренней пограничной мембраны. Хроматический анализ позволяет объективно оценить степень контрастности ВПМ при воздействии витальных красителей. Несмотря на отсутствие регистрации индоцианин зеленого, он обеспечивает лучшую*

Ключові слова: атомно-силова мікроскопія, діамантовий синій G, аналіз кольоровості, хромовитректомія, індоціанін зелений, внутрішня прикордонна мембрана, трипановий синій, вітальні барвники.

визуализацию контрастности по сравнению с другими утвержденными красителями. Атомно–силовая микроскопия дает возможность изучать нативную ВПМ. Таким образом, исследовательской группе удалось создать обширную топографическую карту, включая структуру поверхности, толщину и жесткость ВПМ. Было выявлено, что структура поверхности, жесткость и клеточная адгезия асимметричны между двумя противоположными поверхностями мебраны и подобная асимметрия может быть воспроизведена для других базальных мембран глаза с помощью данной ультраструктурной модели базальной мембраны.

Chromovitrectomy and macular surgery

Vitreomacular traction may be associated with significant visual disturbances, such as acute visual loss and central scotomata [1]. It is associated with a multitude of related macular conditions including epiretinal membrane formation [2], retinal surface folds [3], wrinkling of the ILM [4], loss of the foveal contour [5], and can result in an alteration of neurosensory retinal layers with intraretinal cystic degeneration [3], can lead to persistent cystic macular edema [6] and may ultimately result in the formation of macular holes [7]. Macular holes tend to lead to sudden and often complete loss of central vision. Vitreomacular traction is finally regarded as an important risk factor for the development of exudative age-related macular degeneration [8], the most prevalent cause of severe vision loss in the industrialized world [9].

Surgical relief of vitreomacular traction by simple vitrectomy was first described a quarter century ago [10]. Vitrectomy frequently does not fully eliminate traction, however, as parts of the vitreous cortex may remain attached to the retinal surface and act as a scaffold for fibrocellular and fibrovascular proliferations [11, 12], resulting in significant patient morbidity and surgical failure [13]. Surgical removal of the ILM had initially occurred unintentionally [14], but inasmuch as patients fared well even if large fragments of ILM had been stripped [14], ILM removal was proposed as a therapeutic option for vitreomacular traction [15]. ILM removal allows a more complete relief of both vertical and tangential traction, essential for surgical success and the prevention of recurrence [16], by creating a new vitreomacular interface through the elimination of vitreous cortex and proliferative tissue [13]. Vitrectomy with removal of the ILM has, thus, evolved into the standard treatment for diseases related to vitreomacular traction [17]: At the end of a vitrectomy procedure, the surgeon grasps the ILM with a fine forceps and carefully peels it off the underlying retinal layers. The procedure is extremely delicate as the ILM is transparent, extremely thin and in direct contact with highly damageable retinal structures. It represents an enormous challenge and responsibility for the surgeon and may make for a panoply of complications [16, 18,19,20]. A particular technical difficulty lies in the initiation of the ILM peel and the visualization of the border or edge of the membrane once ILM peeling has been started [21]. It can be difficult to continue an ILM peel if the edge is lost and to determine the total extent of the peel [22, 23]. Difficult visualization of the ILM and firm attachment of the ILM to the underlying retina can, thus, present technical challenges even for experienced vitreoretinal surgeons.

A number of years elapsed after the first report on an intentional surgical ILM removal [15] before a refinement of surgical technique was addressed in an effort to improve its safety and ease: A diamond dusted cannula was introduced to facilitate lifting of an initial ILM flap [24]. But it was not until Kadonosono's bold intravitreal application of indocyanine green (ICG) to stain the ILM that such refinements were felt to, indeed, contribute greatly to safety and practicability of the intervention [19]. Within a short period of time, many previously skeptical surgeons would trust themselves to engage in macular surgery. Peeling of the stained ILM soon gained worldwide acceptance [17] and went on to be known as Chromovitrectomy [25]. It was during the author's years as a resident in Munich, that a local group attracted global attention by pouring cold water on this initial excitement among vitreoretinal surgeons, describing worse functional outcomes after ICG-assisted ILM peeling in macular hole surgery [26]. Both the importance and the challenge of rendering ILM removal a safer procedure, which have sparked the author's scientific interest until the present day, had become apparent.

Until the present time, the photochemically active tricarbocyanine dye ICG is approved only as an intravenous substance in the context of ICG angiography for ophthalmic purposes. Intravenous application represents an off-label use [17]. Kadonosono's report was published shortly before data on ICG's specific staining capacity for the ILM became available from a donor eye study [21] and safety data were limited to the author's own unpublished transmission electron examinations of rabbit eyes previously injected with ICG. In so far, the multitude of concerns over ICG's safety for intravitreal application issued in the following years came as no surprise and involved dose-dependent in vitro toxicity on various retinal cell populations [27–29], severe histologic damage to the inner retina after ICG in combination with intraoperative light exposure [30], a shift in cleavage plane [26], optic nerve damage [31] and clinically apparent unexpected visual field defects and unfavorable visual acuity outcomes [26]. Surgeons were left with the difficult choice between operating with insufficient visibility and exposing their

patients to a potentially harmful adjuvant which was not approved for intraocular use. They were faced with an urgent need for new resources to improve safety and efficacy of ILM-removal. The key to such new resources resided in an improved understanding of ILM material properties and their influence on the efficacy of chromovitrectomy, which has evolved as the core theme of the present research project.

Ilm material properties in chromovitrectomy

Safety and efficacy of a chromovitrectomy intervention are governed by how well the target tissue can be identified, how well a point of vantage for lifting the initial ILM flap is recognized, the precision with which the ILM is grasped with the forceps, adhesion strength between the forceps and the ILM, the thickness, stiffness and elasticity of the ILM and on how well the completeness of ILM removal can be monitored. Thus, apart from the surgical skills of the ophthalmologist, successfully carrying out a chromovitrectomy involves the understanding and proper instrumentalization of its physical features such as contrast behavior and bio-anatomical and bio-mechanical properties including micromorphology, thickness and stiffness distribution.

Contrast behavior

The year when the present research project was formally launched was marked by the introduction of the synthetic triphenylmethane **brilliant blue g** (BBG) as the first biostain **approved for intravitreal use** in the European Union [17]. BBG's potential as an ophthalmic dye had previously been recognized when it was found to stain the lens capsule in low concentrations in an animal model [32]. The new product was announced as a vital dye with a favorable preclinical safety record [33] and no obvious adverse effects had been observed in one small clinical case series at this point [34]. For usage in the posterior segment of the eye, staining was proclaimed to be specific for the ILM [34] and the expectation was that the vitreoretinal community would avidly adopt the long awaited approved adjuvant. In reality, however, acceptance of BBG turned out to be more than sluggish.

In order to better understand the reservations against the new vital dye, as well as its actual potential, we evaluated its staining properties, safety and surgical outcome [17] in a retrospective, non-comparative multicenter clinical case series, based on our first consecutive 17 applications of the new product.

Removal of the ILM was successfully completed in 15 of the 17 cases. In two interventions, additional ICG was applied to allow safe ILM peeling. Staining strength was measured based on post-operative subjective surgeon ratings and revealed **weaker contrasts** with BBG than with ICG. A complete extraction of the ILM could be confirmed by OCT in 16 out of 17 interventions, one case displayed mild residual perifoveal ILM. Signs of apparent dye toxicity were not encountered and postoperative functional outcome was excellent with best corrected visual acuity unchanged in one and improved in all other patients. OCT scans of central retinal thickness were improved in all but one patient, reflecting reduced vitreoretinal traction and edema. BBG's inferior contrast ratings in this small series, underscored by the need for additional ICG in individual cases, was interpreted as a likely explanation for the substance's low acceptance and for ICG's continued popularity. It was felt that more objective data were needed to compare contrast behavior of available biostains.

New method to objectively quantify ILM contrast

To this end, a novel methodology was developed by our group to **objectively quantify** the intraoperative color **contrast** between the stained ILM and the underlying unstained retina as it is perceived by the human visual system [35]. This method, termed chromaticity analysis, is based on intraoperative videos. Individual images displaying good viewing quality and maximum staining within the vascular arcades are selected. In each image, two regions of interest (ROI) representing maximum contrast are signaled: One ROI is marked in an area with maximally stained ILM. A second ROI of similar dimensions is chosen in an area where the ILM has already been removed during the course of the procedure, exposing the unstained retina. Based on the wavelengths captured by the charge-coupled device (CCD) of the camera, a custom made software application calculates the average color of each of the two ROIs. Fig 1.

In order to comprehensively describe how well the contrast between these two colors can be perceived by the human eye, a mere comparison of their wavelengths would be insufficient, however, because the contrast sensitivity of the human visual system is wavelength-dependent. In other words: Some color-contrasts can be better recognized than others, although the numerical difference in wavelength might be identical. For this reason, the software application is equipped with a function to project the measured wavelength-differences into a color space organized according to **human color perceptiveness**. A color space is a diagram representing all visible colors: Based on large scale systematical empirical analyses of human visual sensitivities to color differences, the spectrum of visual light has been arranged in what is called the CIE 1931 color space [36]. In this chromaticity diagram, a classical vector space, regions containing all colors indistinguishable from the color located in the center of the region were defined [36]. These regions feature an ellipsoid shape. The regions in this original CIE 1931 color space vary in size and orientation depending on their center color, turning their interpretation relatively unpractical. An attempt to create a less distorted representation lead to the development of the CIELAB (CIE 1976 L*,a*,b*) color space. A transcription of the original ellipses to the

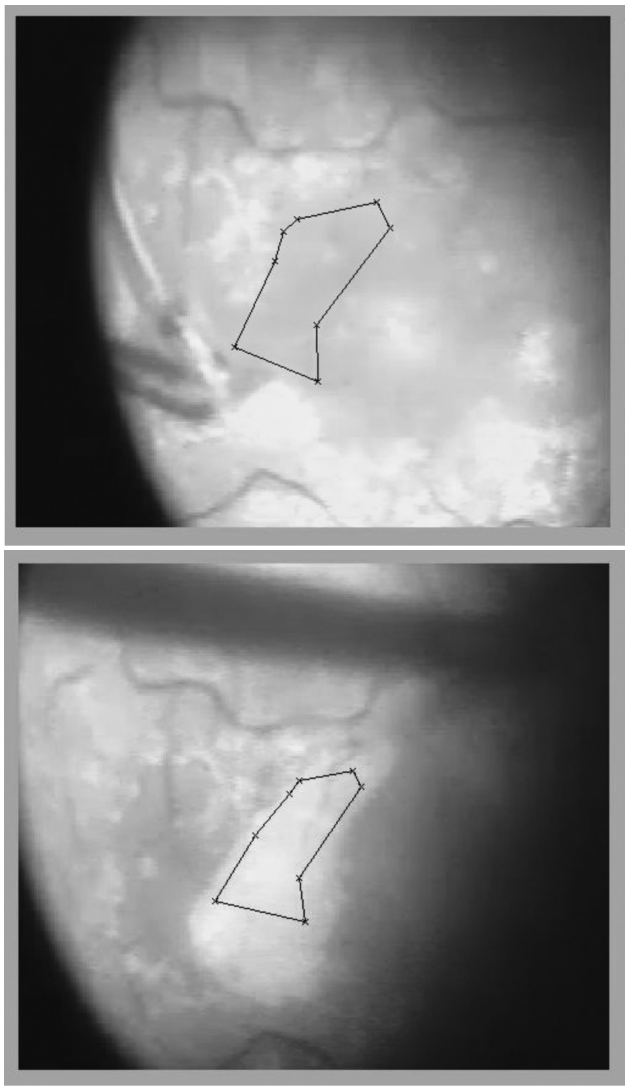

Fig 1: Chromaticity analysis screenshot.

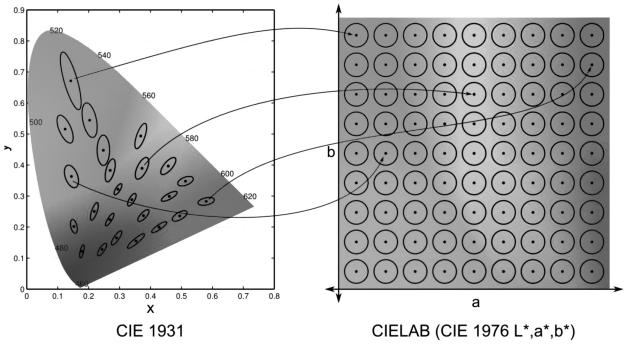

Fig 2: Left: In the CIE 1931 color space, regions of colors which cannot be distinguished by the human eye appear ellipsoid; right: In the more uniform CIELAB color space, the same regions are circular and distances within the color space are proportional to their discriminability by the human visual system.

new CIELAB color space results in an almost circular distribution of indistinguishable colors. Changes of the same visual importance are reflected in an identical distance in the CIELAB color space. This distance is referred to as a chromaticity score and can be regarded as a direct measure for the strength of perceived contrast [37]: The higher the chromaticity score, the better the surgeon will recognize a particular contrast. The accuracy of this novel method has recently been confirmed by Kadonosono, the initial descriptor of ICG chromovitrectomy [38]. Fig 2.

Objective comparison of staining capacity of vital dyes BBG's staining strength inferior to that of ICG

The new methodology allows the comparison of different vital dyes and can monitor the effectiveness of refinements in surgical technique in terms of staining strength. In a first step, chromaticity analysis was employed for a **head-to-head comparison of the staining comportment of BBG and ICG**. Twenty-six consecutive routine chromovitrectomy interventions for diseases related to vitreomacular traction were enrolled in a retrospective, multicenter, nonrandomized clinical case series [35]. Fourteen subjects underwent ICG and 12 BBG chromovitrectomy. One hundred thirty-three individual measurements were performed across the study sample and yielded **significantly better staining results for ICG compared to BBG** with median chromaticity scores of 14.7 and 6.8, respectively. These objective data forcefully corroborated subjective data from the previous study supporting that unsatisfactory contrast characteristics could contribute to the slower than expected acceptance BBG in chromovitrectomy, despite its obvious advantages over ICG with respect to approval status and toxicity profile. Our data have recently been confirmed by other researchers [38].

TB's staining strength equivalent to that of BBG

While vitreoretinal surgeons in Europe were now equipped with an approved — albeit relatively inefficient — vital dye, their colleagues in the United States remained defrauded of any legally recognized option in chromovitrectomy altogether. The only medical adjunct approved for intravitreal use both in the United States and Europe, the toluidine derivative Trypan blue (TB), is labeled for the removal of epiretinal membranes, but not for staining of the ILM. TB's outstanding affinity to epiretinal membranes is undisputed [39, 40]. Epiretinal membranes are progressive fibrocellular proliferations on the vitreal surface of the ILM. Different from the ILM, which is a basement membrane, epiretinal membranes are characterized by glial cells, hyalocytes, retinal pigment epithelial cells, and transdifferentiated fibroblast-like cells [41]. It is, thus, not surprising that epiretinal membranes and the ILM should be stained by different substances. On the other hand, TB had been successfully used for staining of the anterior capsule,

also a basement membrane, in cataract surgery for over a decade [42]. Indeed, several clinical studies now suggest that TB may be more useful for ILM-removal than customarily conceived [43, 40, 44–47]. We therefore decided to apply chromaticity analysis to shed light on TB's role as a potential biostain for both epiretinal material and the ILM [48].

In a retrospective, multicenter clinical case-series, we analyzed 50 consecutive chromovitrectomy interventions in 50 patients. Twenty-one patients were operated using BBG, 14 with ICG and 15 with TB. In total, 317 measurements were performed. As the specificity for the ILM of both ICG and BBG is uncontroversial, a distinction of images displaying epiretinal membranes or ILM was made only in the TB group. This differentiation was made during a post-operative review of each video with the surgeon. We believe, that this protocol guaranteed a high degree of accuracy, even in the absence of histologic confirmation, as we had demonstrated previously, that intraoperative visual distinction of epiretinal material and ILM is possible with a high degree of precision [49]. A clinically useful staining, sufficient for epiretinal material and/or ILM removal, was observed in all interventions. To our surprise, **TB** turned out to effectively **stain epiretinal material and the ILM equally well**. Whether TB stains the ILM directly in analogy to its affinity to the anterior lens capsule or if fine cellular debris overspreading the ILM is responsible for its tinge [50, 51] cannot be deduced from our results. What can be said is that TB should be included in the list of available vital dyes for chromovitrectomy, particularly as **TB's staining strength for the ILM** turned out to be **equivalent to that of BBG**. Recently, a novel combination biostain containing both TB and BBG received CE-approval (MembraneBlue-Dual™, DORC International, Zuidland, The Netherlands) [52]. From a theoretical viewpoint, it can be expected that the staining effects of both substances in this preparation may add and augment each other. This expectation is backed by our clinical experience, but further research is needed for verification.

«Heavy BBG» is practical but it's staining strength not significantly improved over traditional BBG

Although vitreoretinal surgeons were now vested with two approved biostains for chromovitrectomy poor staining strength continued to compromise clinical usefulness. Further refinements were urgently anticipated. Various new dyes, including patent blue [53, 54] , bromophenol blue [55], infracyanine green [55], triamcinolone [54], fluoresceine [54], aniline blue [56] and methyl blue [56] are in preclinical evaluation but cannot be recommended for routine use at this time. Another way of improving intraoperative staining might consist in upgrading existing substances with the intention of improving their efficacy. One such upgrade has recently become available and consists in a heavier than water solution to achieve higher BBG concentrations on the retinal surface while avoiding unnecessary dye exposure of other ocular structures. To this end, 0,065ml or 13 % deuterium (D20) were added to the hydration shell of the commercially available 0,25mg/ml BBG solution by the manufacturer. Deuterium atoms replace, thus, part of the hydrogen atoms in the water contained in the preparation. The resulting increase in nuclear mass elevates the specific weight of the solution to 1,018 g/cm^3. As the dye molecule was not changed, a design modification was sufficient to retain CE approval. Deuterium is a hydrogen isotope with numerous effects on metabolic processes. Toxicity has been described at much higher exposures, usually higher than 20 % of the body water [57].

The introduction of deuterium may have initially gone unnoticed by many surgeons, as the original preparation was replaced by the new one under the same brand name and in a largely unchanged package in June 2010. An experimental study has shown that the new compound effectively sinks onto the retina more readily after injection and that 4,5 fold higher concentrations are reached near the retina compared to conventional BBG [58]. Initial clinical reports, although based on small samples of 8 and 3 patients, respectively, attest favorable outcomes and no evidence of dye-related complications [58, 59]. The injection of the dye resulted in rapid collection at the posterior pole and smooth dye-removal. The authors had the impression that staining results were superior to conventional BBG, although a direct comparison was not performed. Larger clinical trials or objective measurements were not available justifying a quantitative study based on the chromaticity analysis method:

In a nonrandomized, prospective clinical study, 71 consecutive chromovitrectomy interventions were analyzed [60]. In 21 patients conventional brilliant blue G was used, 50 patients underwent heavier than water BBG (**BBG-D20**) chromovitrectomy. In total, 193 chromaticity measurements were performed. Removal of the ILM was possible in all interventions without the application of additional vital dyes. Evidence for dye-related toxicity or complications was not seen. BBG-D20 was observed to sink to the retinal surface upon injection more readily than conventional BBG, which invariably dispersed throughout the vitreous cavity. Chromaticity measurements showed a slightly superior staining capacity for BBG-D20 than for conventional BBG, yet statistical significance was not reached. The revised preparation is, hence, appreciated for improved dye collection on the retinal surface, while **staining strength is not significantly enhanced.**

Another way to improve intraoperative ILM contrast might lie in the combination of chromovitrectomy with the use of filtered intraoperative light. The favorable effect of tinted glasses on visual function has been known since ancient times [61]. Yellow and orange filters have been described to increase contrast perception by a reduction of ocular media light scatter and decreased

chromatic aberration [61]. Spectral filters are available in many commercially available endoilluminator devices, as they allow the attenuation of retinal and retinal pigment epithelium light toxicity through the reduction of the endoillumination short-wavelength spectrum [62]. Only few surgeons rely on the use of spectral filters for chromovitrectomy and a medline search reveals only two publications on the protective effects of endoillumination filters in the last three decades [62, 63]. We used chromaticity analysis to examine the influence of spectral light filters in connection with intraoperative endoillumination on intraoperative ILM contrast. Fifty-nine consecutive brilliant blue G chromovitrectomy interventions were evaluated. Altogether, 324 measurements were performed [64]. We found that **available spectral filters do not significantly change the strength of color contrast perception** during chromovitrectomy. On the one hand, these results failed to prove an increase in color contrast with spectral filters. Yet, one may argue that the finding of an equivalent color contrast is encouraging: The significant reduction in retinal light toxicity offered by spectral filters without a reduction in color contrast represents a potential benefit. We suggest further research, however, before a general recommendation for routine use of spectral filters can be made, as other factors, such as luminance, influence intraoperative visibility of the target tissue, apart from color contrast.

The search for an approved vital dye to enhance intraoperative contrast during chromovitrectomy is ongoing and will require further research. We believe that chromaticity analysis will be instrumental in objectifying the staining capacity of existing and alternative dyes and surgical protocols.

Micromorphology

Apart from staining characteristics, both ILM thickness and biomechanical features influence peeling behavior [65]. However, little is known on these intrinsic material properties of the native ILM [66]. The epithelial/mesenchymal interface of most tissues is marked by the presence of a basement membrane. These thin, uniform insoluble sheets of highly specialized extracellular matrix proteins are found in all multicellular organisms [67], but vary in composition in a tissue-specific manner [68]. Basement membranes serve as substrates for epithelial cells, endothelial cells and myotubes [69]. They also contribute to the mechanical strength of their neighboring tissues [70]. The ILM is located at the vitreal border of the retina and is one of 6 basement membranes of the eye [69]. Our current knowledge of topographic variations within the ILM is mostly based on light microscopic [71] and transmission electron microscopy (TEM) studies [72, 73] of dehydrated and fixated specimens. These studies may, however, not accurately portray the native ILM, as the fixation process induces artifacts, resulting in a 30–50 % reduction in ILM thickness and 30 % increase in stiffness [69]. A reliable concept of the true dimensions of the thickness profile and biomechanical footprint of the native ILM was urgently anticipated.

Atomic force microscopy reveals ILM material properties under native conditions

We applied atomic force microscopy (AFM) as a unique tool to visualize, manipulate, and quantitatively assess structural and biomechanical characteristics of native biological samples at nanometer scale resolution [74, 75]. This technique is based on the deflections of a mechanical probe as it is rasterscanned across a sample surface while the applied force is controlled by a piezoelectric element (Figure 3a). The probe is composed of a flexible cantilever with an ultrasharp tip (<15nm in diameter) at its free end [75]. A laser beam that is reflected off the back of the cantilever is used to monitor cantilever bending. Several different modes can be used. The most widely used modes are contact mode, where the cantilever tip is in constant contact with the sample surface, and force indentation mode, where the sample is indented with a specific force and stiffness is

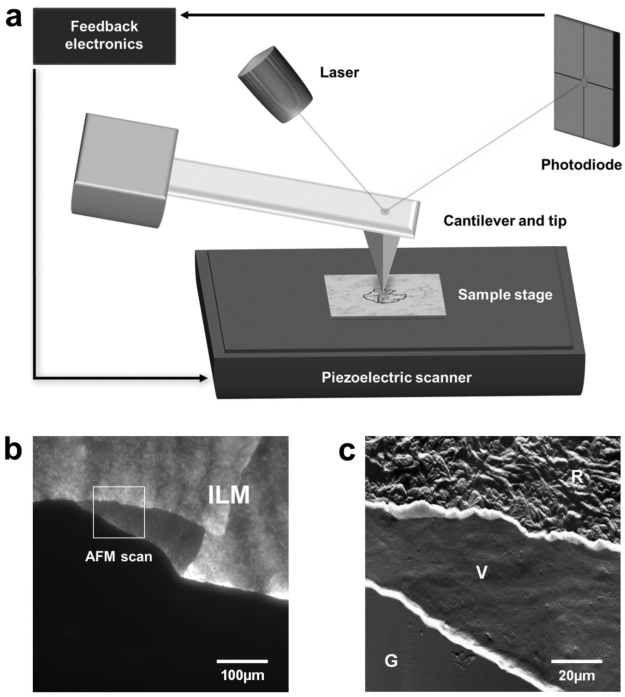

Fig. 3: (a) General overview of the principle of atomic force microscopy. The piezoelectric elements allow tiny but highly precise movements on electronic command. (b) A segment of human ILM freshly stained for laminin and visualized by fluorescence microscopy. The area analyzed by AFM is highlighted and illustrated in (c): A piece of retinal surface (R) is flipped over a segment exposing the vitreous-facing surface (V) and reveals the major topographical differences between both sides. The triangular space in the left lower quadrant shows the glass substrate (G), which is used as a reference.

calculated from the recorded force-distance curve. The signals of piezoelectric element displacement are used to generate a three-dimensional profile of the sample surface [75]. Different modes can be used. The most widely applied modes include contact mode where a laser beam reflected off the back of the cantilever is used to image the surface of the specimen based on cantilever bending, and force indentation mode where sample stiffness can be extrapolated from the piezoelectric force needed to impinge on the sample surface. The signals generated by either the cantilever deflection or the vertical piezoelectric scanner displacement can also be used to generate a three-dimensional profile of the sample surface [75]. Although AFM has been available for more than two decades [76], it has been underused in vision sciences [77] and we set out to employ this technique to plot the first comprehensive map of topographic and nanomechanical variations of the fully hydrated native human ILM (Fig. 3).

ILMs from the posterior pole of 10 postmortem human eyes were prepared as flat mounts and investigated by AFM under physiological conditions. Structural analysis was complemented by transmission electron microscopy [65]. The ILM could be imaged by AFM at unprecedented detail (Figure 3c). Three-dimensional AFM images showed a characteristic ILM orientation with dissimilar surfaces. **The vitreal side displayed a smooth surface aspect while the retinal side was marked by a conspicuous bold relief** with structures appearing like mountains and ridges separated by valleys and crypts. The ILM overlying the **fovea centralis showed a calderas-like architecture**. The central 350–400 μm appear thin and smooth. Partially round-arched, relatively steep walls rise around the center and appear to correspond to additional layers ramped up on a smooth basic substratum.

Thickness distribution

The thickness of the native **ILM averages to 3488nm**. It is, thus, roughly **four times thicker than previously described** in TEM analyses. Topographic variation of ILM thickness is found notably around the fovea: Based on morphology and roughness, this basic stratum is contiguous with the vitreal surface of the ILM. Average thickness is minimal in this immediate center of the fovea (138nm) and peaks to more than 4000 nm at a foveal distance of roughly 1000 μm. Outside the fovea, thickness distribution was relatively uniform and close to the overall average. Although mean ILM thicknesses were similar, standard deviation was higher on the retinal than on the vitreal side, indicating greater roughness. This thickness profile suggests that best staining results during chromovitrectomy, as well as favorable conditions for grasping the ILM with intraocular forceps, can be expected at a foveal offset of roughly 1000 μm, owing to increased thickness. The results are also in accordance with the current concept of macular hole formation [44], involving the peculiar fragility and thinness of the foveal retina in combination with the insertion of native vitreal collagen fibrils into the collagen network of the foveal ILM. The ILM has been described to be the main contributor to mechanical stability of the retina and vitreoretinal border [45]. The particular frangibility of the thin foveal ILM can, thus, be regarded as a fundamental factor for the formation of macular holes.

Stiffness distribution

A stiffening effect of the use of photochemically active vital dyes in combination with intraoperative endoillumination has been suspected due to possible cross-linking effects [78]. As enhanced ILM stiffness facilitates lifting up an initial ILM flap during chromovitrectomy a hardening effect could represent an additional intraoperative utility of vital dyes [65]. In recent *in vitro* studies, we could corroborate significant stiffening through the combination of both ICG and BBG with intraoperative endoillumination for the lens capsule [79] and the ILM (unpublished data). No stiffening was observed in the absence of light for either vital dye.

The meaning of such effects of vital dyes is difficult to interpret, however, without a generic understanding of the biomechanical properties of native basement membranes in general and of the ILM in particular. Available AFM stiffness data were limited to measurements on samples taken from outside the clinically most relevant posterior pole region [80]. A general description of stiffness distribution along the native vitreoretinal interface was not provided. These studies where instructive, however, in showing that AFM is superior to other techniques by eliminating the requirement for a dehydration step in the fixation procedure, which induces an increase in stiffness of at least 30 % [80].

Force indentation measurements were, thence, included in our donor eye study [65]. ILM stiffness analyses grossly matched thickness findings with respect to anatomical distribution, with significantly higher stiffness values in the central segment compared with the more peripheral quadrants. These findings underscore that ILM peeling may be most easily commenced within a foveal distance of roughly 1000 μm [65]. Indentation analysis of the **retinal side** yielded an overall mean **stiffness of 224 kPa**. Vitreal side stiffness measurements averaged 44 kPa. **Compared with the retinal side, the vitreal side proved 4.9 times softer**. Interrelated TEM and AFM analysis allowed a correlation of the heterogeneous stiffness distribution and extracellular matrix density. In line with the bipolar stiffness distribution, a significantly higher extracellular matrix density was found in TEM analysis in the retinal compared with the vitreous layers of the ILM [65].

The ILM as a model for basement membrane architecture in general

The observation of asymmetrical morphological appearances and biomechanical features of the ILM's vitreal and retinal side emerged to reach far beyond

the scope of chromovitrectomy. The current basement membrane model proposes a single-layered extracellular matrix sheet that is composed of mostly laminins, collagen IVs and proteoglycans [81]. Yet, our results seemed difficult to conciliate with a monolayer basement membrane concept. Our recent AFM and cell adhesion analysis of the lens capsule, Descemt's membrane and ILM of 20 human cadaver eyes revealed that the proteins and peptide domains within adult human basement membranes are organized in at least two distinct sublayers [82]. This layered distribution provides basement membranes with side-specific properties, including a characteristic tendency to roll in a side-specific pattern. Biomechanical testing confirmed that the epithelial side of basement membranes is stiffer than the stromal side across different types of basement membranes. Cell adhesion assays revealed that epithelial cells adhered to the epithelial side of basement membranes only. The data indicate that **basement membranes are asymmetric from the outset**. We propose that this functional asymmetry is essential to build and maintain multicellular organisms with alternating layers of epithelial and connective tissues.

We are convinced that atomic force microscopy opens new possibilities for investigating native biological tissues under physiological and pathological conditions and will contribute to an improved understanding of basement membrane material properties in the context of chromovitrectomy and beyond.

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