Moser Center for Leukodystrophies
at Kennedy Krieger Institute

2023 LBSL RESEARCH PROGRESS REPORT

I am delighted to provide our 12-month progress report on the Moser Center's LBSL Research Program. We have made exciting progress beyond what was anticipated and have gotten extremely encouraging results from our gene therapy and experiments with the gene that causes LBSL.

In summary, 1) we show that AAV9 (Adeno-associated virus 9) carrying *DARS2* and injected into the brain ventricles improves behavioral function in our previously reported mouse model; 2) we have developed a new spinal cord mouse model and are testing the therapy in this new model as well; 3) we show that ASO (antisense oligonucleotide) therapy improves neuronal function in the dish and is taken up by neurons in the brain when injected into the mouse nervous system; 4) we have patented these technologies and with the help of Johns Hopkins Technology Transfer are exploring several options to find a commercial path towards clinical development of these therapeutics. Meanwhile, we continue to make significant progress on our clinical research collaborations to obtain data needed to design the right clinical trials in LBSL. I am extremely grateful for our amazing research team's hard work and our donors' continuous support.

Definitions that may be helpful:
LBSL - Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation

AAV9 – Adeno-associated virus 9 is a virus engineered to deliver a gene of choice. These viruses are safe, can stably integrate and type 9 has an affinity for cells of the brain. We, along with collaborators at the University of Maryland, have designed a ***DARS2*-AAV9** which will deliver the healthy *DARS2* gene to cells.

ASO – Antisense oligonucleotide is a short synthetic sequence of RNA or DNA that can modify a person's genes or proteins. We have designed a sequence to specifically improve the amount of healthy *DARS2*, the gene that causes LBSL.

DARS2 – *DARS2* is the gene that is responsible for causing LBSL when a mutation is present. This gene makes a protein (a tRNA synthetase) that connects aspartic acid, a specific amino acid, to its tRNA to help construct proteins, which are the building blocks of our cells and our bodies in general. Capital italics *DARS2* refers to the human gene, while *Dars2* refers to the mouse gene. DARS2 refers to human protein.

Isogenic cells – Isogenic cells are cells that we use as a comparison to our LBSL patient cells. These cells were made by a process that uses a complicated technology to remove the *DARS2* mutation from patient lines. These 'corrected' cells are now identical to patient cells, without the LBSL mutation. By comparing these cells with our patient cells, we know that all differences are a consequence of the LBSL-causing mutation.

Mitochondria – Mitochondria are the organelles, or structures within a cell, that enable all of our cells to respire, or make energy, to maintain our bodies.

Please find below a summary of our progress over the last year:

MILESTONE 1: GENE THERAPY PROJECT

a. *Complete studies that helped us determine if AAV9 could restore the amount of DARS2 in LBSL patient cells to healthy levels and improve cell function.*

We continue to get promising results using the *DARS2*-AAV9 on our patient brain cells: proper gene copies are produced and **function (total outgrowth) is improved** (Figure 1).

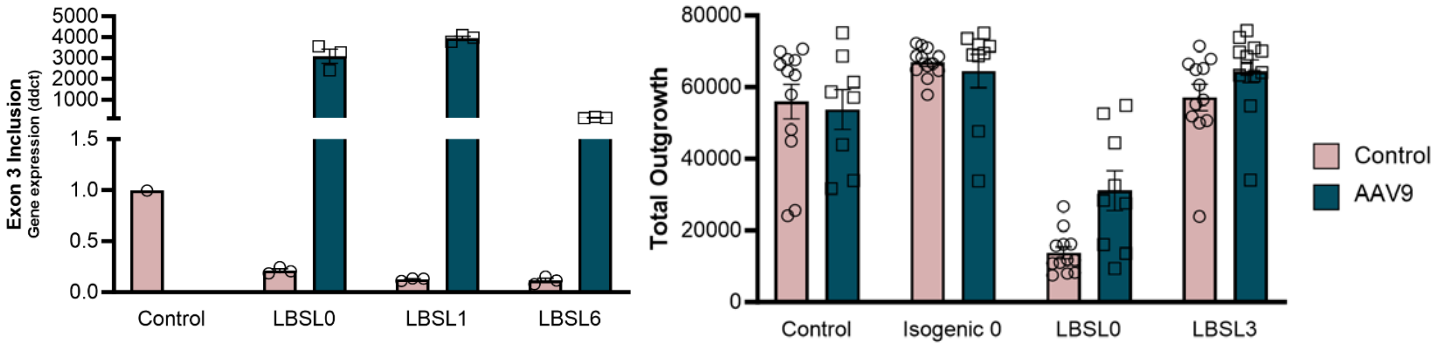


Figure 1. Mature LBSL brain cells have fewer full-length copies of DARS2 (lower Exon 3 inclusion in above left graph) compared to control. Treatment with AAV9 significantly increases the number of copies including Exon 3, relative to control (dark blue bars). Similarly, total growth of cells is shown for control, isogenic, and two LBSL patient lines. For both patient lines, AAV9 therapy improves growth after 10 days of treatment (data shown above right).

b. Complete initial studies delivering our engineered AAV9 to mice to determine where the virus traveled in their brains.

LBSL mice that show a progressive loss of cells within the brain were given *DARS2*-AAV9 at 8 weeks of age, through a direct brain injection. AAV9 is used as a vehicle to deliver full-length *DARS2* to cells within the central nervous system. We have started preliminary analysis of the biodistribution (where the AAV9 travels in the brain) of *DARS2*-AAV9. This particular virus glows green under a microscope, allowing us to track where it is in the brain and body. While this work is ongoing, after 6 months, we observe cells that express the green protein in multiple areas of the brain. Importantly, we only injected the AAV9 into one side of the brain and are able to observe expression on both sides (Figure 2). Based on the shape of these cells, we believe them to be neurons, the cells that send and receive messages to and in the brain, however future analyses will verify cell types. In Figure 2, green indicates that the virus is present.

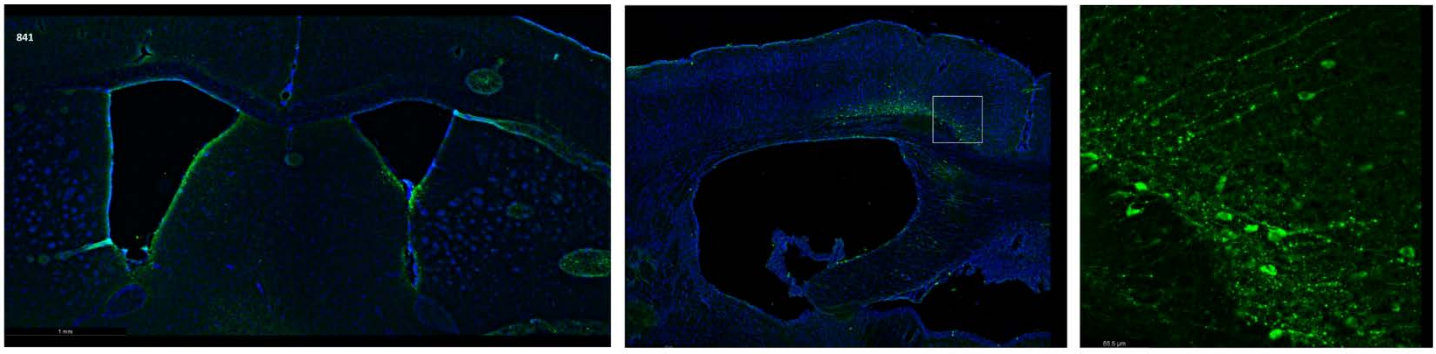


Figure 2. Forward regions of the brain show green signal in both sides of the brain, indicating circulation of the virus (left). Further back in the brain, green signal can be observed in areas of the brain where cells usually die within this model (middle image). A higher magnification image in this region (right) reveals green signal to be within neurons, our brain cell of interest.

The next steps for this project include analysis of total brain area and thickness to determine if gene therapy was sufficient to reduce cell death. Secondly, we have designed a method to identify where *DARS2* exists in

the brain, and which copies originated from the mouse and which originated from the delivered virus. These results will help us to understand how much DARS2 needs to be present in order for a change to occur.

c. Complete initial studies on how delivering AAV9 into a mouse's brain alters its behavior

As published in 2020, in mice that lack *Dars2* in the brain, a behavioral change is evident at five months of age. When given the AAV9 carrying healthy *DARS2*, the gene mutated in LBSL, we show that this behavior is improved compared with untreated mice (Figure 3). The dose given was based on literature values and was administered as a one-time-only treatment. While this therapy did not reverse the behavior completely, **these results show clearly that AAV9 treated animals are less severely affected** and we will validate these results in future experiments using optimized drug dosing and routes.

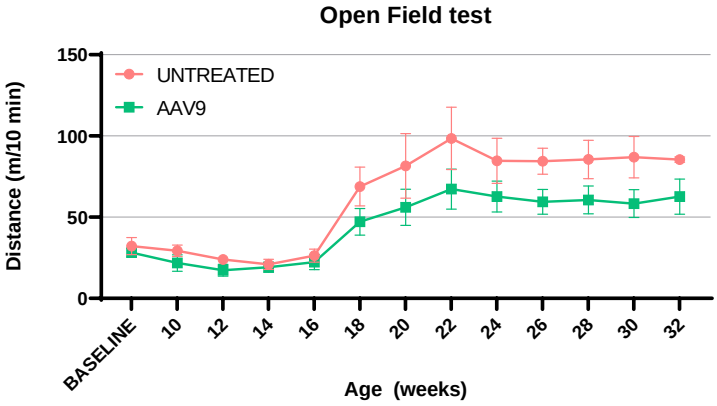


Figure 3. All mice included in this experiment were *Dars2* brain deletion mice. Some received treatment (*green line*) and some received saline *(untreated; red line)*. Activity was measured over a 10-minute period every other week for six months. A clear difference can be seen between animals receiving treatment and those that did not. The red, untreated, line in Figure 3 very much resembles the degree of behavioral change (hyperactivity) we observed with these mice in previous experiments (published 2020).

A NEW LBSL MOUSE MODEL

During this time we have also made significant progress on a new LBSL mouse model. This model has reduced *Dars2* within the dorsal root ganglia, or the populations of sensory specific cells that are located just beyond the spinal cord. These mice show a significant motor deficit, characterized by hind limb dysfunction and smaller size. When the distance between their front or hind legs is measured, they show a wider stance (referred to as base of support, BOS) compared to healthy mice (see Figure 4).

We administered *DARS2*-AAV9 to these mice, as a single injection into the spinal cord, when they were either 5 or 7 days old. At one month of age, we tested how these mice walk and found their BOS to be less variable (Figure 4), and more similar to healthy mice than to untreated mice. Similarly, their weight loss was less severe, and again, more closely resembled the control group than the untreated mice. These results again suggest that replacement with healthy *DARS2* can rescue the deficits caused by *Dars2* dysfunction.

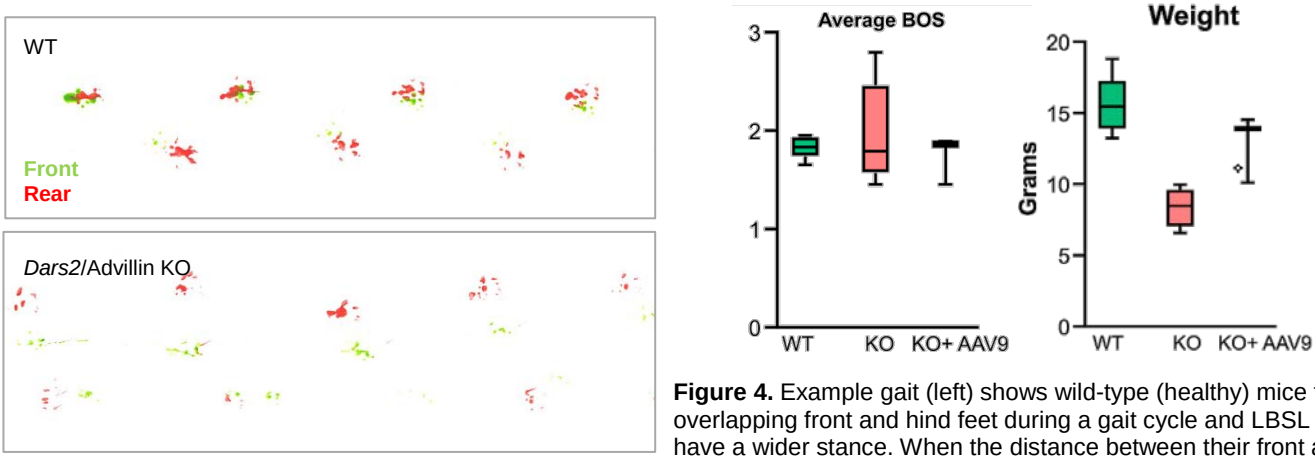


Figure 4. Example gait (left) shows wild-type (healthy) mice to have overlapping front and hind feet during a gait cycle and LBSL mice have a wider stance. When the distance between their front and hind legs is averaged, LBSL mice show greater variability in their step. Delivery of AAV9 reduces this variability and rescues their weight loss (graphs, right).

MILESTONE 2: ASO (antisense oligonucleotide) PROJECT

a. Complete efficacy studies to assess whether delivery of ASO to LBSL patient cells can restore DARS2 to healthy levels and improve cell function.

We designed a short, *DARS2*-specific, synthetic RNA sequence (antisense oligonucleotide, ASO) that when delivered to patient cells can identify and "patch" a common patient mutation. We have previously demonstrated the effects of ASO therapy, specifically showing that ASO can increase production of healthy copies of *DARS2*, the gene that causes LBSL. **New results show functional improvements in brain cells after ASO therapy in terms of energy production (Figure 5), and brain cell growth (Figure 6). Additionally, we show successful uptake of ASO into brain cells when plated in a dish (Figure 7).**

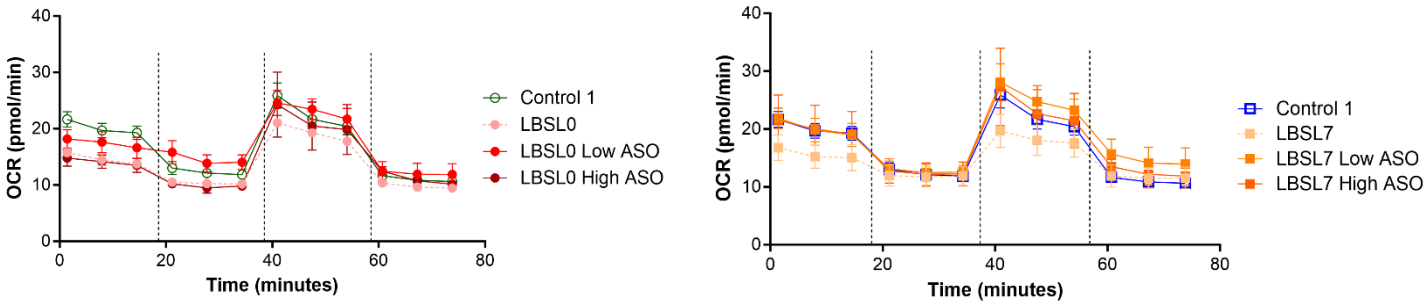


Figure 5. The oxygen consumption rate (OCR) was measured over several minutes during a 'mitochondrial stress test'. Results from time zero to 20 minutes indicate baseline cell respiration. In both LBSL cell lines shown above, the dotted patient lines show lower baseline energy production compared to ASO treated and control. From minutes 40-60, the cells are pushed to their maximum respiration and again, treated patient cells show higher levels of respiration compared to untreated cells.

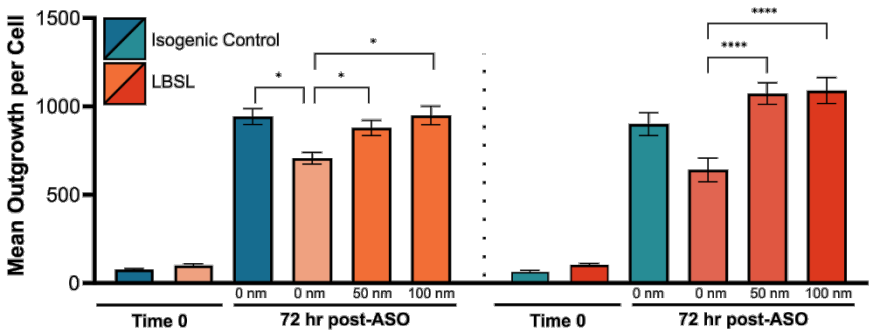


Figure 6. The average outgrowth (growth of brain cells) is shown over a period of several days. Time zero demonstrates that early after cells are placed into dishes, the growth of LBSL cells is similar to control cells. After 5 days in a dish and 72 hours after ASO treatment, it is shown that untreated (0 nm ASO) LBSL cells have the shortest growth, and treated patient lines resemble control levels.

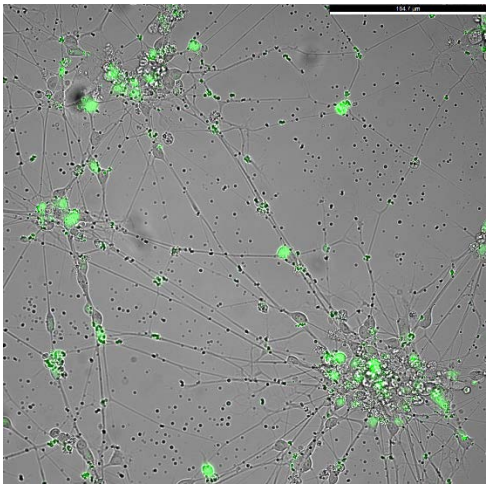


Figure 7. We imaged brain cells that were treated with a green-tagged ASO. This ASO was also tested in functional studies to ensure that the tag does not interfere with normal function of the ASO. This image was taken hours after treatment, and uptake is rapid.

b. Complete initial studies of ASO biodistribution in mouse brain and organs

In adult healthy mice, we injected ASO either to the brain (intracerebroventricular), or to the spinal cord (intrathecal) and observed distribution (spread) after 6 days. From these mice, we collected liver, kidney, heart, brain, spinal cord and blood. Preliminary data show that the ASO is taken up by many brain cells when the ASO is injected to the brain (Figure 8, top). When delivered to the spinal cord, we see some signal within the brain and expect that repeated dosing would result in greater observation of the ASO signal within this tissue (Figure 8, bottom). Higher magnification imaging of the ASO within regions of the brain of brain-injected mice show that the ASO (green) overlaps with the neuron (red) signal (Figure 9). To check, we also identified astrocytes, an abundant and important cell within the brain, however we did not observe overlap in signal (pink) with ASO. **We were pleased to observe ASO within the brain, as we believe that brain cells in LBSL are the most susceptible to DARS2 deficiency.**

Importantly, no signal is observed within the kidney, liver, or heart (see Figure 10 and 11, respectively) indicating the ASO remained localized to the brain. Analysis of blood serum showed no toxicities. These studies will be repeated following a longer treatment with ASO.

c. Engage GMP manufacturer for large scale clinical grade ASO production for human trials

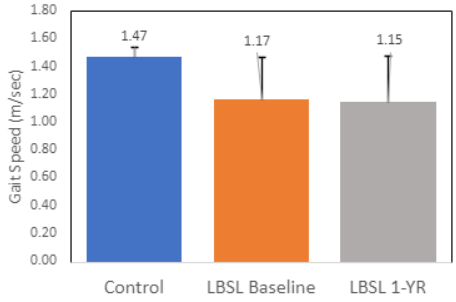
We have engaged a GMP (Good Manufacturing Practices) manufacturer who has provided a cost quote for creating a GMP manufactured ASO product for us. This will be necessary once we complete the first round of toxicity studies of our ASO. The FDA (Food and Drug Administration) will likely require us to test the GMP manufactured products in two species before giving it to patients. We hope that these efforts can be conducted on the commercial side as we have patented our products and are currently searching for a company to license the technology and move forward towards clinical development.

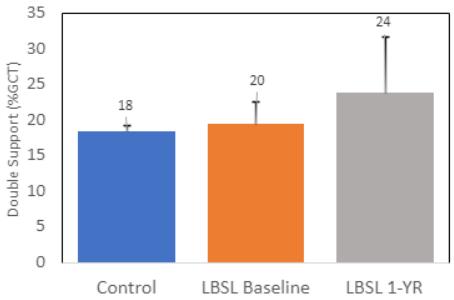
MILESTONE 3: CLINICAL RESEARCH

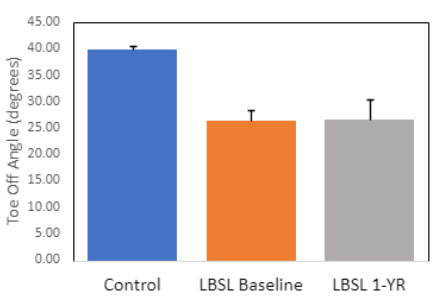
a. Complete interim analysis of follow up data from US and Netherlands (AMC)

One year follow-up data from a subset of LBSL patients in the Netherlands has been transferred and analysis is in process. The Netherlands group has also collected a large dataset of healthy control data for comparison of performance on the walking and balance tests. The series of graphs below represent control and patient performance and on the 6MWT (6-minute walk test, fast-as-possible pace), the Timed Up and Go Test, and 4 standing balance tests (Feet Apart Eyes Open, Feet Apart Eyes Closed, Feet Together, Eyes Open, Feet Together Eyes Closed).

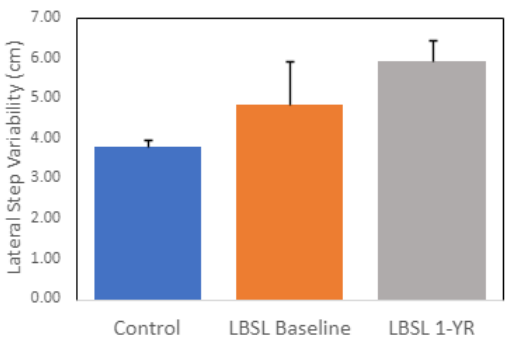
6-minute Walk Test. AMC LBSL patient gait speed was decreased by a large, clinically meaningful margin (0.3 m/s) at baseline compared to age- and sex-matched healthy controls. However, after one year, LBSL gait speed did not decline further relative to baseline. Double Support (the percentage of time spent during the gait cycle with both feet on the ground) was increased in LBSL vs. Controls at baseline, and further increased after one year, indicating a decline in gait stability. Similarly, the lateral step variability, a proxy of gait ataxia or a variable stepping pattern, further increased during the interim. The toe off angle is the angle of the foot during step off as a stride is initiated, and a smaller angle can be seen in association with leg spasticity, weakness or impaired proprioception (the sense that allows us to know where our body is in space). The toe off angle was significantly decreased in the LBSL cohort by 15%, and this change was stable after 1 year.

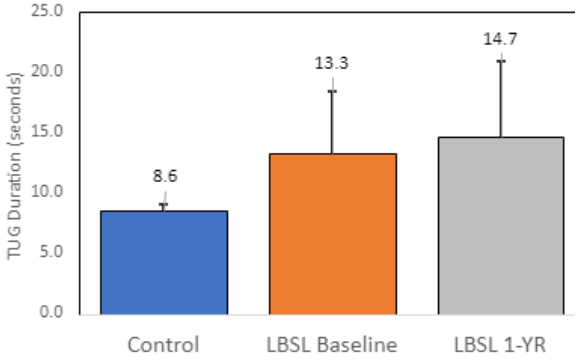




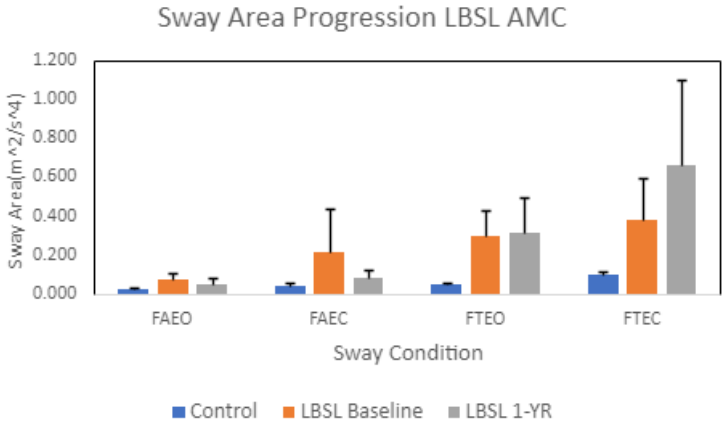


Timed Up and Go Test. In this fast-as-possible three-meter walk test, participants rise from a chair, walk, turn and sit down. The TUG duration (seconds) was significantly increased for LBSL participants, with a further minimal increase after one year.





Sway Tests. Similar to our findings in the Kennedy Krieger LBSL cohort, the sway area during a 30-second standing balance test was greatest in the feet together, eyes closed (FTEC) condition. Although not statistically significant, there is a trend of further increased sway in the FTEC condition after one year, and the mean sway area is six-fold larger than the age and sex-matched control sway area. **Importantly, these results bear promise that sway with feet together, eyes closed may be an early indicator of disease progression and could be used as an outcome measure in a trial.**



b. Start obtaining data from Finland

Our research collaborators at the University of Helsinki (PI Dr. Emil Ylikallio) have been enrolling patients since Spring 2023, four subjects have been enrolled and tested so far, with continued interest generated through social media and their clinical practice. Our data transfer agreement is pending, and we anticipate sharing interim analysis results as part of the Spring 2024 six month report.

Our newest research collaboration at the Hospital Pequeno Principe in Curitiba, Brazil (PI Dr. Josiane Souza) is in progress. Final site IRB edits were made in October 2023. Seven interested patients have been engaged through clinicians working with Dr. Souza or by CureLBSL social media and contact registry efforts. Two Opals kits have already been purchased and are being stored in the U.S. until we determine a vendor to facilitate the most secure, cost-effective means to deliver the equipment to our partners, anticipated by the end of October 2023.

c. Initial engagement with FDA regarding a Patient-Focused Drug Development (PFDD) vs listening session

We are excited to announce that a very successful FDA patient listening session was held on May 31, 2023. This is thanks to the excellent efforts of the CureLBSL Team, and Melody Kisor in particular for hard work to engage the patients and families and capture their voices in such a compelling format. Dr. Amena Smith Fine provided a clinical overview and has advised Melody on the post-meeting FDA executive summary report. The full known spectrum of LBSL was represented (infantile, juvenile and adult onset), the signs and symptoms, and the impacts on patients and families through a series of interviews, brief videos and educational slides. The concluding question was "What is the primary symptom you're looking to address?" (Answers: Balance, stability, seizures, breathing weight management). Attendance was high, and FDA participants included representatives from the Office of the Commissioner, Center for Biologics Evaluation and Research, Center for Devices and Radiological Health, Center for Drug Evaluation and Research, and many non-FDA attendees from foundations including the NCATS (National Center for Advancing Translational Sciences) of the NIH (National Institute of Health). We are meeting with CureLBSL October 20, 2023 to start planning engagement for Critical Path Innovation (CPIM) and PFDD meetings in summer or fall 2024.

d. Identify regulatory consultant for FDA IND (Investigational New Drug) filing

We are considering a short list of consultants and determining an appropriate budget for these next steps. Current plans for the PFDD (FDA Patient-Focused Drug Development) are that this meeting would be spearheaded by CureLBSL (perhaps jointly with NORD (National Organization for Rare Disease), with invited clinical speakers from Kennedy Krieger and Amsterdam. NORD is preparing a quote for PFDD planning services and communications. The Critical Path Innovation meeting will be led by our research team at Kennedy Krieger, with support from CureLBSL. We are gathering information on consultants who are experienced in working rare disease patient advocacy groups.

Thank you again for all your support and your trust in our work. We are eager to continue our important work and are inspired by our donors' dedication and commitment to find a cure for LBSL. You can make a safe and secure online donation here.

Sincerely,

Ali Fatemi, MD, MBA
Chief Medical Officer
Blum Moser Endowed Chair, Kennedy Krieger Institute
Professor of Neurology & Pediatrics, Johns Hopkins School of Medicine

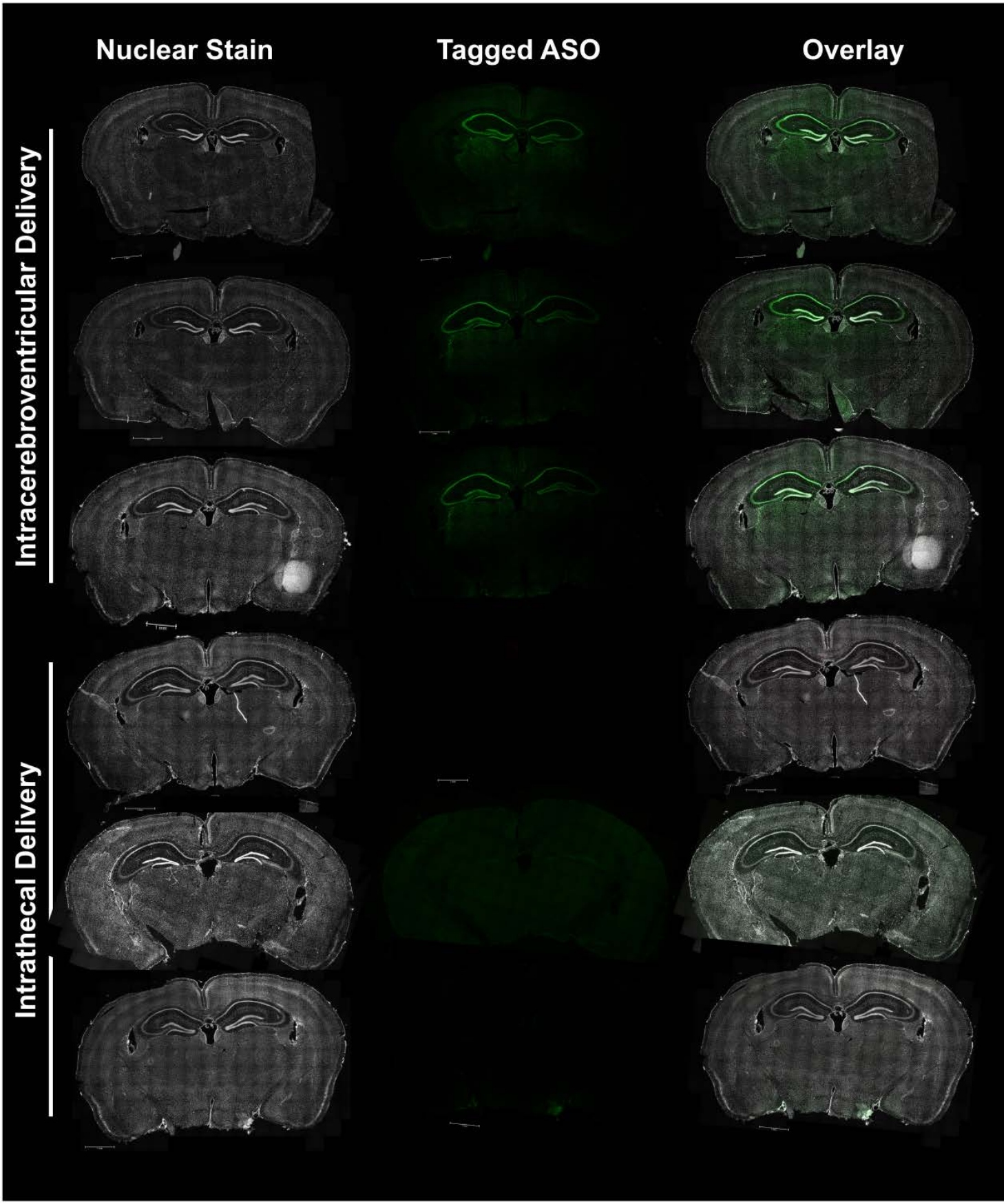


Figure 8. Brain sections from six healthy control mice are shown, three with brain injections (top) and three with spinal cord injections (bottom). Images on the left show brain structures (level of the hippocampus) using a stain for the cell nucleus. The middle column shows the green signal of ASO, and the third column is the overlay.

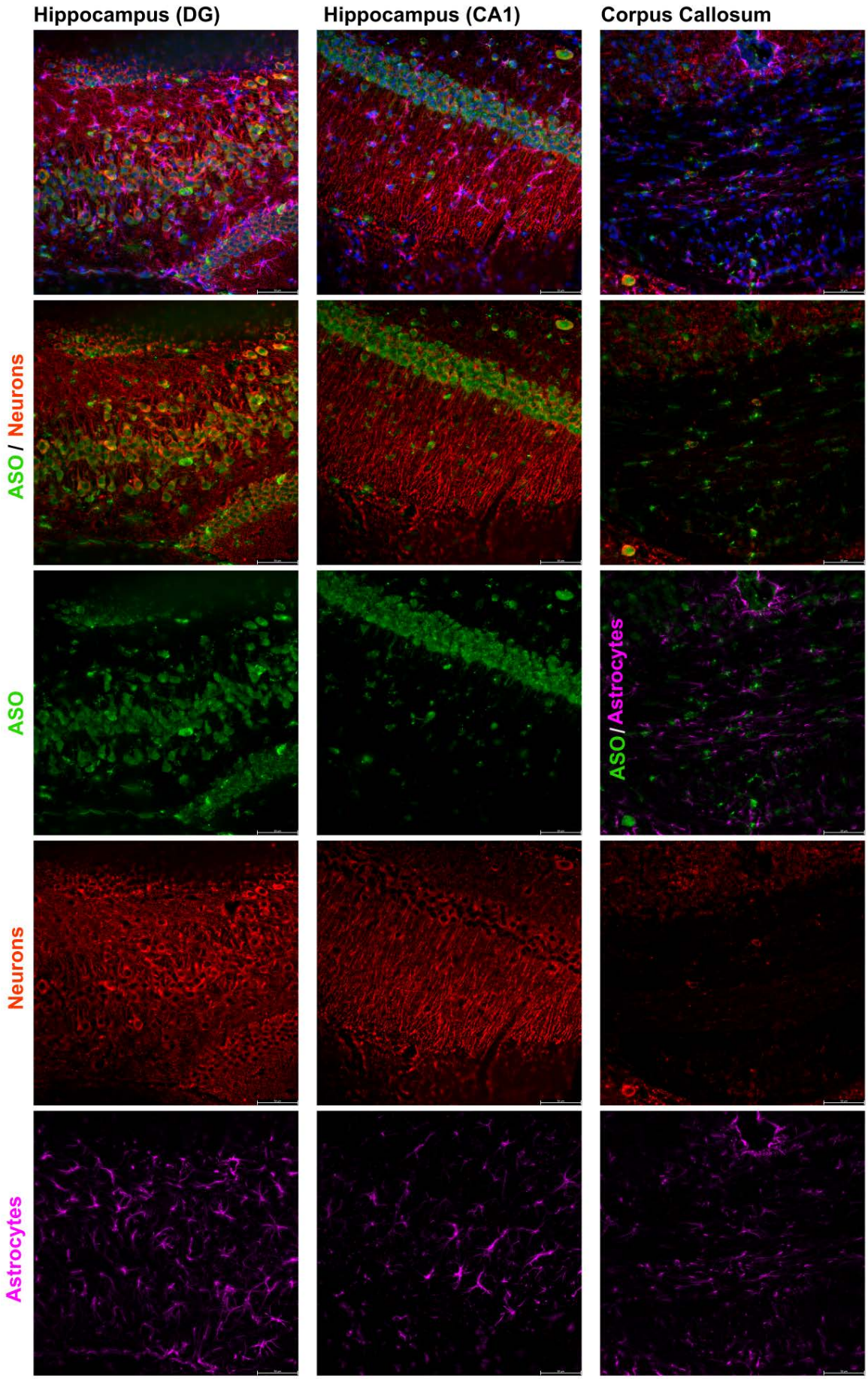


Figure 9. Higher magnification images of different brain regions show ASO (green) to overlap with neurons (red).

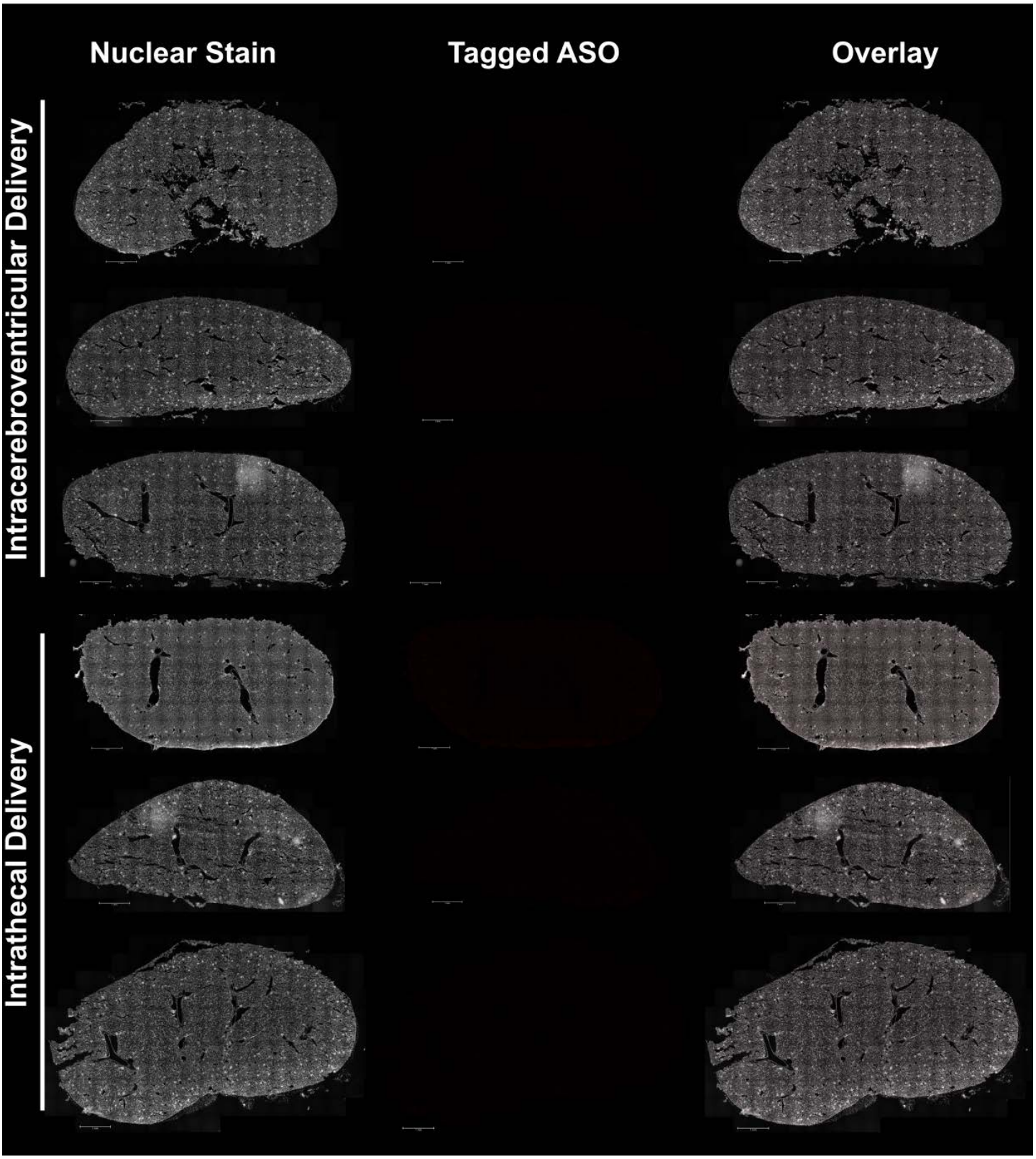


Figure 10. Kidneys from the same six ASO injected animals are shown and no green (ASO) signal is observed within these tissues. General morphology of the kidney looks normal. Blood tests were sent to test for kidney function and were returned normal; however validation of these data are necessary.

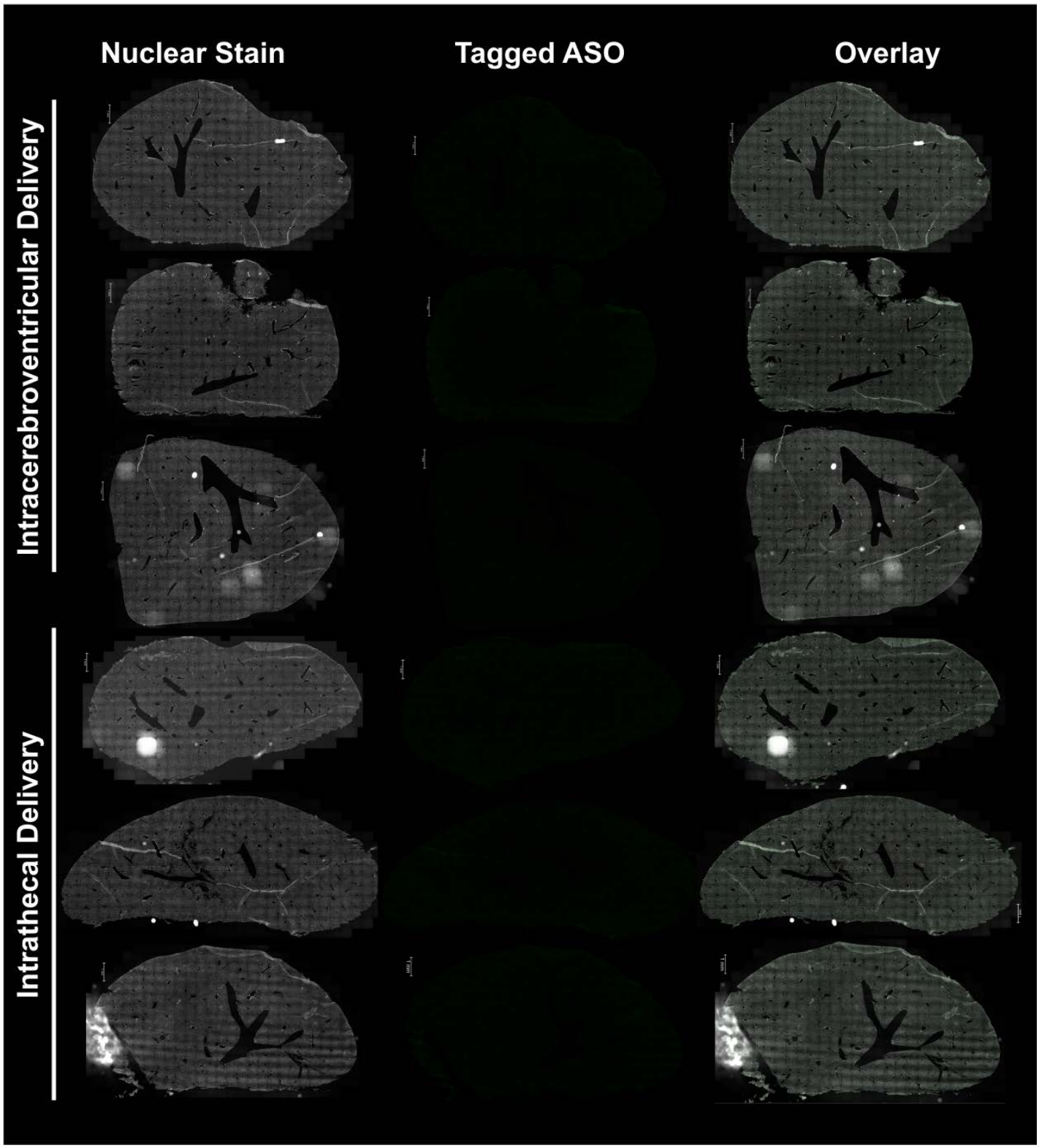


Figure 11. Livers from the same six ASO injected animals are shown and no green (ASO) signal is observed within these tissues. General morphology of the liver looks normal.