Supplementary Material:
Thickness and closure kinetics of the suprachoroidal space following microneedle injection of liquid formulations
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Ex vivo injection procedure
The conjunctiva, fascia, and extraocular muscles were carefully dissected off the rabbit eye. The eye was pressurized to a physiological intraocular pressure (IOP) of 10-12 mmHg by penetrating a 25 gauge needle through the optic nerve, which was connected to a water reservoir raised to ~14 cm above the eye. A 750 µm-long, 33-gauge hollow microneedle (kindly provided by Clearside Biomedical, Alpharetta, GA) attached to a 250 µL glass chromatography syringe (National Scientific, Rockwood, TN) was used to make injections. All injections were performed 3 mm posterior to the limbus at the 12 o’clock position (superior) to be as far as possible from anatomical barriers that impede circumferential flow. Depending on the experimental condition, each injection consisted of 25, 50, 75, 100, or 150 µL of 0.5% (w/v) red-fluorescent particles suspended in Hank’s Balanced Salt Solution (HBSS; Gibco, Life Technologies, Carlsbad, CA). After each injection, the needle was held in place for 1 min to minimize reflux.

Ultrasound imaging to determine SCS thickness
A high-frequency ultrasound (U/S) probe (UBM Plus, Accutome, Malvern, PA), with a minimum axial resolution of 15 µm, was used to generate 2D cross-sectional images of the SCS in rabbit eyes ex vivo after injecting volumes ranging from 25 to 150 µL. An U/S probe cover (Clearscan, Eye-Surgical-Instruments, Plymouth, MN) was attached to the UBM Plus to facilitate U/S image acquisition. Three minutes after injection, the U/S probe was used to acquire eight sagittal views around the eye (12, 1.5, 3, 4.5, 6, 7.5, 9, and 10.5 o’clock). Post-processing of the U/S B scans was performed to find the thickness from the outer sclera to the inner retina at 1, 5, and 9 mm posterior to the scleral spur (Figure S1). The mean, median, and standard deviation for each eye were calculated.

3D cryo-reconstruction to determine 3D distribution of particles and fluorescein
Microneedle injections of 25 – 150 µL containing red-fluorescent particles were performed in pigmented rabbit eyes, as described above. The eyes were frozen 3 min post-injection using ethanol chilled to -80°C. Frozen eyes were put into cryomolds loaded with Optical Cutting Temperature embedding medium (OCT; Tissue-Tek, Sakura Finetek, Tokyo, Japan) and India Ink (Higgins Ink, Leeds, MA). The cryomolds were then half-submerged in liquid nitrogen until the OCT was solid. The eye was removed from the plastic mold for sectioning. The pigmentation in the eye and the India Ink in the OCT prevented fluorescence from out of plane during imaging (see below).
Figure S1 – Calculation of SCS thickness in ultrasound B scans. A line segment perpendicular to the sclera and choroid, from the outer sclera to the inner retina, is found. The conjunctiva is excluded from the measurement. The tissue thickness is found and subtracted out, resulting in the SCS thickness.

Each cryomold was connected to the mount of a cryostat (CryoStar NX70 cryostat, Thermo Fisher Scientific, Waltham, MA or Leica CM 3050 cryostat, Wetzlar, Germany). A digital SLR camera (Canon 60D, Canon Inc., Melville, NY) with 100 mm prime lens was positioned on a tripod such that the camera was along the longitudinal axis of the cryo-block (to minimize apparent motion of the sample as it was cut and any keystoning effect). Camera parameters were held constant at shutter speed = 1/15 s and aperture = F/2.8. The camera was placed close enough such that the sample occupied >80% of the image sensor.

One red fluorescent image of the cryo-block was obtained every 300 µm by slicing the sample with the cryostat (Figure S2). Since the cryo-block was locked in place for each image and the camera was stationary, determining the orientation of one image relative to other images was simplified compared with other reconstruction methods. To visualize the location of the red-fluorescent particles, a red filter was placed on the lens, and the sample was illuminated with green LED light (HitLights, Baton Rouge, LA). Care was taken not to shift the camera when connecting the filter. After every 300 µm of tissue was removed by the cryostat, the procedure was repeated (including imaging measurements) until the entire eye was imaged.

Image stacks consisting of red fluorescence images were imported into a custom Matlab (Mathworks, Natick, MA) script. The image stack was preprocessed by spatially transforming each image so the four corners of the mold matched up, using the Matlab functions ‘cp2tform’ and ‘imtransform’. The spatial transformation accounted for angular deviations (e.g., roll around the longitudinal axis of the sample), linear deviations (e.g., shifting the camera), apparent changes in sample size due to changes in distance from sample to camera (i.e., as the sample was cut), and perspective deviations (i.e., viewing the sample from off the longitudinal axis).

The eye was modeled as a sphere and discretized into an array of 100 x 100 pixels. For each of these 10,000 pixels, a ray originating from the centroid of the eye through each point was identified (Figure S2B). The intensity along each ray was used to map the SCS thickness, as determined by the distribution of red particles, in the SCS (Figure S2C). The SCS thickness was determined by finding the distance between the first appearance and first disappearance of red fluorescence, as determined with an absolute intensity threshold and the first derivative of the intensity (to ensure an edge). This procedure was repeated for all
10,000 rays to yield a 2D map projection or a 3D surface plot (Figure S2D and S2E). Note that only SCS thickness per ray was calculated, not particle concentration.

A low-pass filter was used on the 100 x 100 array to remove aberrant signals. The resulting array was viewed as a surface plot in Matlab. Key parameters from each sample included distribution of thickness (5th, 25th, 50th, 75th, and 95th percentile, mean, and standard deviation) excluding thicknesses < 25 µm, thresholded area, and thresholded volume.

![Diagram of 3D cryo-reconstruction methods. Microneedle injection of particles suspended in HBSS is made in the suprachoroidal space (SCS). (A) Eye is cryo-sectioned. Every 300 µm, fluorescence images of the block are taken. (B) Image stack is uploaded to Matlab script. The centroid of the eye is identified manually by visual inspection, and 100 x 100 rays originating from the centroid are calculated. (C) The intensity along each ray is used to calculate the SCS thickness. (D) The SCS thicknesses for each of the 10,000 rays are calculated and displayed as either a (D) spherical representation or (E) 2D equatorial map representation. Similar to a Mercator projection of Earth, the regions near the anterior and posterior pole in (E) are greatly distorted. Injection site indicated with yellow arrow.](image)

**Mechanical testing of sclera-choroid attachments**

To assess the mechanical properties of attachments between the sclera and choroid, a peel test (modification of ASTM D1876 method) was performed on sclera/chorioretina strips with and without microneedle injection of HBSS into the SCS (N=8 per condition). Albino rabbit eyes *ex vivo* were used for this experiment.

Either the superior and inferior hemisphere was randomly assigned to receive a microneedle injection of 100 µL HBSS. After injection, two sagittal strips – one with no injection and one with SCS injection – from
the same eye, approximately 10 mm in width and 14 mm in length, were cut from the eye. The actual width was measured with a caliper. A scalpel blade was gently run along the edges of the strip to ensure the edges were clean. The ciliary body was cut posterior to the scleral spur so the choroid could be separated off the sclera. A 5 mm biopsy punch was used to make a hole through the cornea. The strip was mounted on a force displacement station (ESM301 motorized stand, Mark-10 Corp., Copiaque, NY) with a 50 gF force sensor (Series 5 Force Gauge, Mark-10 Corp.). A hook attached to the force sensor was threaded through the hole in the cornea, and the ciliary body was clamped to a stationary platform. The force displacement station was programmed to pull (i.e., peel) the sclera from the choroid at a rate of 60 mm/min. The force readings were collected in real time by the MESUR Lite gauge software. The average force per width of sclera/choroid tissue strip was calculated.

SCS collapse rate with different liquid formulations
The rabbit was anesthetized with isoflurane. Topical proparacaine was given to further anesthetize the eye. To study the effect of viscosity on SCS collapse time, one of the following formulations was injected: [i] 50 µL of HBSS; [ii] 50 µL of Discovisc (Alcon Laboratories, Fort Worth, TX); [iii] 50 µL of 1% carboxymethyl cellulose (CMC; 700 kDa high viscosity) dissolved in HBSS; [iv] 50 µL of 3% CMC in HBSS; [v] 50 µL of 5% CMC in HBSS; and [vi] 25 µL of 5% CMC in HBSS. High-frequency ultrasound B-scan was used to determine the rate of SCS collapse. Eight sagittal views over the pars plana were acquired: (a) supranasal, over the injection site; (b) superior; (c) nasal; (d) supratemporal; (e) temporal; (f) infratemporal; (g) inferior; and (h) infranasal. Image acquisition for these formulations occurred for up to 28 d \textit{in vivo}.

Off-line post processing was performed on the U/S views to measure the SCS thickness. The U/S probe used has a minimum axial resolution of 15 μm. For each U/S view, a line segment 5 mm posterior to the scleral spur and perpendicular to the sclera was created (Figure S2). The line started at the outer surface of the sclera and ended at the inner surface of the retina. The sclera and chorioretina were included in the measurement to ensure the line was perpendicular. SCS thickness was then calculated by subtracting the tissue thickness from the measured line length. Curve fitting was done to determine the rate of SCS collapse.

Determination of SCS clearance kinetics by fundus imaging
To study the effect of viscosity on fluorescein movement in the SCS, a 50 µL microneedle injection of the following formulations were tested: [i] 0.025% (w/v) fluorescein sodium (25% AK-Fluor, Akorn, Lake Forest, IL diluted with HBSS); and [ii] 0.025% fluorescein sodium (25% AK-Fluor, diluted with HBSS) and 5% CMC in HBSS.

The approximate clearance rate of injected fluorescent material from the SCS was found by taking fluorescein fundus images over time in the rabbit eye \textit{in vivo}. Topical eye drops of tropicamide and phenylephrine (Akorn, Lake Forest, IL) were administered prior to each imaging session to dilate the eye. A RetCam II (Clarity Medical Systems, Pleasanton, CA) with the 130° lens attachment and the built-in fluorescein angiography module was used to acquire the images. Multiple images were taken with the blue light output from the RetCam II set at 0.0009, 1.6, and 2.4 W/m². In an attempt to capture the entire interior surface of the ocular globe, nine images were captured: central, supranasal, superior, supratemporal, temporal, infratemporal, inferior, infranasal, and nasal. This allowed imaging into the far
periphery. Imaging was done immediately after injection, at 1 h, every 3 h for 12 h, and every two days post-injection.

The total clearance time, which we defined as the first time point in which fluorescence was not detectable by visual observation, was determined for all eyes injected.

Results

Figure S3 – Fluorescent image slice of pigmented eye (A) and albino eye (B). In the albino eye, there is no pigment to absorb the fluorescence from out of plane (arrow). Since the fluorescent glow was difficult to segment appropriately, pigmented eyes were used.

Figure S4 – Area of SCS coverage with increasing injection volume. Injection volumes ranging from 25 – 150 µL were performed in ex vivo rabbit eyes and the area of circumferential coverage was determined by 3D cryo-reconstruction. Values represent mean±SEM. Linear regression performed on data indicated that area was directly related to injection volume ($R^2 = 0.92$).
Figure S5 – Time course of SCS thickness following microneedle injection of (A) 50 µL Discovisc and (B) 50 µL 5% carboxymethyl cellulose in HBSS. Injection was performed supranasal and U/S B scans were acquired at eight positions around the ocular globe. Each time point represents the mean of 3-5 replicates. X-axis divisions at Inf. = inferior; Nas. = nasal; Sup. = superior; Temp. = temporal. SCS thickness (y-axis) marked every 1 mm.
References