High-Frequency Ultrasound Imaging of the Duck Embryo Retina as a Near Real-time Preclinical Platform to Study Advanced Proliferative Diabetic Retinopathy

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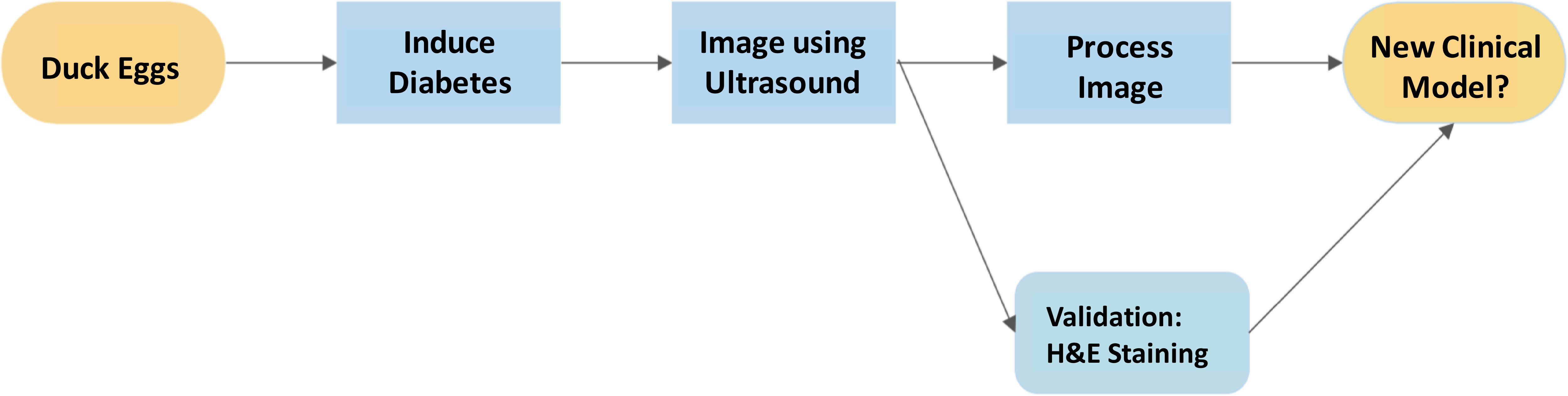
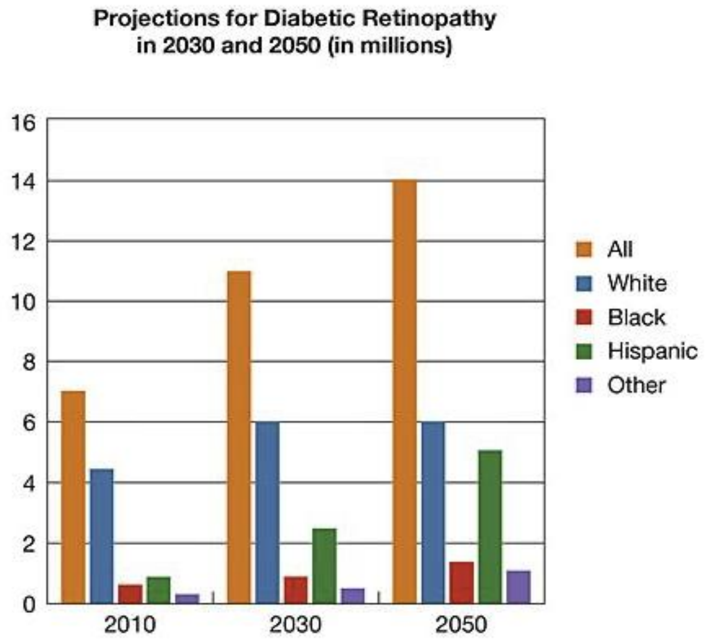
Study Overview

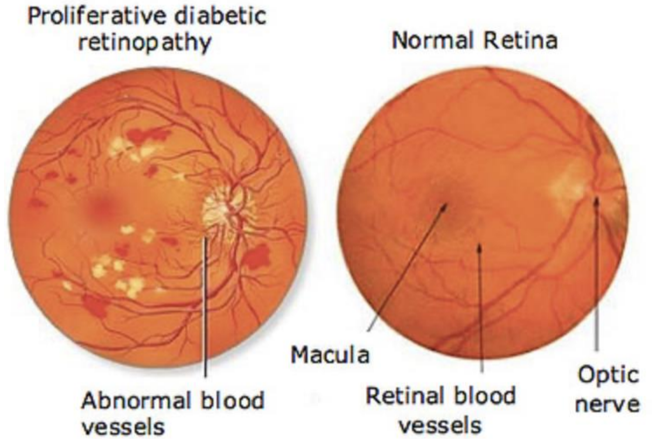


- Serves as a proof-of-concept study for future experiments
- Ultimately, establish a platform to evaluate DR therapies and drugs

Diabetic Retinopathy (DR) is a leading cause of blindness in the working class

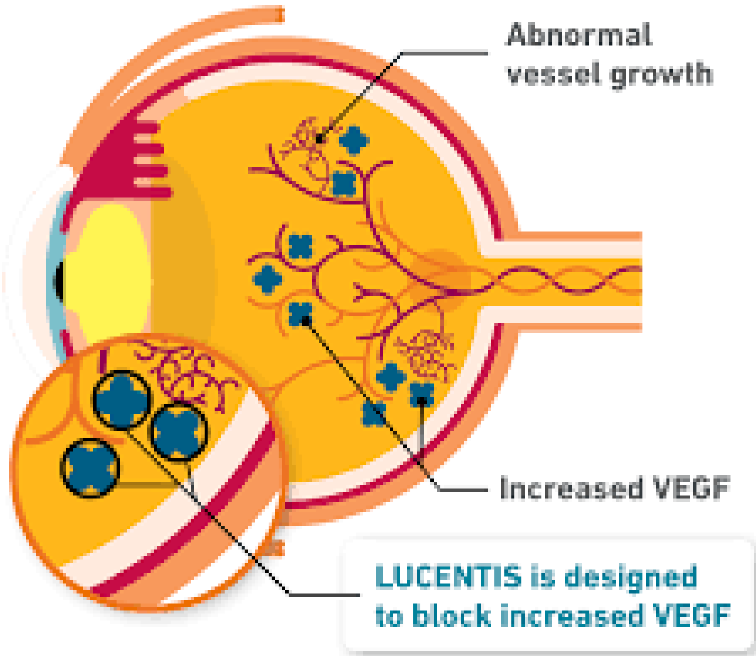
- Excess amounts of sugar in blood blocks existing blood vessels
- Proliferative DR causes neovascularization
  - Angiogenesis or vasculogenesis
- Mild vision problems progressing to blindness
- Thinning of retinal layer, separation of epidermal membrane, cataracts





Various limitations exist for current models and technologies

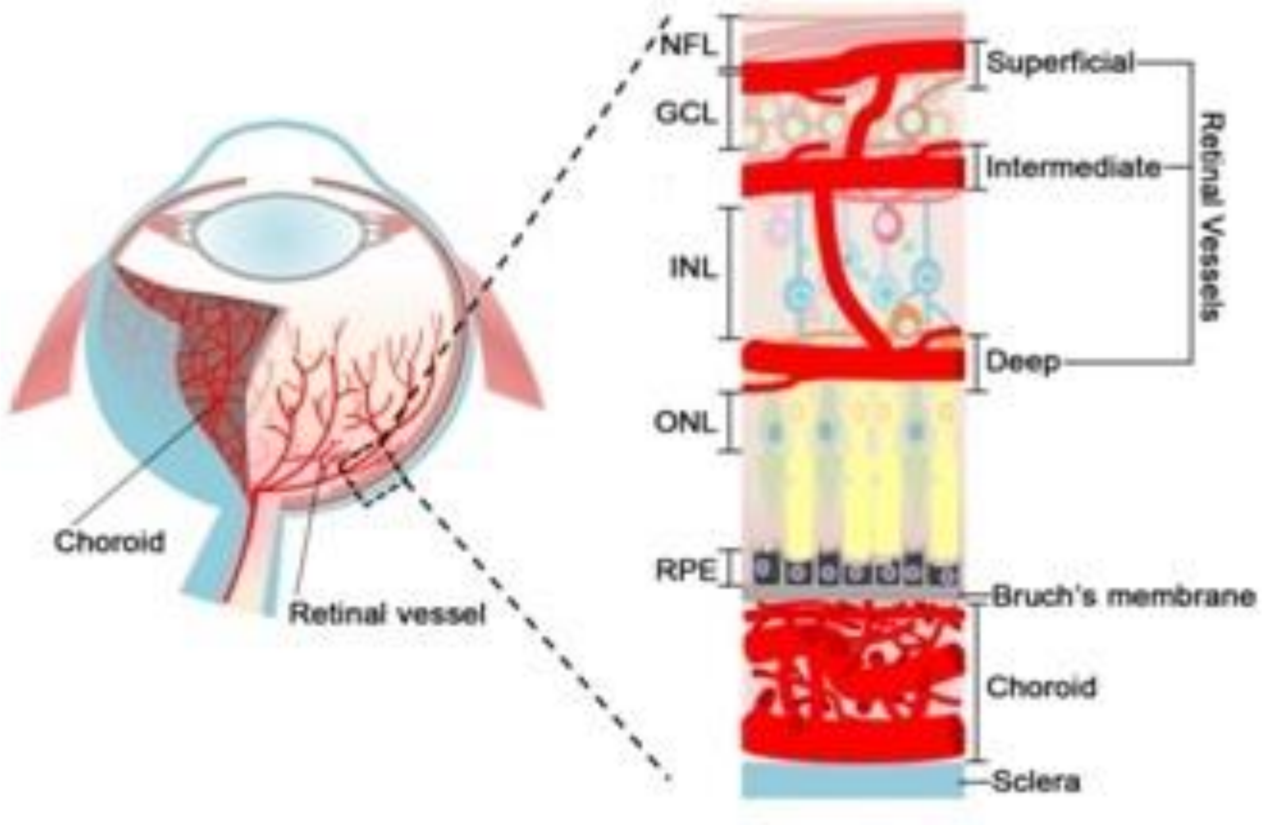
- Anti-angiogenic and anti-VEGF drugs are tested in animal models
  - Ranibizumab (Lucentis), aflibercept (Eylea), bevacizumab (Avastin)
- Histology to evaluate perfusion is resource intensive and not real time
- Ophthalmic imaging requires specific skills and qualifications
- Duck embryo and ultrasound as a potentially better platform for drug evaluation



Example of anti-VEGF drug

Retinal and choroid blood vessels have the same response

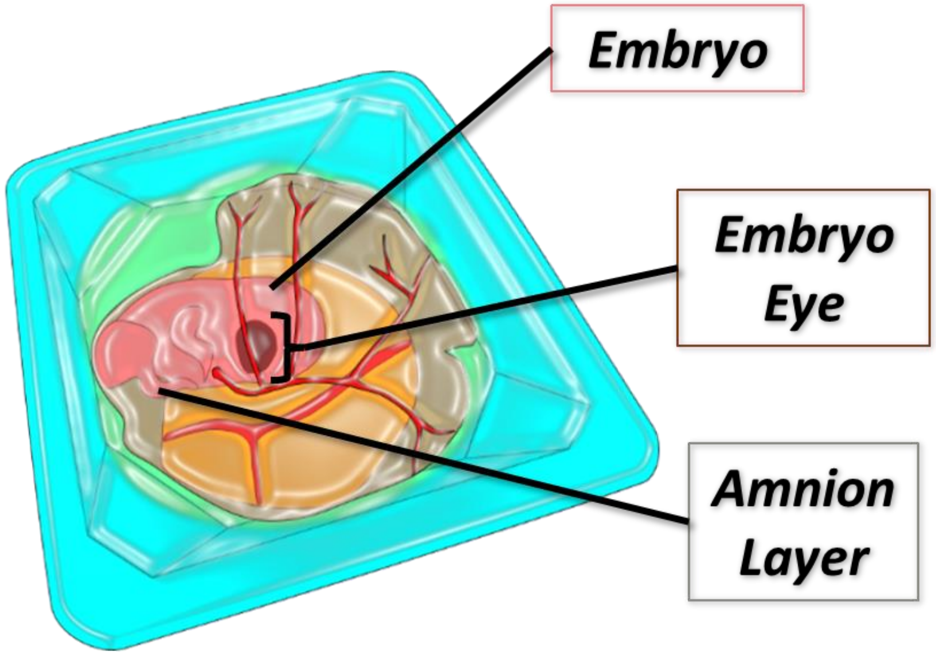
- 3 retinal blood vessels + choroid
- Impacts both choroid and retina in the same manner
  - Neovascularization
- Intravitreal injection Anti-VEGF
  - Prevention of growth of new blood vessels
  - Reduces swelling
  - Improves central vision



Anatomy of the eye

Chick embryo eyes can be used as physiologically accurate models of human eyes

- Chicks are diurnal with complex colour vision
- Has the three relevant photoreceptors
- Has a macula
  - Homogenous response to damage/inflammation
- Cone-dominant avian species
- Abundance of retinal tissue

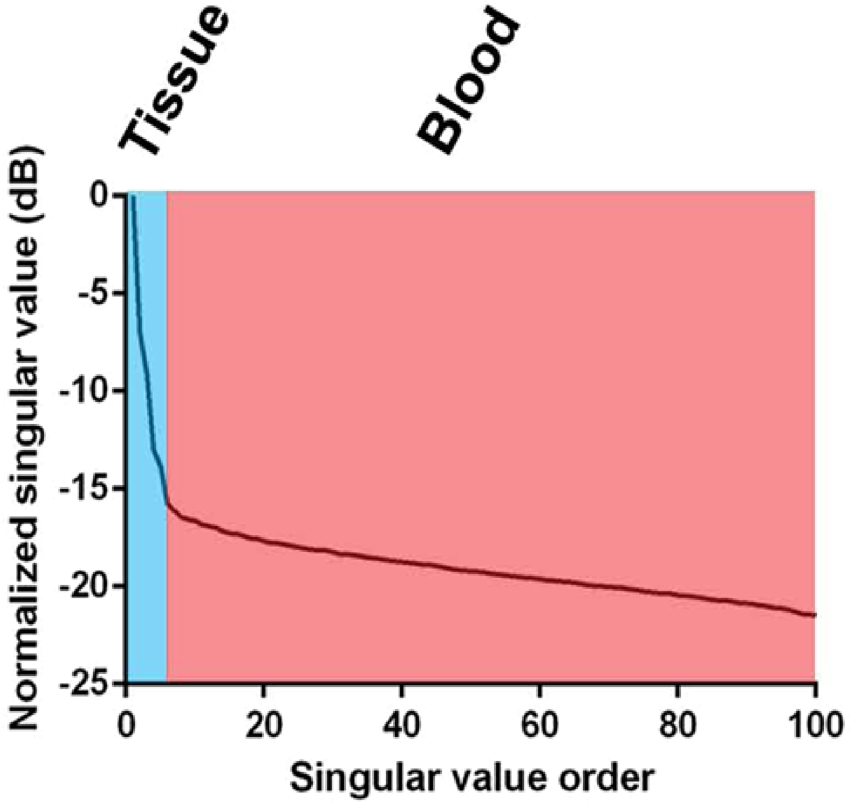


Ex ovo chick embryo

Using Ultrasound Microvessel Imaging (UMI) to delineate retinal & choroidal blood vessels

- Huang et al. developed UMI and applied it to highlight vasculature of tumours
- Uses SVD-based algorithm
- Quantify using MATLAB
- Translate to use on retina and choroid
- Limitation: Physiological Motion

$$Vascularity\ Index\ (VI) = \frac{Area_{vessel}}{Area_{ROI}}$$



SVD Lambda Curve

SVD-based UMI can detect an *increase* of *vascularity* in diabetic duck embryo retinas and choroids vs. healthy

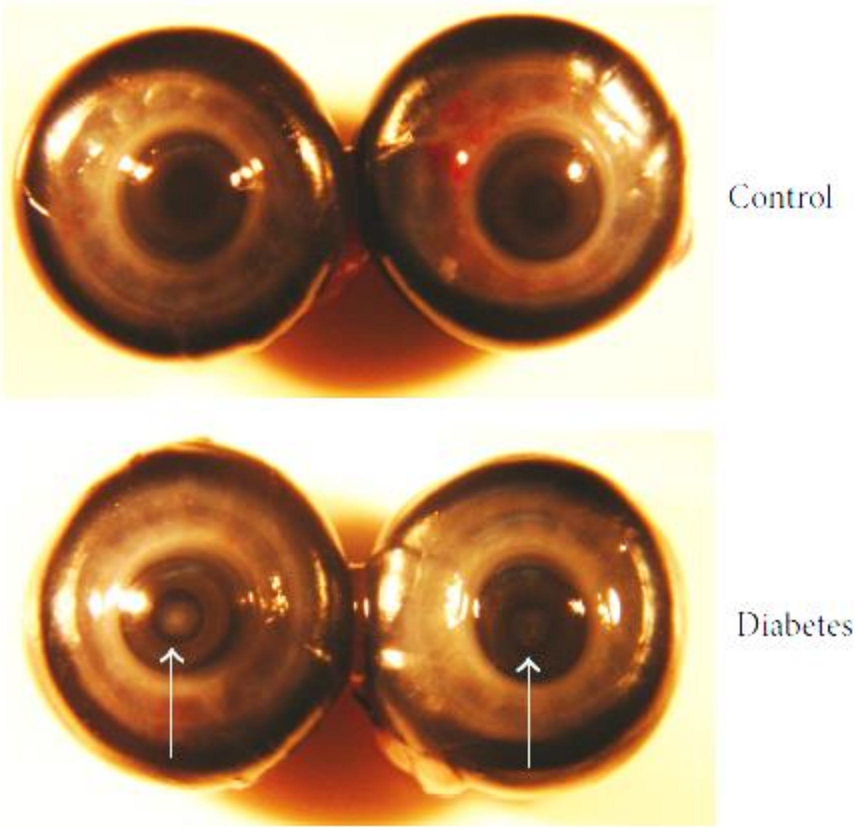
Objectives:

- To induce diabetic retinopathy in duck embryos
- To use HF-US coupled with UMI to highlight vasculature in the retinal and choroidal region of duck eyes
- To partially validate diabetic retinopathy using histology

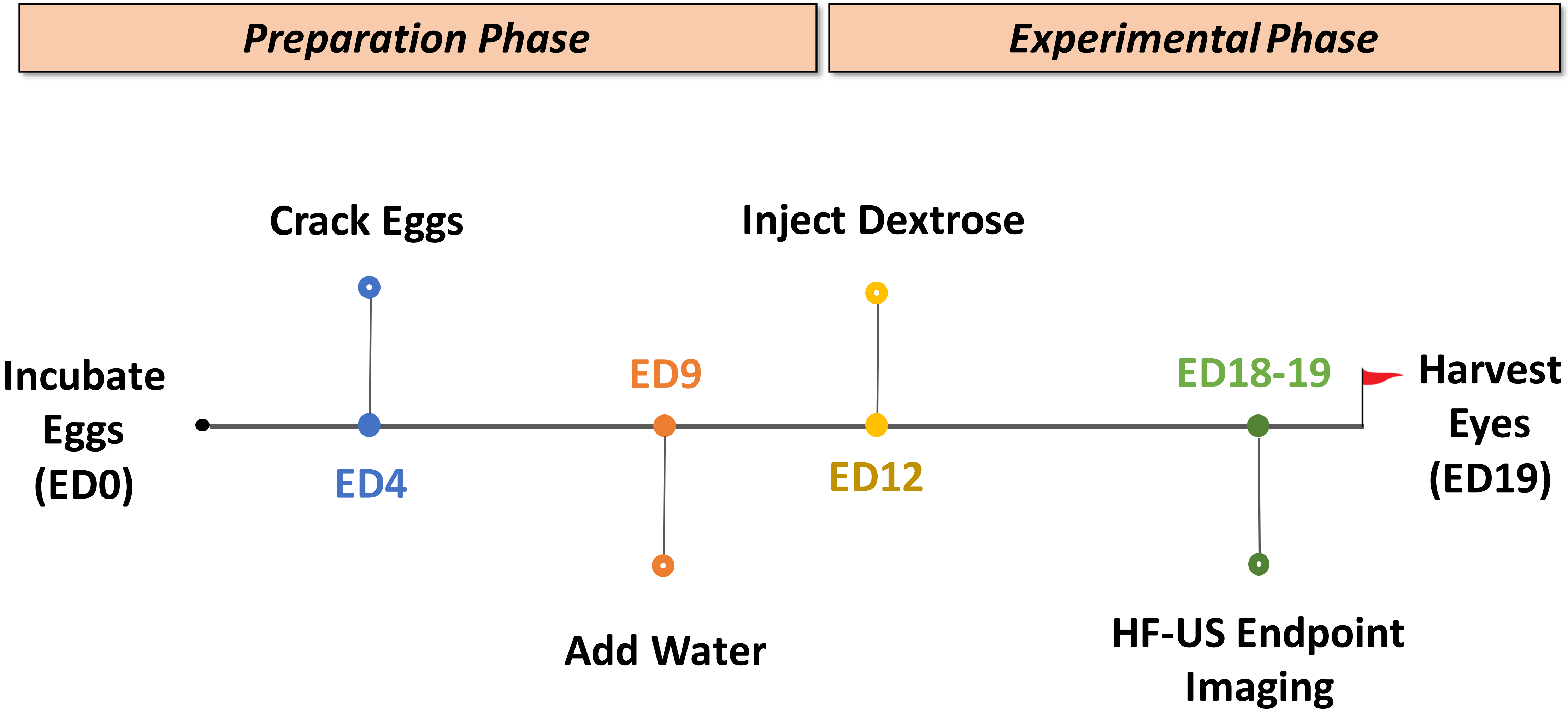
Type-I diabetes and DR can be successfully induced in the chick embryo

- Underdeveloped pancreas in the chick embryos
- Induced diabetes by injecting streptozotocin (STZ) or glucose
- Blood-glucose levels, molecular markers, ERG waves, cataracts, and thinning of retinal layer

- Followed a similar protocol with duck embryos, which have slower but same development cycles



Cataracts



Experimental Timeline

Duck eggs were weighed, incubated, and cracked on ED4

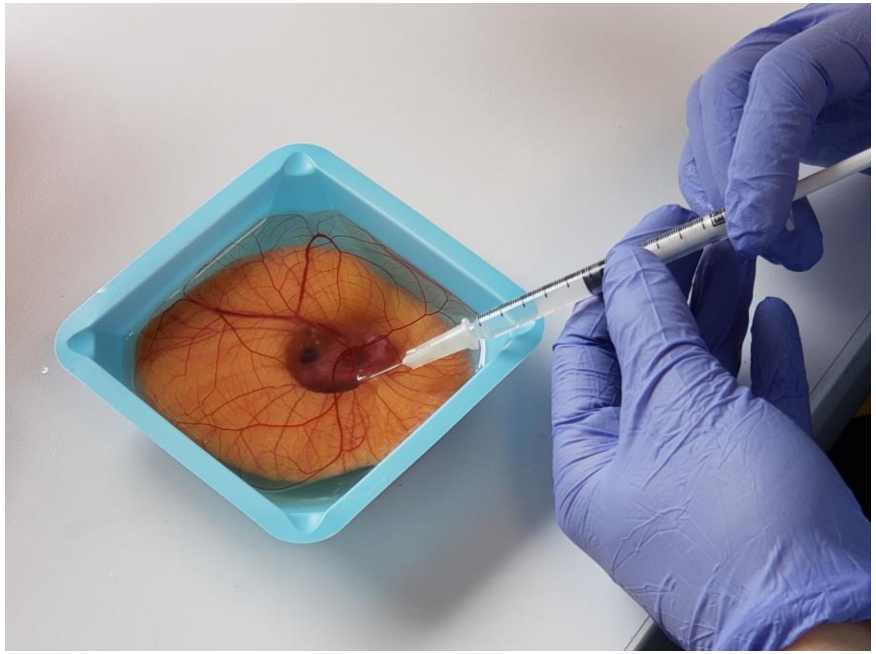
Duck eggs (Top L), Cracking (Bottom L), Incubating (R)

- All the eggs selected were 25g ± 1g
- 15 eggs were incubated for this experiment
- Water was added to container on ED9 to maintain humidity

Incubate **ED4** **ED9** **ED12** **ED18-19** **Harvest**

250 mg/mL D-glucose solution was injected into the amnion layer of the embryos on ED12

- 10 embryos survived and were randomized into Control & Treated
- 5 were injected with 2500 mg of D-glucose per kg of egg into the amnion layer
- Approximately 285 µL of 250 mg/mL solution per embryo



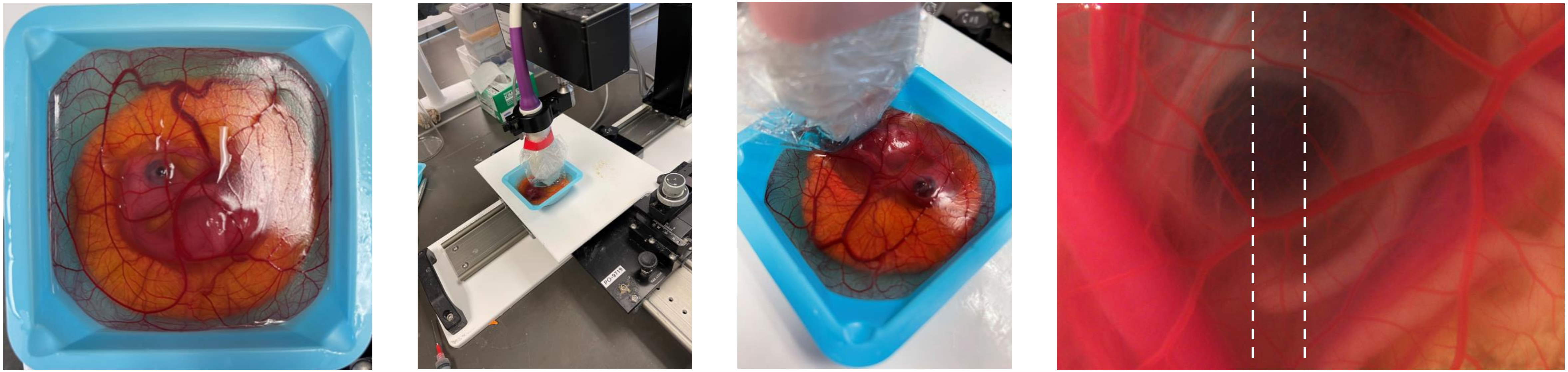
Injection of dextrose into the amnion

Incubate — ED4 — ED9 — **ED12** — ED18-19 — Harvest

If you think you might feel queasy, please look away for the next 2 slides!

Two cross-sectional B-mode images of one eye were acquired per embryo

- 5/5 Controls and 3/5 Treated survived for imaging
- VisualSonics Vevo® 2100 system with MS700 transducer at 50 MHz

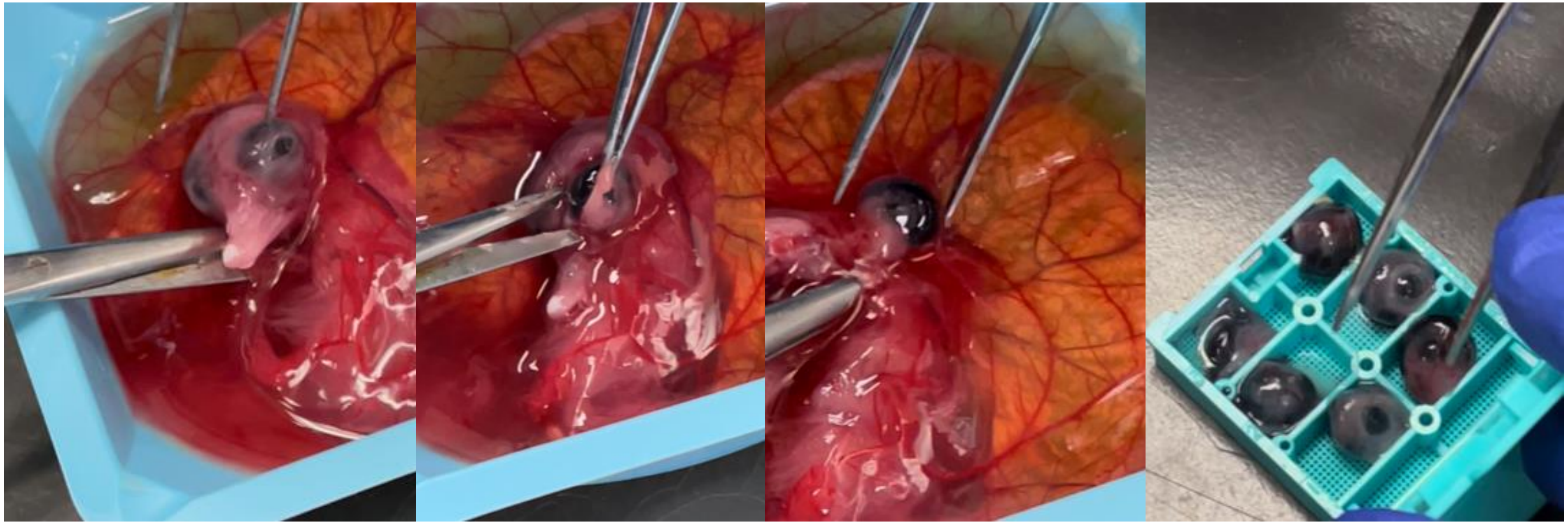


Ex ovo chick embryo (1st), HF-US imaging setup (2nd & 3rd), Cross-sectional profile of US images (4th)

Huang *et al.* Scientific Reports. *Nature* (2019).

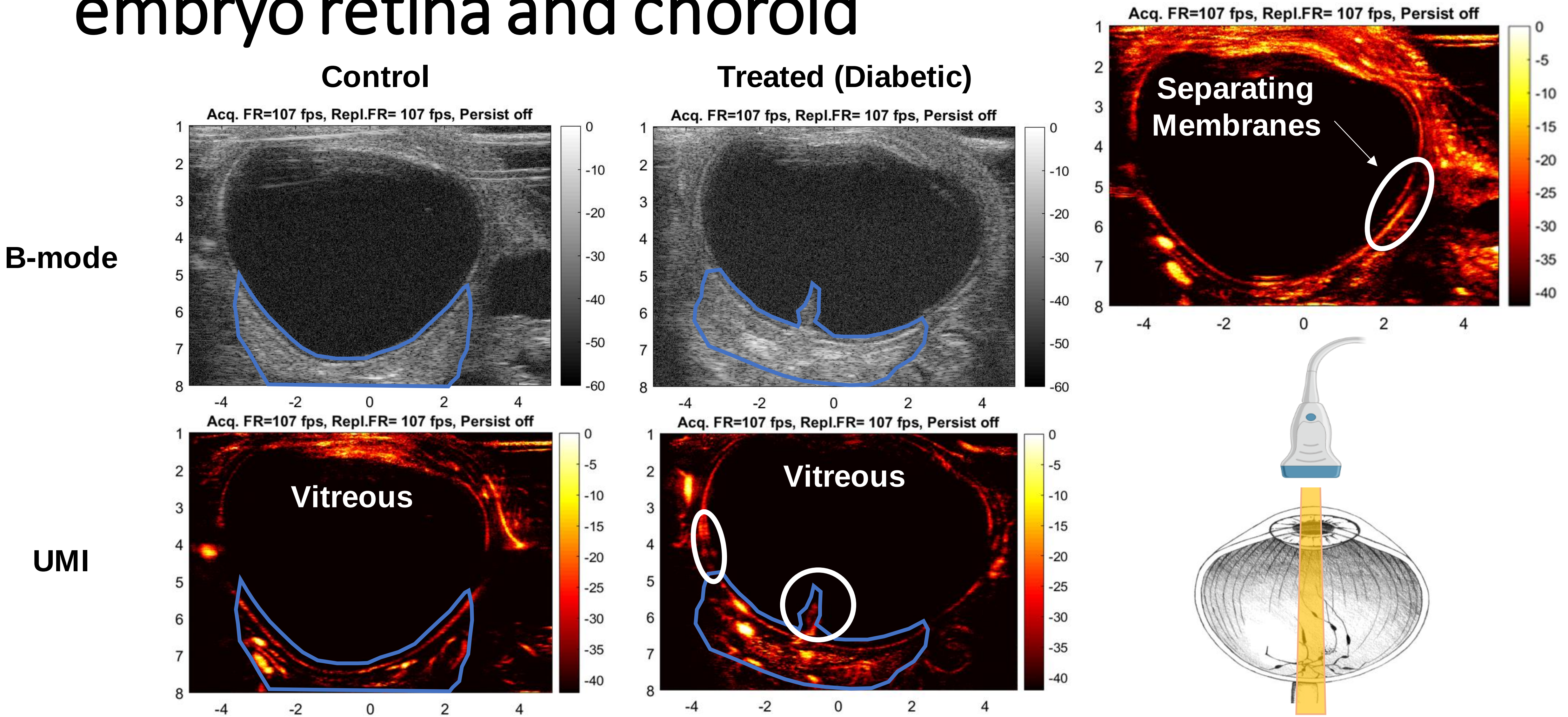
Both eyes were harvested and stained with Hematoxylin & Eosin (H&E)

- 3 pairs each of Control and Treated eyes
- Validation of diabetic retinopathy by seeing thinning of retina

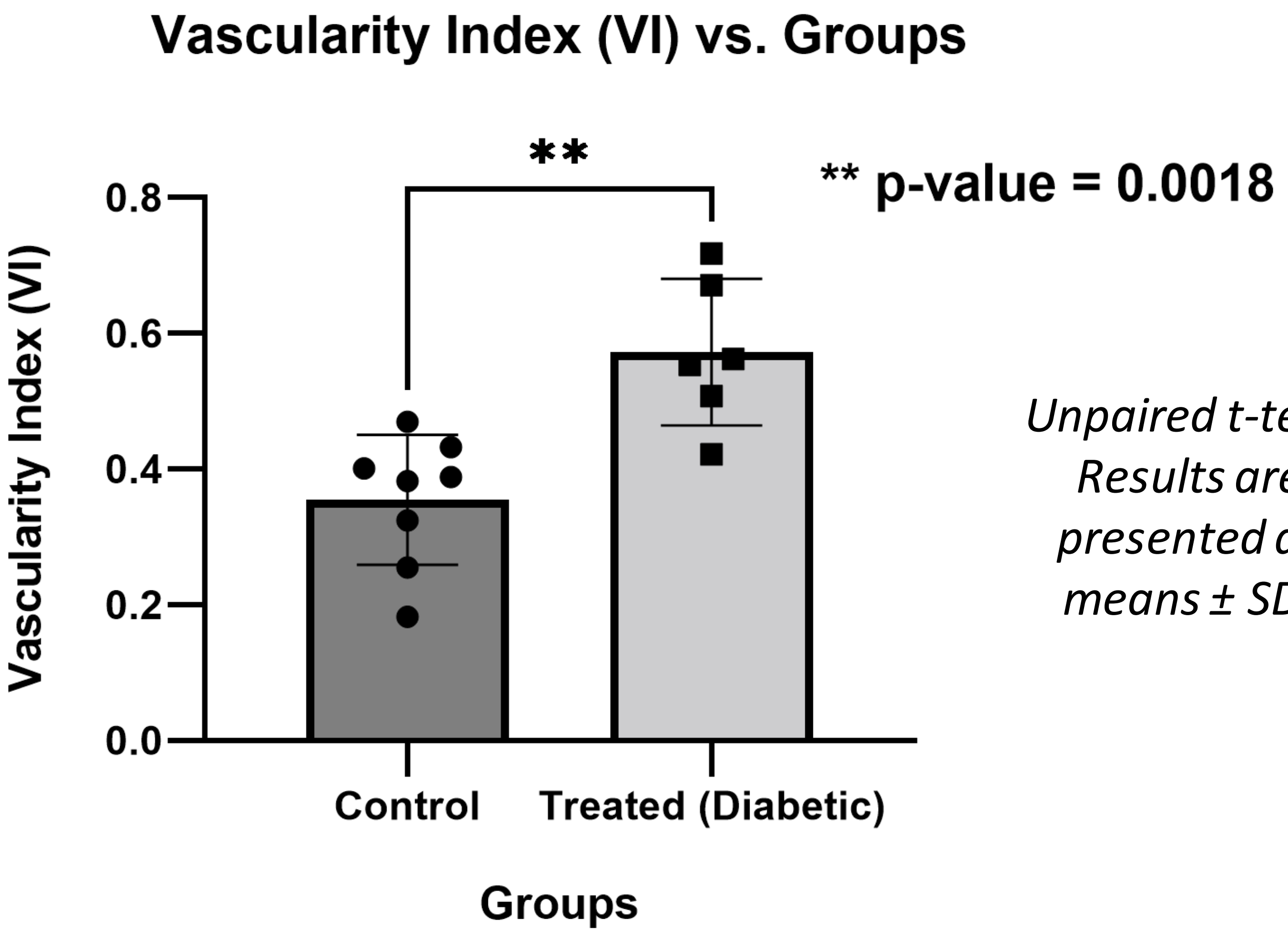


Incubate ED4 ED9 ED12 ED18-19 Harvest

UMI can detect microvasculature in the duck embryo retina and choroid

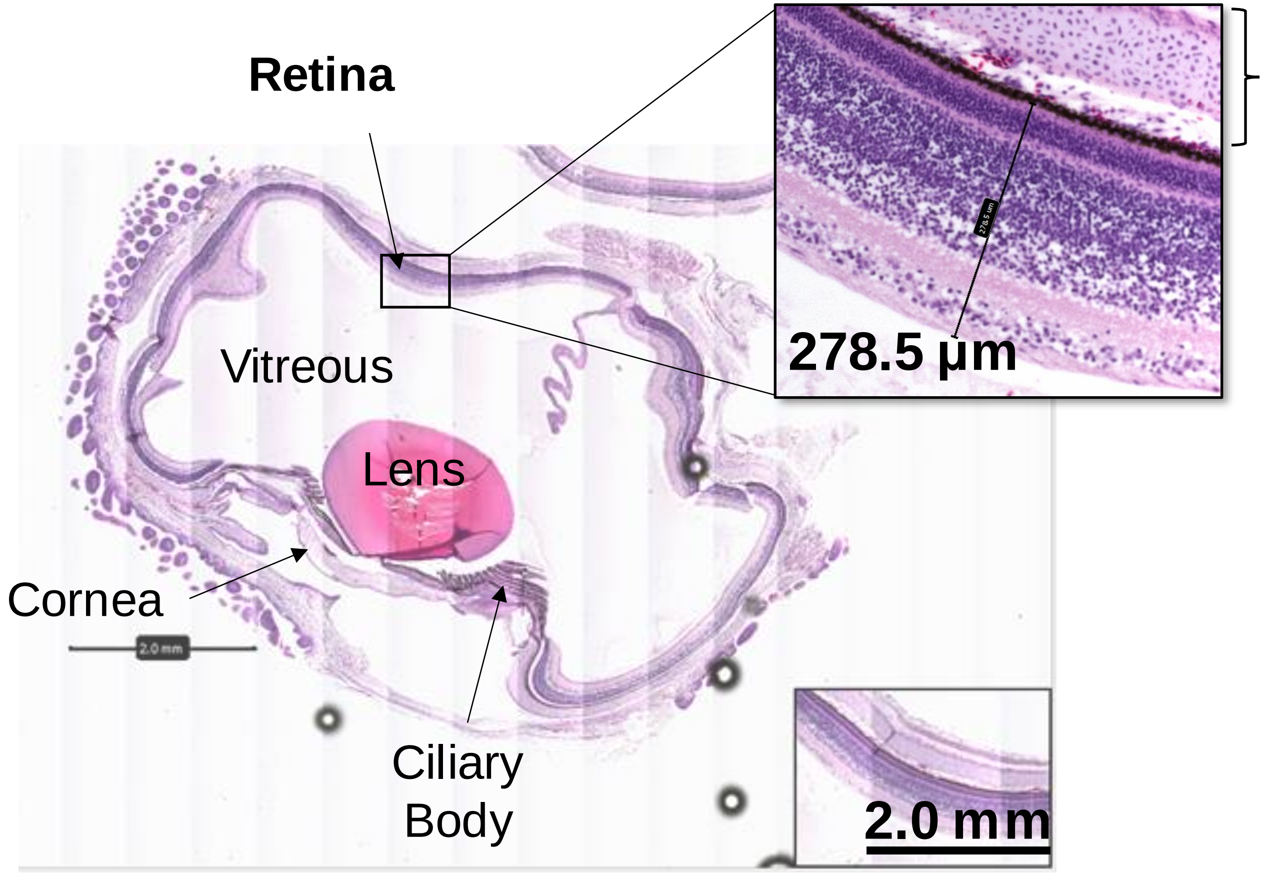


Diabetic duck retinas and choroids exhibits a greater vascularity index

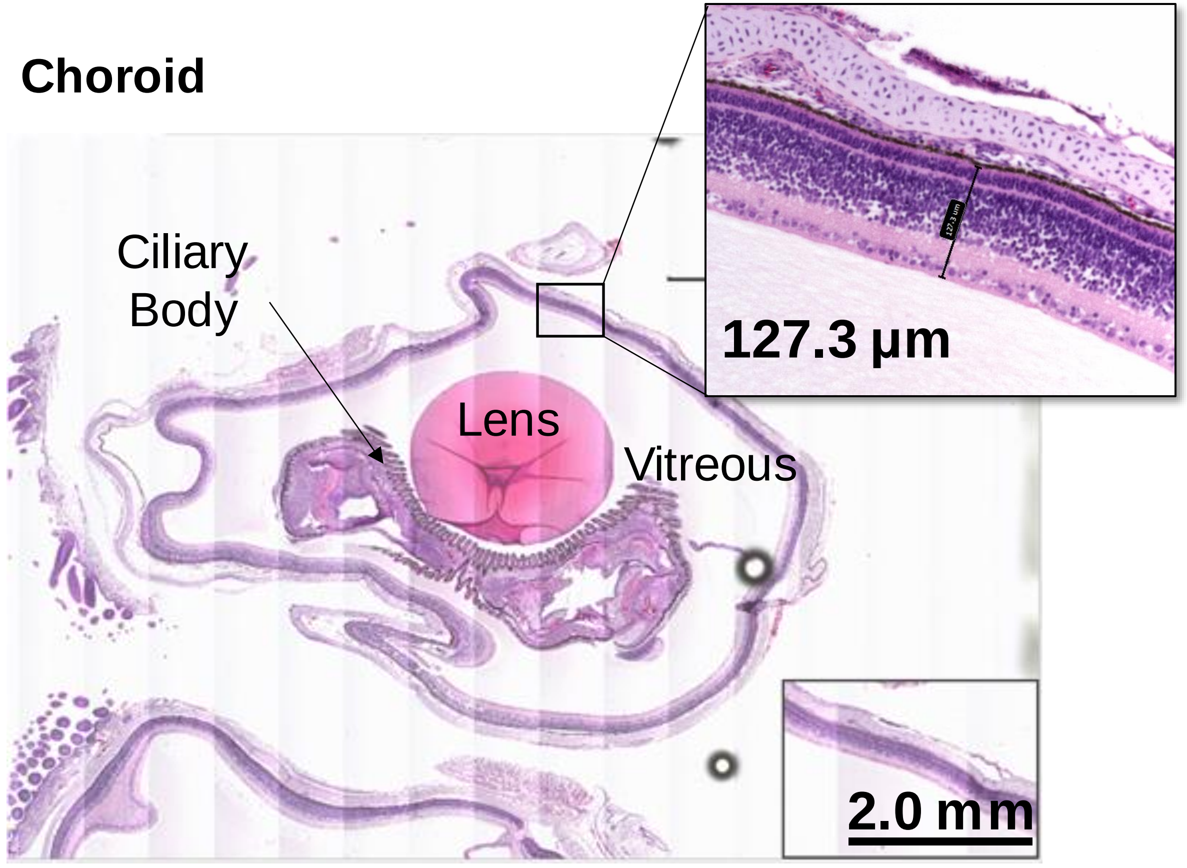


Unpaired t-test. Results are presented as means ± SD.

H&E staining showed a thinning of the retinal layer in diabetic embryos

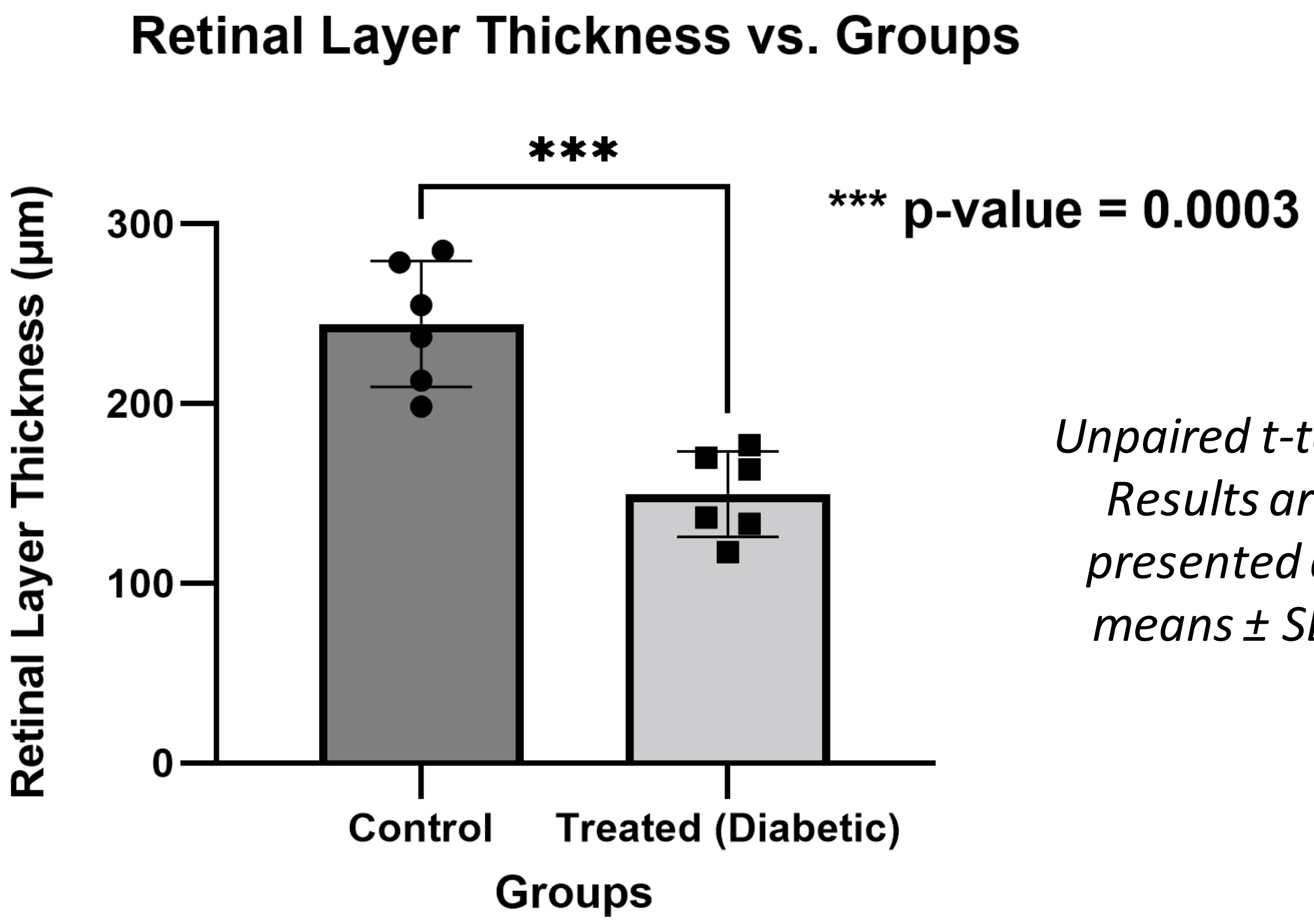


Control



Treated (Diabetic)

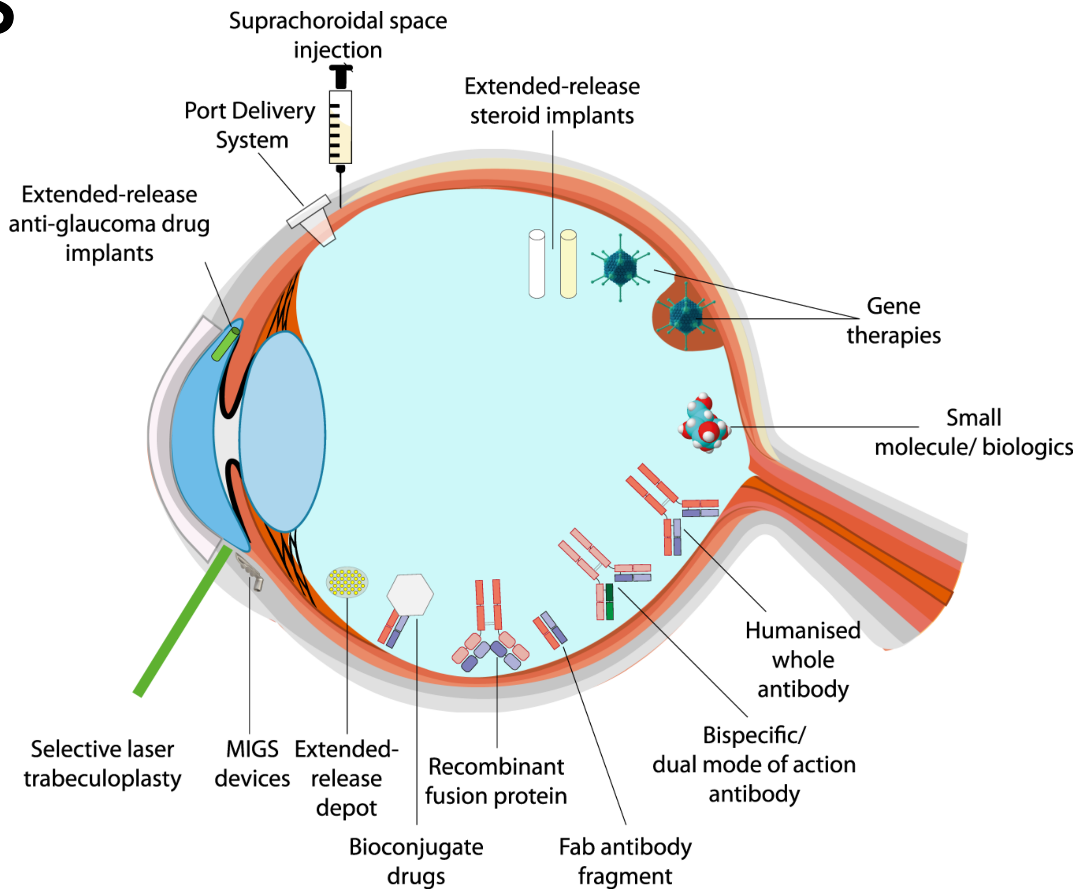
H&E staining showed a thinning of the retinal layer in diabetic embryos



Unpaired t-test. Results are presented as means ± SD.

UMI of the diabetic duck retina can be used to evaluate therapeutic agents

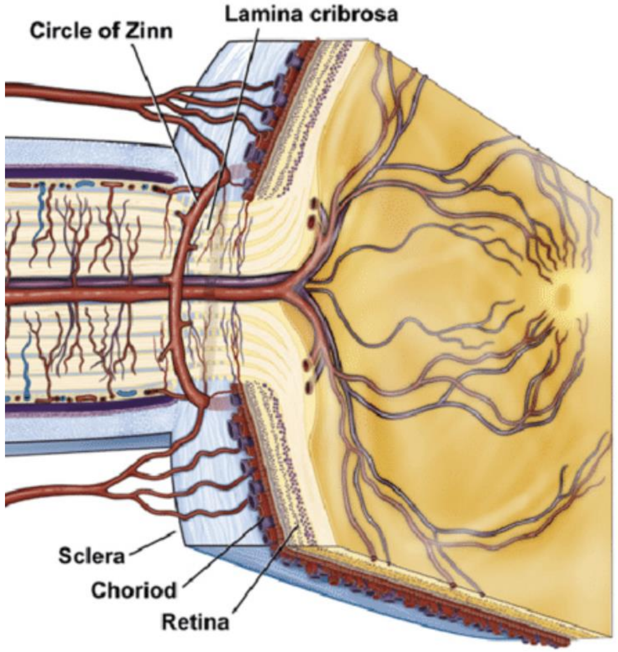
- UMI could detect microvasculature in the duck embryonic retina and choroid
- H&E results confirmed the presence of DR via retinal thinning
- We may be able to use UMI to evaluate new anti-angiogenic drugs / therapies



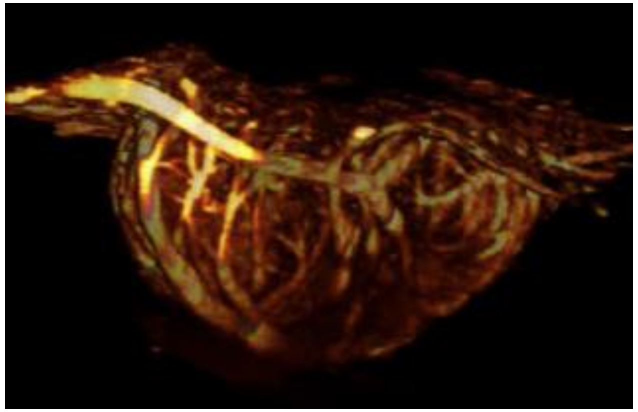
Current delivery systems for therapeutic agents in the eye

Project Limitations

- Small sample size for diabetic (n=3)
- Limited validation methods available to us for successful induction of diabetes and DR
- Retina spherical vascular profile - 3D image which captures 90+ slices with step sizes
  - Unable to accurately measure retinal vascular density of entire eye
  - However, drugs have same impact on choroid and retina so doesn't invalidate data



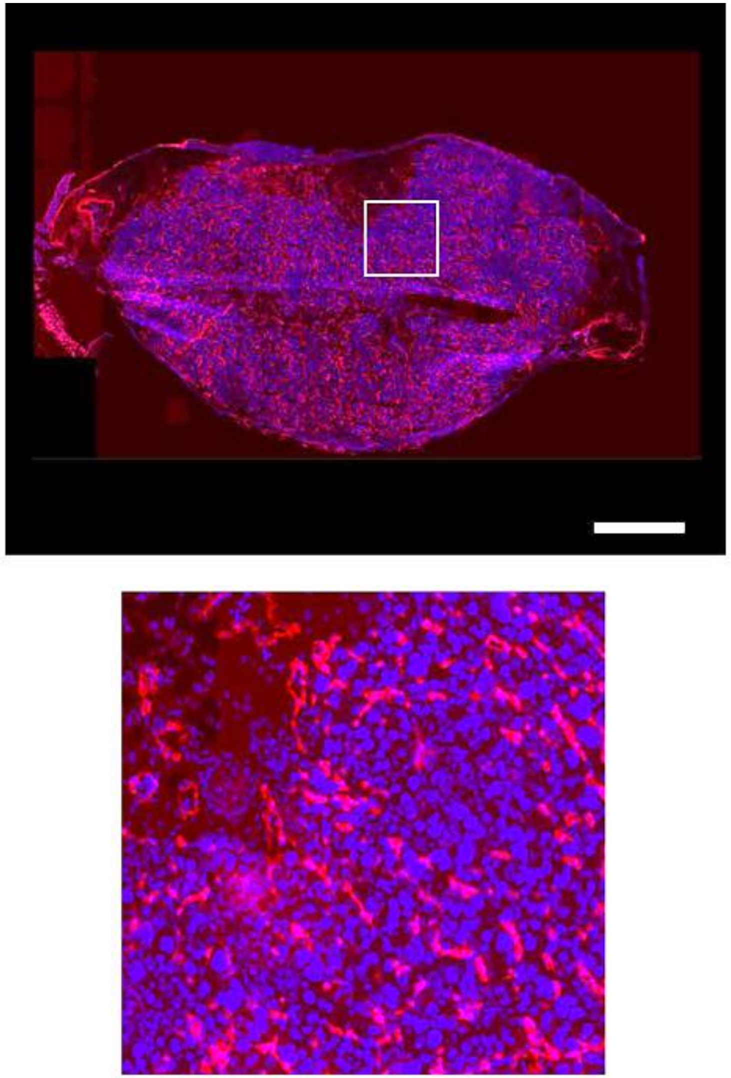
Retinal vessels are on the top of the retinal layer



3D tumour rendering from Huang et al.

Future Steps

- Highlight functional vasculature by injection of lectin rhodamine of GFP eggs
- Tuning image acquisition/processing parameters
- More time for diabetic disease progression
- STZ would increase survival rate and cataract formation
- Consult people with experience in duck eye and ophthalmic imaging modalities



Vasculature in RENCA tumor stained by lectin rhodamine

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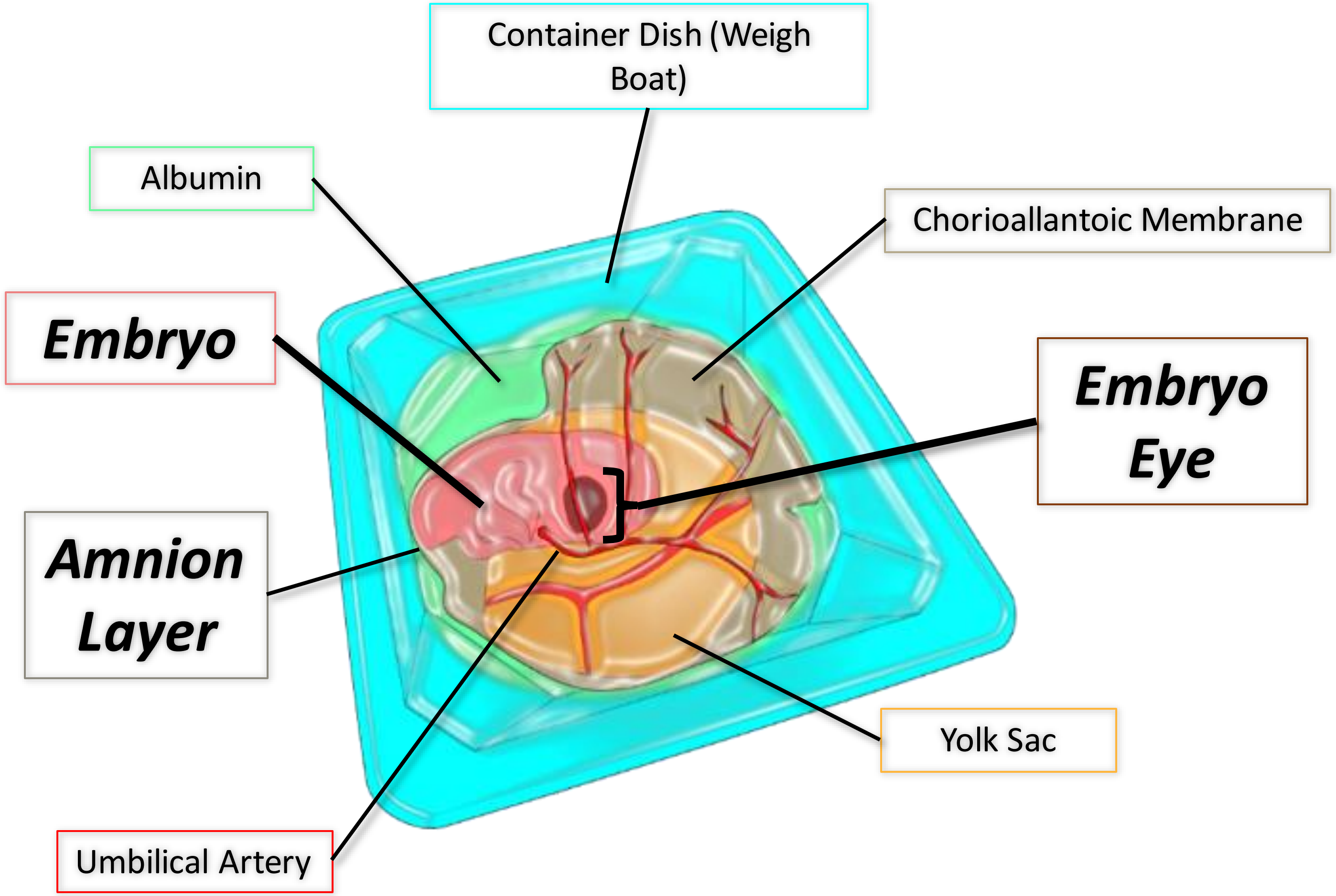
Supplementary Slides

Chick embryo assays are used to study eye-related diseases

- Retinal neovascularization (Glaser et al., 1980)
- wAMD treatment testing (Samkoe et al., 2007)
- Model diabetic retinopathy (Shi et al., 2014)



Ex ovo chick embryo

Animal Model Limitations

- Rodent retinas do not have a macula, cannot serve as an adequate model of diabetic macular edema
- Chronic inflammation contribute to DR but mouse models poorly mimic human inflammatory diseases
- The rod-dominant nocturnal retinas is not the best model to address the dysfunction and apoptosis of cones in human DR.

Model	**Zebrafish**	**Dog**	**Rat**
Limitations	- Resource Intensive - Not closely related to humans	- Resource intensive - Long time to develop diabetes	- Not representative of the human eye - Lack macula, rod-dominant

Robinson *et al.* Disease Models & Mechanisms (2012); Lopes de Faria *et al.* Hypertension (2011);

Some details about HF-US B-mode acquisition and image processing parameters

- 35 µm nominal axial, 70 µm nominal lateral
- TxFrequency: 50,000,000 Hertz (50 MHz)
- TxCycles: 1
- TxPower: 100
- RxGain: 21.9758
- LineDensity: High
- LinePitch: 1.9000e-05
- NumFocalZones: 1
- NumSamples: 584
- NumLines: 512
- FocalZonesPos: 0.0075
- NumFrames: 630
- DepthMin: 1.0000e-03
- DepthMax: 0.0080
- Width: 0.0097
- SamplingFrequency: 64,000,000
- SoundSpeed: 1540
- FrameRate: 107.3829
- AttenuationParam: 0.04
- InterpType: Cubic
- PyramidLevels: 3

Statistical analysis results for VI

Table Analyzed	Data 1
Column B vs. Column A	Treated (Diabetic) vs. Control
Unpaired t test	
P value	0.0018
P value summary	**
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.997, df=12

How big is the difference?	
Mean of column A	0.3545
Mean of column B	0.5723
Difference between means (B - A) ± SEM	0.2178 ± 0.05450
95% confidence interval	0.09908 to 0.3366
R squared (eta squared)	0.5710
F test to compare variances	
F, DFn, Dfd	1.275, 5, 7
P value	0.7416
P value summary	ns
Significantly different (P < 0.05)?	No
Data analyzed	
Sample size, column A	8
Sample size, column B	6

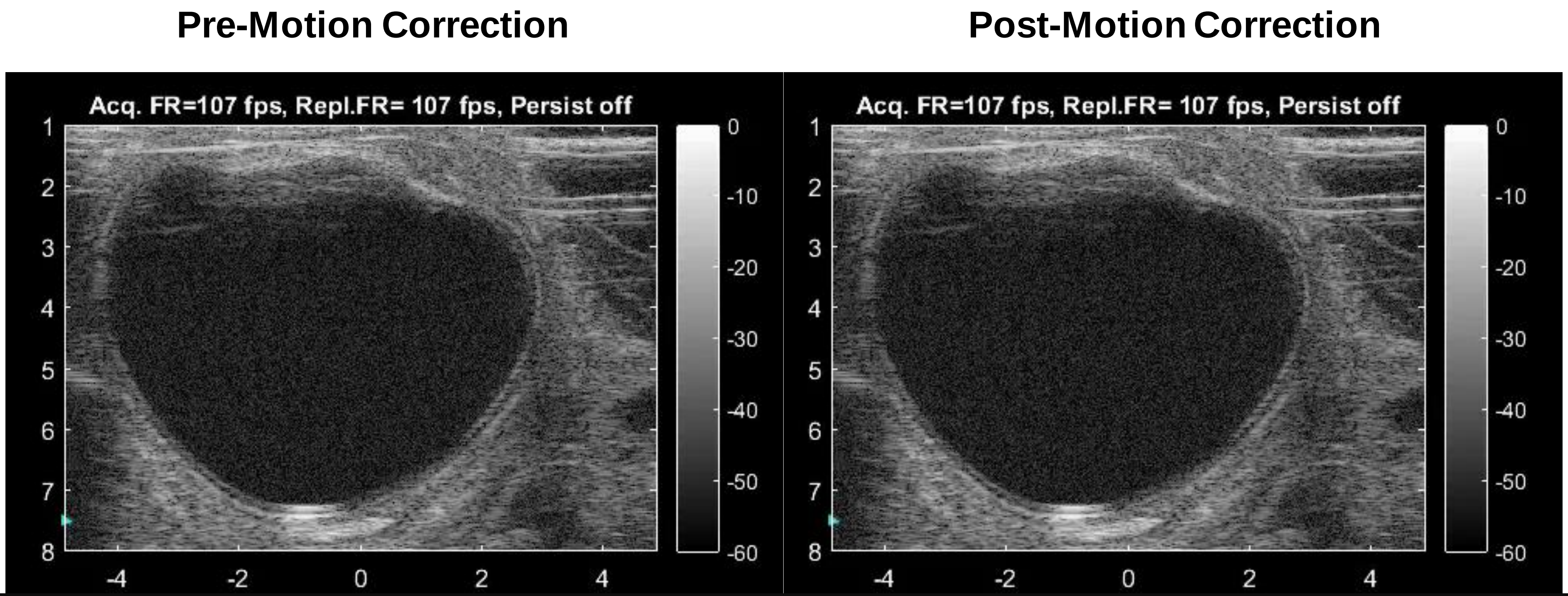
Statistical analysis results for thickness

Table Analyzed	Data 2
Column B	Treated (Diabetic)
vs.	vs.
Column A	Control
Unpaired t test	
P value	0.0003
P value summary	***
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=5.502, df=10

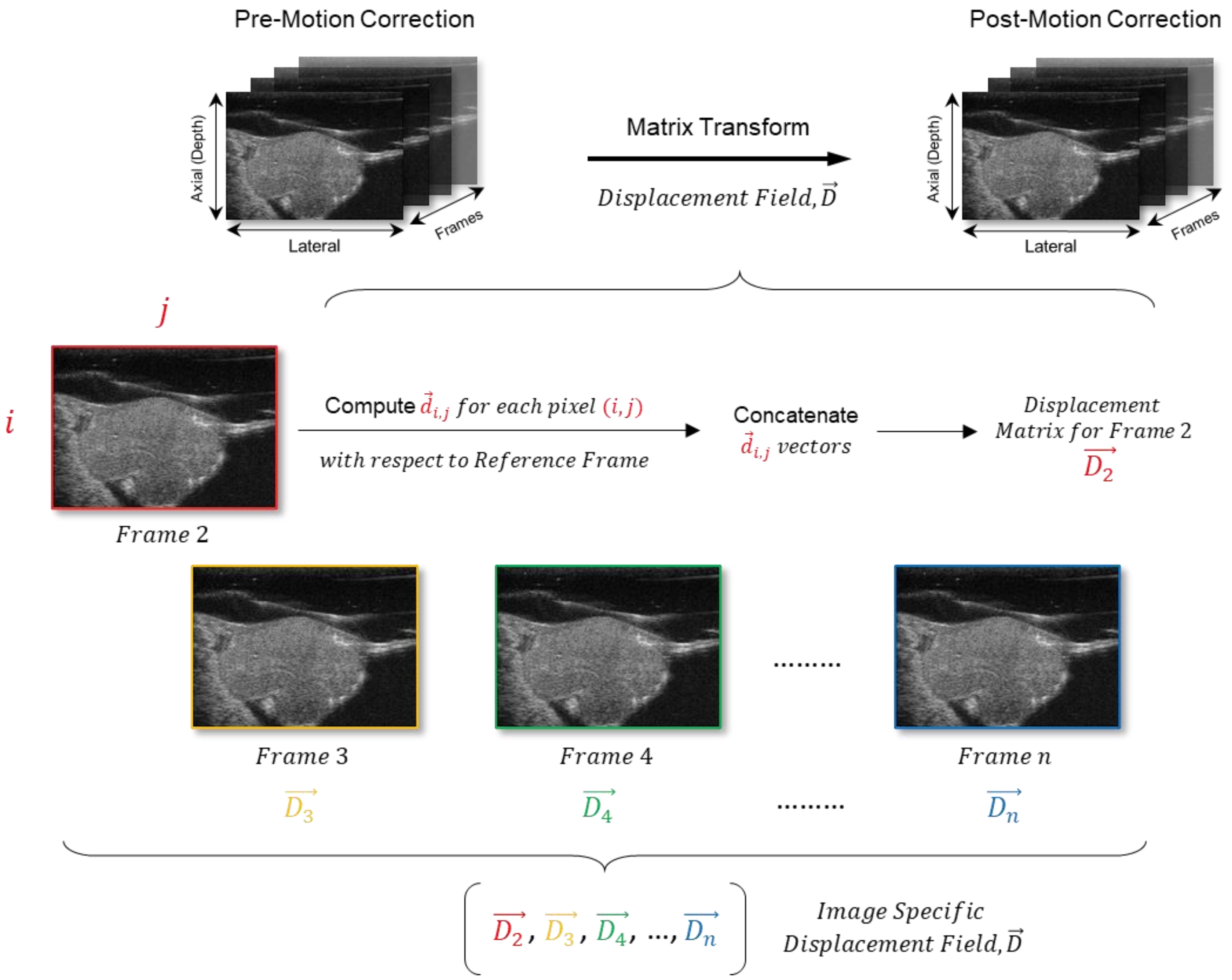
How big is the difference?	
Mean of column A	244.5
Mean of column B	149.6
Difference between means (B - A) ± SEM	-94.93 ± 17.26
95% confidence interval	-133.4 to -56.49
R squared (eta squared)	0.7517
F test to compare variances	
F, DFn, Dfd	2.177, 5, 5
P value	0.4134
P value summary	ns
Significantly different (P < 0.05)?	No
Data analyzed	
Sample size, column A	6
Sample size, column B	6

HF-US images were compensated for some motion from embryonic cardiac activity

- Some motion compensation using MATLAB function (overcome limitation of Huang et al., 2019)



In-plane Motion Compensation via MATLAB using non-rigid image registration (imregdemons)



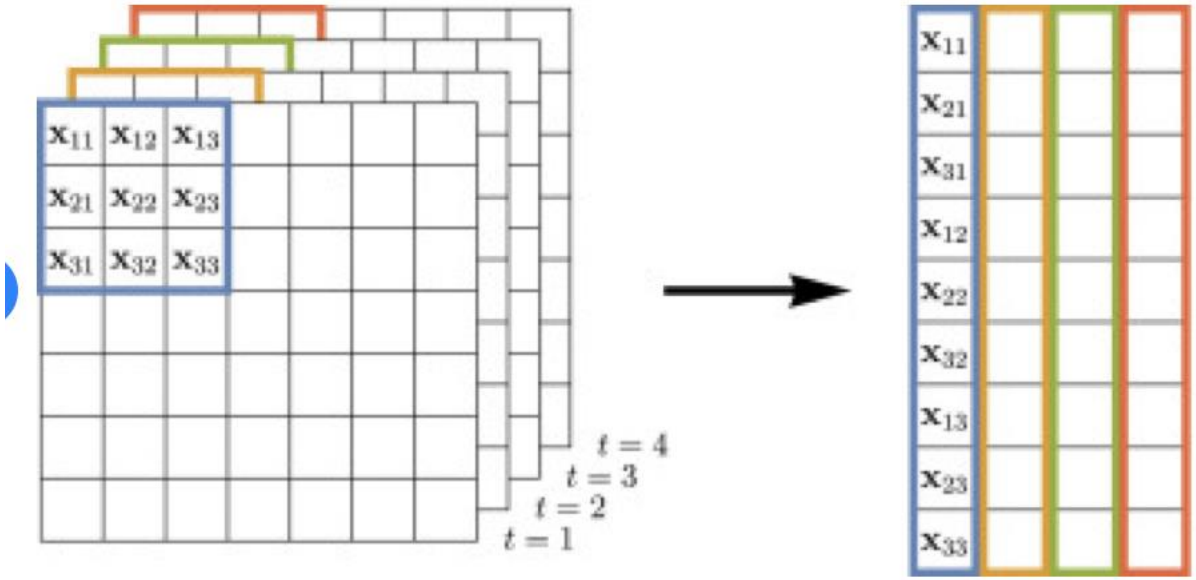
Vessel Diameter

- Vessel Diameter from CAM should be approximately 10-50 microns
  - Compared CAM Tumour vessel diameter to Rat Tumour Vessel diameter
    - 1- 10 and <12 microns respectively
    - Fairly equivalent
  - Proportionally we then figured out the retinal vessel diameter
    - Small arterioles in rat retinas have a diameter of 10-50 microns
    - Compared to this value, vessel diameter should be approximately 10- 50 microns

- Humans retinal blood vessels - 135.73+/- 15.64 microns
- CAM retinal blood vessels - approximately 10-50 microns in diameter

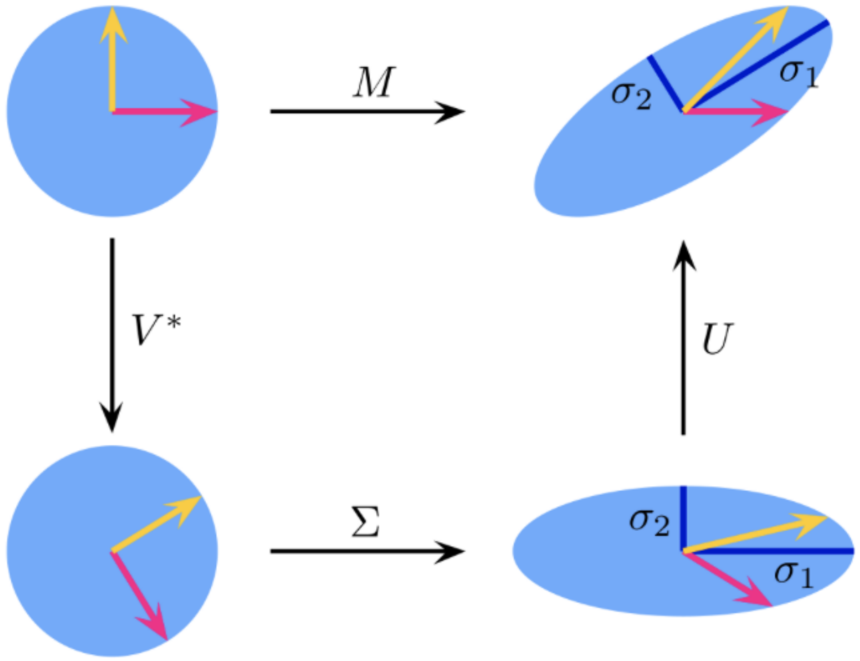
Forster *et al.* Hypoxia. *Dove Press* (2017); Leeson *et al.* Can. J. Ophthalmol. (1979);

Casorati Matrix



$$\mathbf{C} = \begin{pmatrix} \mathbf{x}_{111} & \mathbf{x}_{112} & \cdots & \mathbf{x}_{11T} \\ \mathbf{x}_{211} & \mathbf{x}_{212} & \cdots & \mathbf{x}_{21T} \\ \vdots & \vdots & \ddots & \vdots \\ \mathbf{x}_{xy1} & \mathbf{x}_{xy2} & \cdots & \mathbf{x}_{xyT} \end{pmatrix}$$

SVD



$$M = U \cdot \Sigma \cdot V^*$$

SVD transformations and change of basis. (source)

From the graph we see that SVD does following steps:

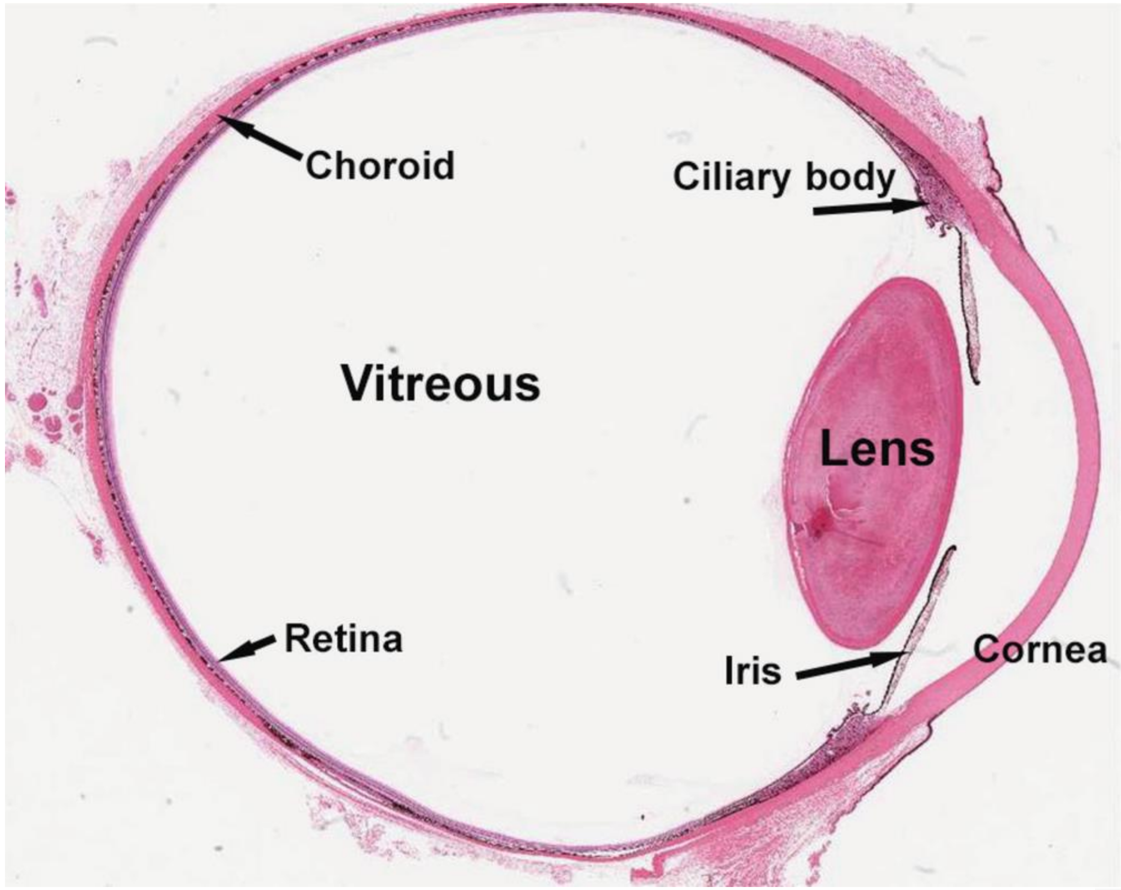
- change of the basis from standard basis to basis **V** (using **V**^{t}). Note that in graph this is shown as simple rotation
- apply transformation described by matrix **Σ**. This scales our vector in basis **V**
- change of the basis from **V** to basis **U.** Because our original matrix M isn't square, matrix **U** can't have same dimensions as V and we can't return to our original standard basis (see picture "SVD matrices")

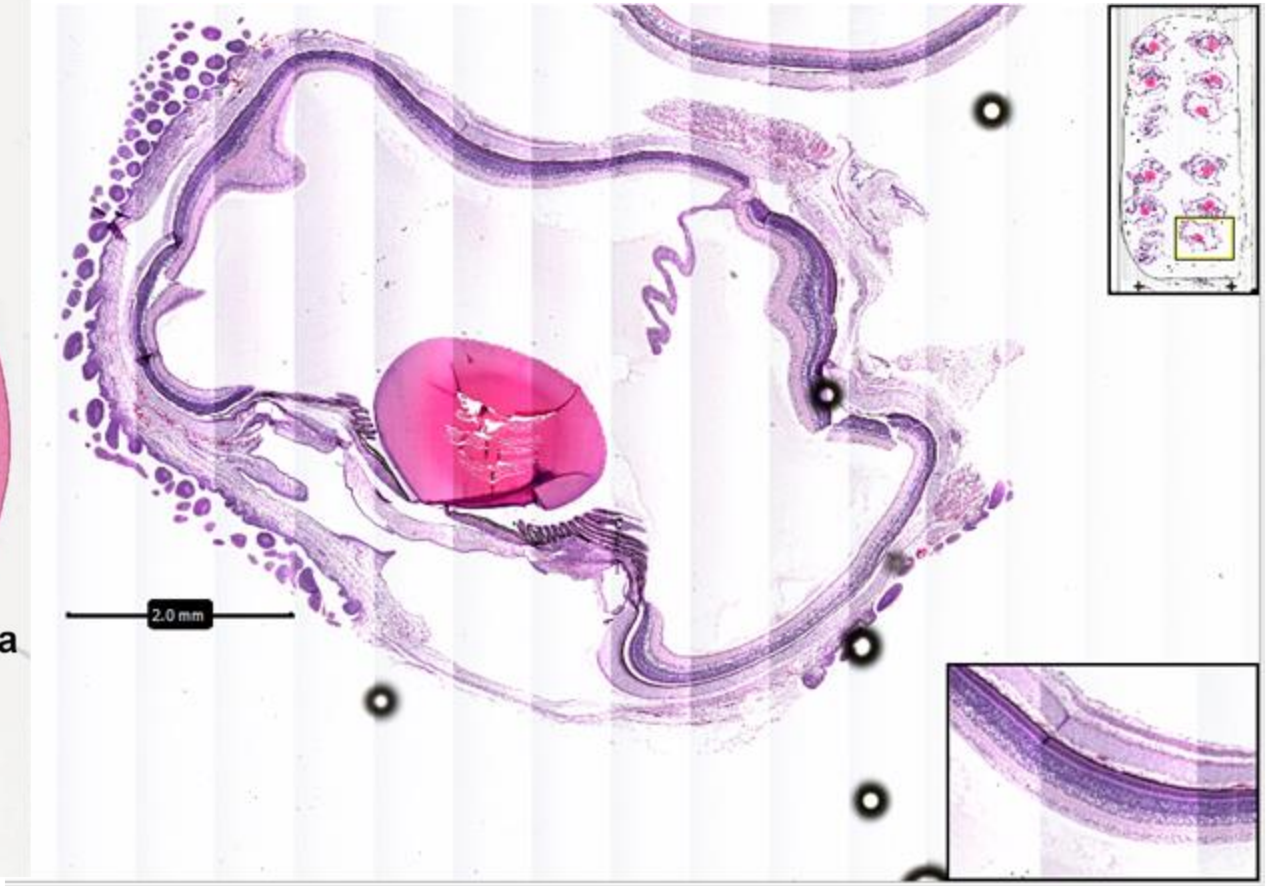
Sectioning Protocol

- Fix the sample in 10%
- Sample is dehydrated (replace with ETOH)
- Sampled is placed into xylene for a couple of times
- Wax is poured into the sample and hardened

- Waxed sample is sectioned using a microtome:
  - Paraffin wax sample is mounted onto the microtome
  - Machine is set to trim at 40um
  - 500um is trimmed off or until the center of the sample is reached
  - Machine then switches to section at 5um and cut sections
  - Cut sections are placed in a water bath at 37 degrees
- Stain with H&E

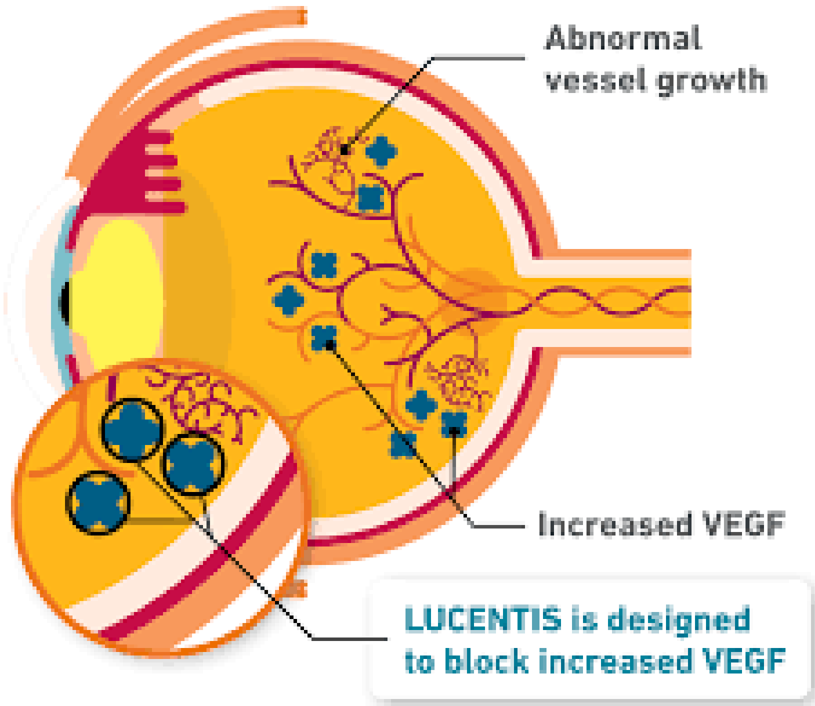
More Sectioning





Ranibizumab

- One of the most common VEGF drugs to use
- Intravitreal injection
- Study shows that it will help with choroidal neovascularization
- All patients improved in visual acuity
- Did not demonstrate deterioration
- Regressed CNV

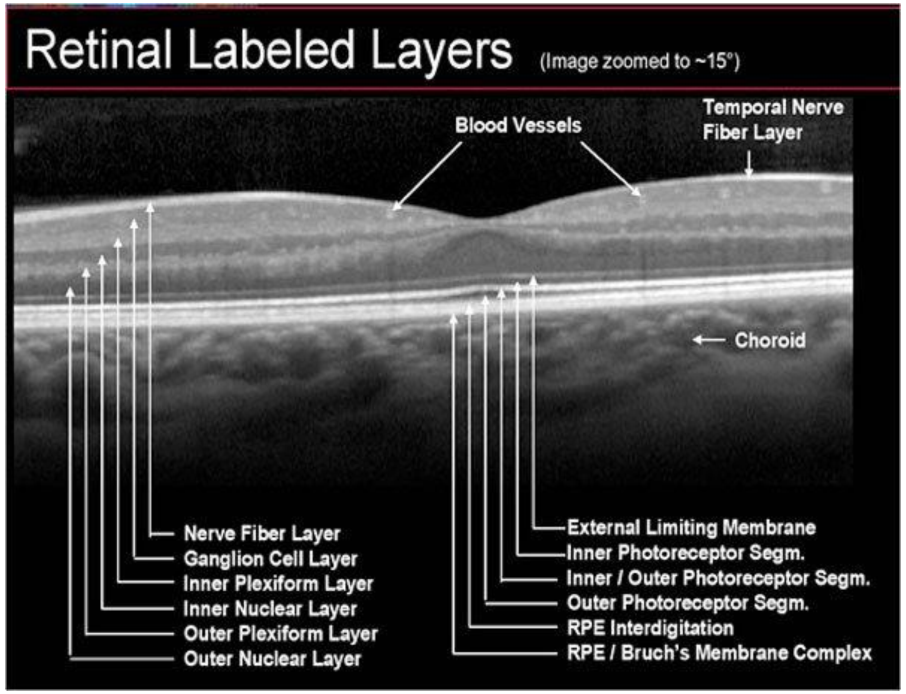


High-Frequency Ultrasound

- 1-MHz continuous ultrasound, with a half-value depth of approximately 2.3 cm, is frequently used to treat deep tissues that are approximately 2.3 to 5 cm deep
  - Issue is the axial and lateral resolution is terrible
- 50 MHz with a depth of 8-9 mm
  - Great axial and lateral resolution
  - Acceptable as the duck eye is approximately 9.1mm in diameter

HFUS and OCT

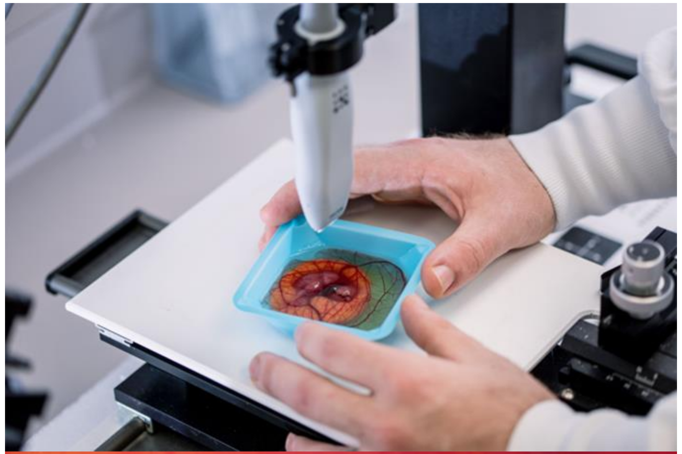
- Higher resolution and can see morphological details in OCT
  - Axial and lateral resolutions are amazing
- HFUS has a much better depth penetration than OCT
- Furthermore, since the OCT is a light-based tech, cloudiness within the vision will negatively impact it
  - Cloudy lenses will reduce image quality - still remains reliable
    - Causing artifacts in viewing
  - Higher signal to noise ratio



Huang et al. Paper Slides

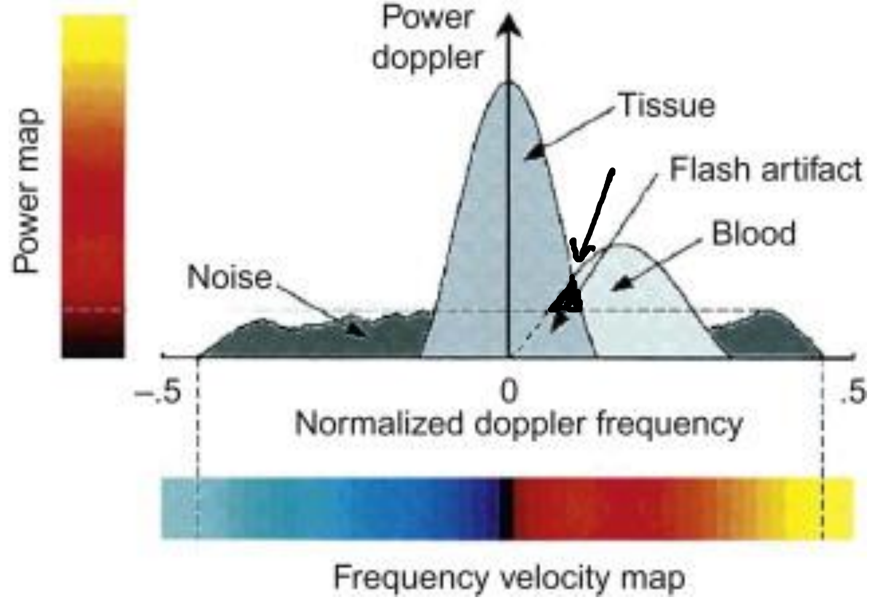
UMI has clinical applications and can be used for hyper-personalized treatment planning

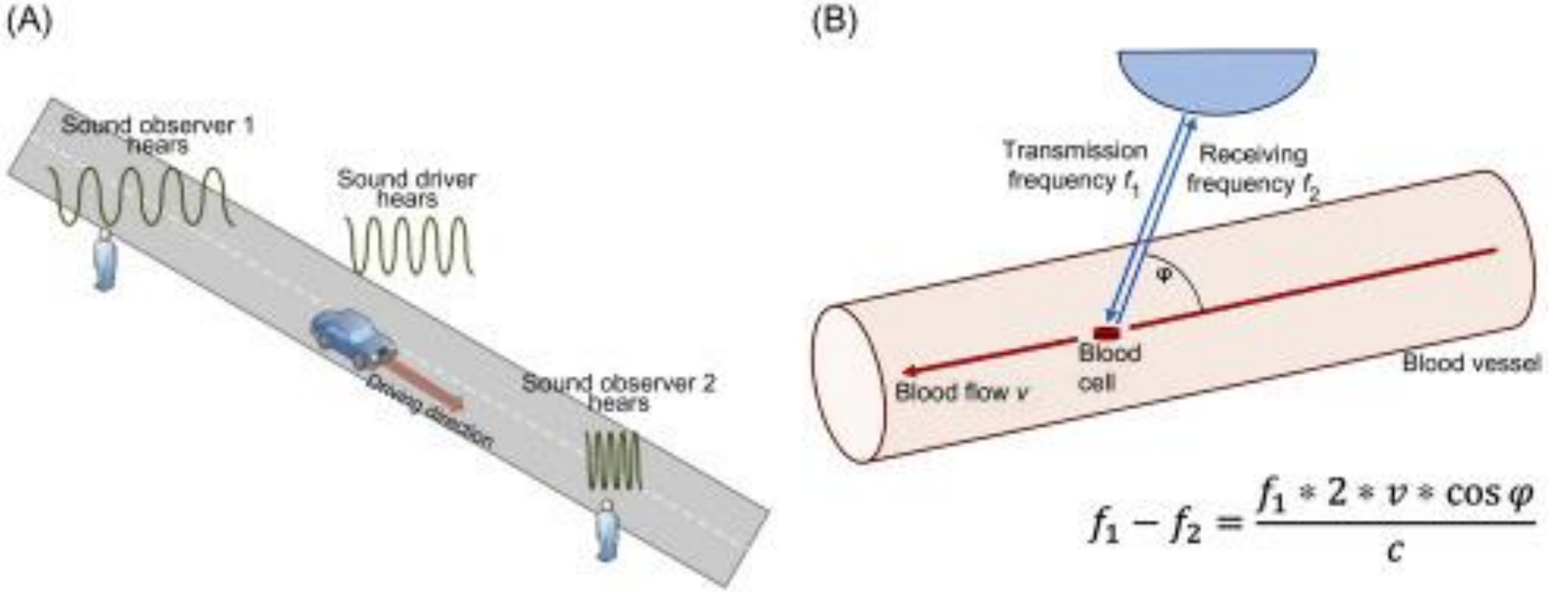
- Clinically significant
  - Noninvasive, contrast-free
  - Commercially available ultrasound machines
- Drug paneling with patient-derived xenografts (PDXs)
  - Rapidly evaluate anti-angiogenic agents
- Retinal vessel imaging in diabetic retinopathy



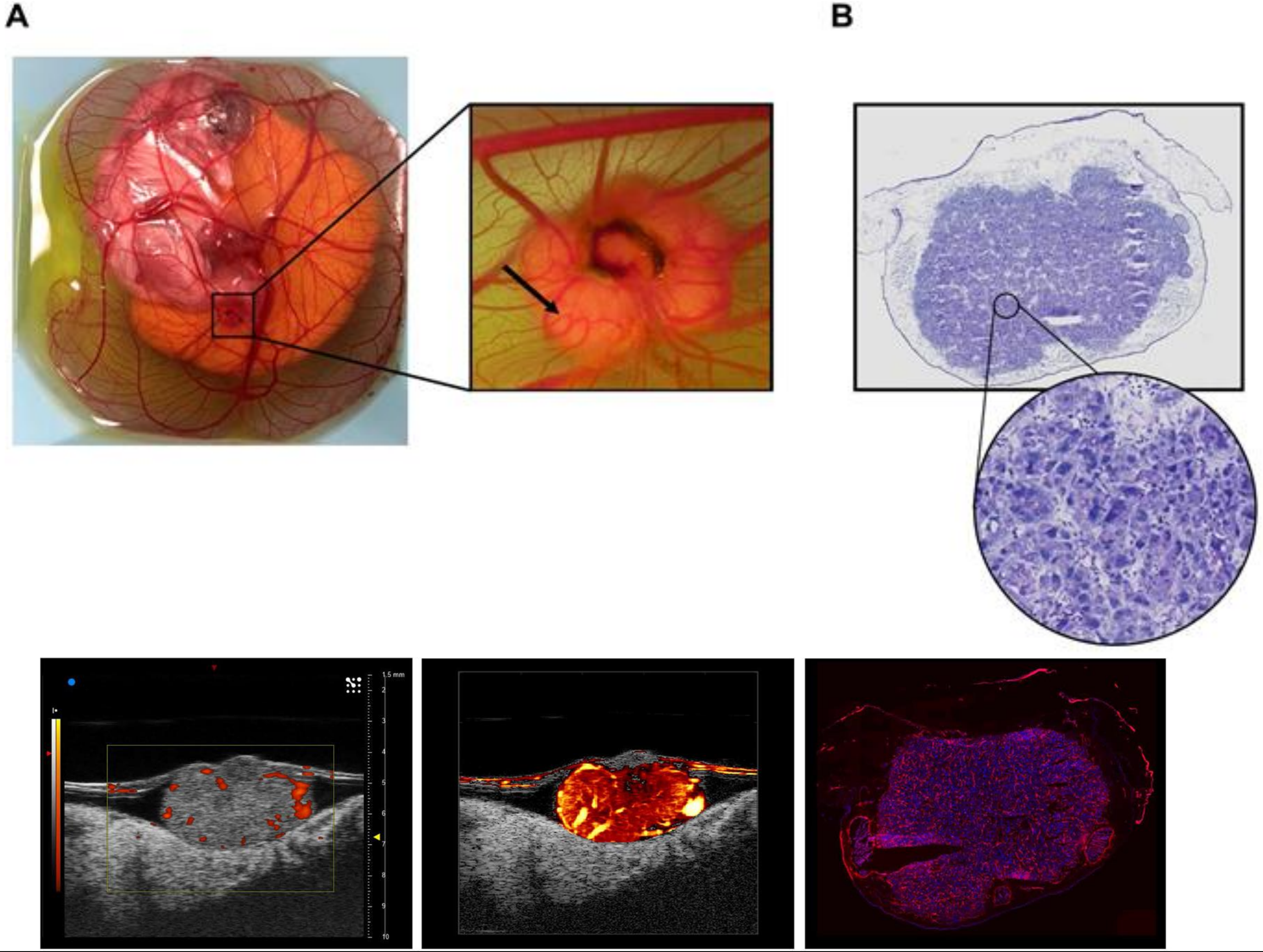
Conventional power doppler struggles with microvessels

- Separating background noise from true flow in slow flowing blood vessels

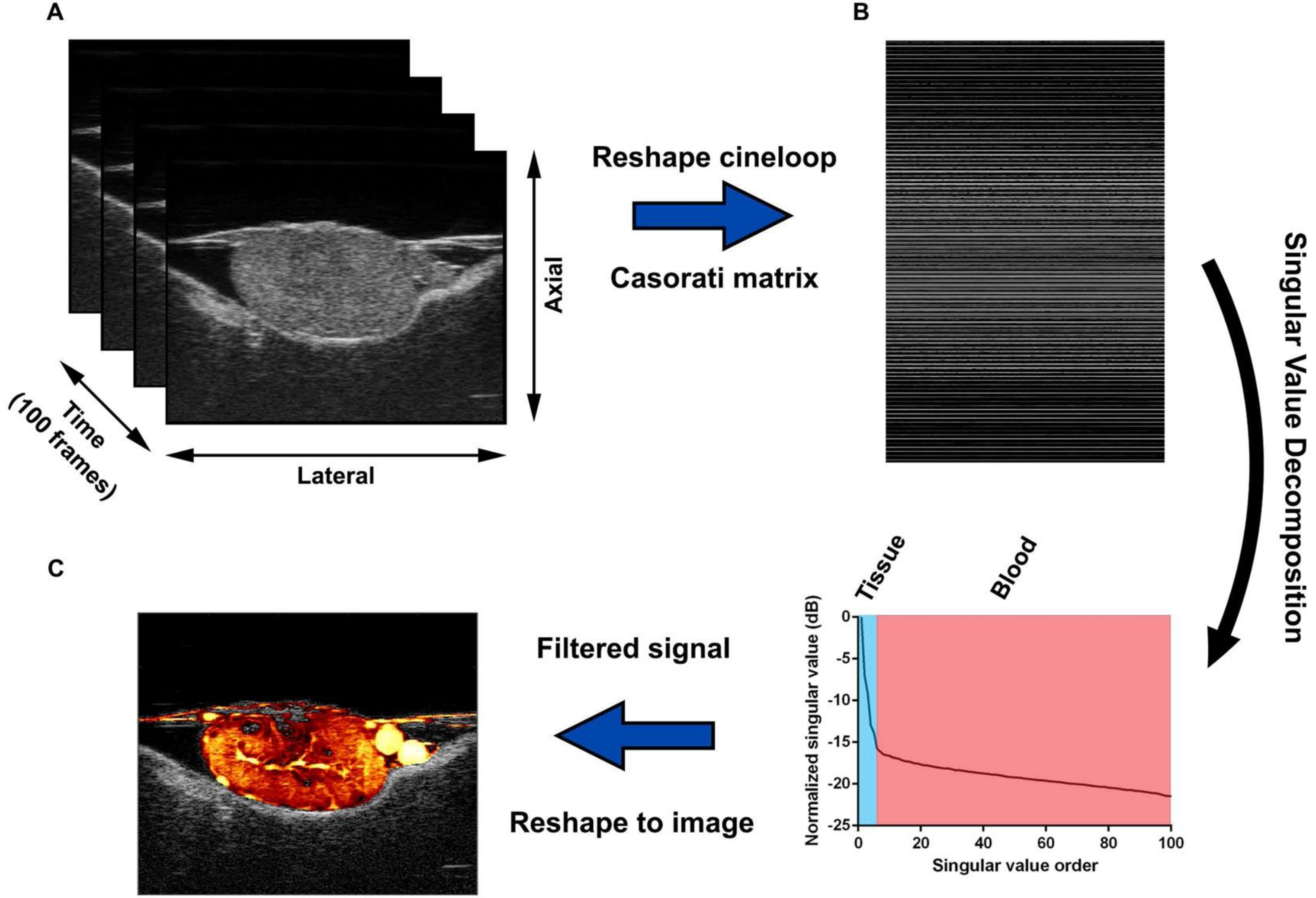




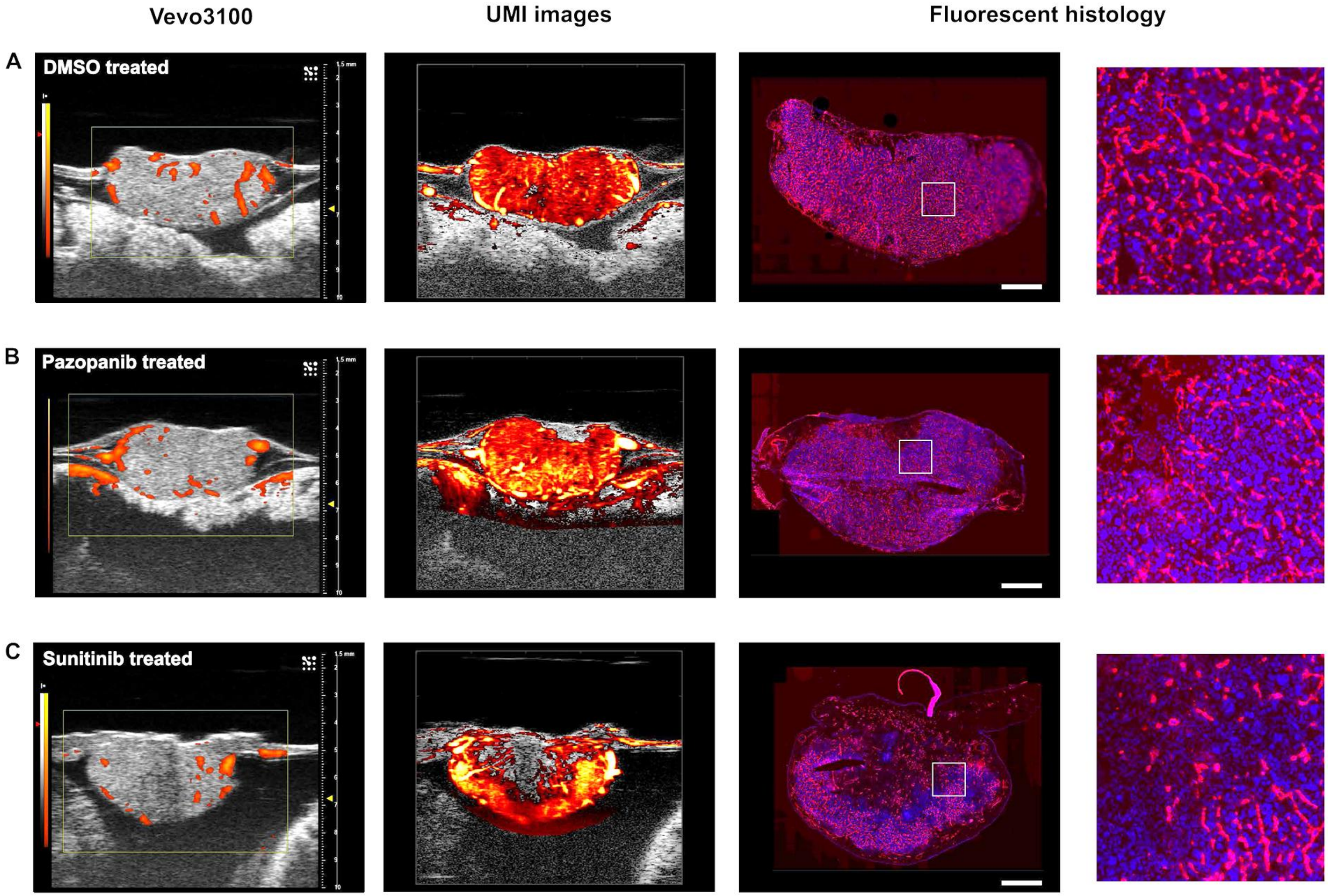
CAM implanted tumours from RENCA cell line are vascularized



SVD-based clutter filtering in UMI delineates microvessels

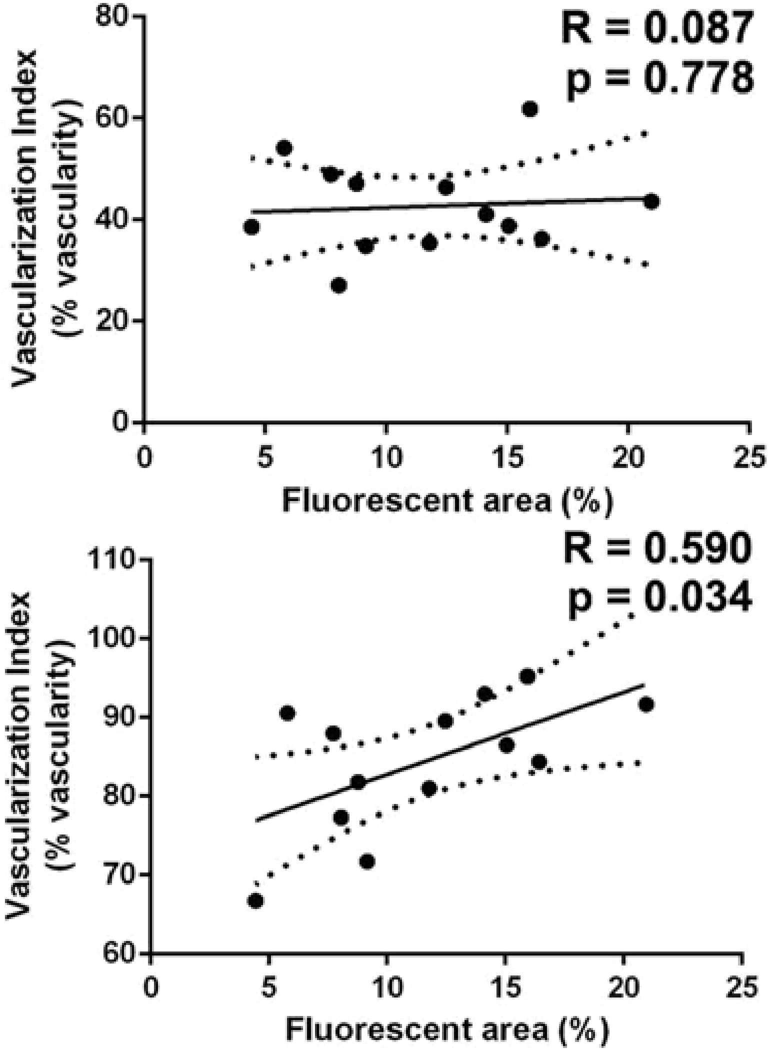


UMI demonstrates superior microvascular detection and reduced tissue background



Huang *et al.* Scientific Reports. *Nature* (2019).

UMI correlates with fluorescent histology



QUANTIFY

$$VI = \frac{Area_{vessel}}{Area_{ROI}}$$

STAINING

- Rhodamin Lectin

CORRELATION

- UMI is moderate but significant
- One-way ANOVA using Holm-Šídák

UMI confirms anti-angiogenic treatment effect as seen in fluorescent imaging