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3D Imaging and Visualization of Gene Therapies in Whole Animals

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INTRODUCTION

With major scientific advancements in gene therapy research, new insights have led to the development and approval of therapies for devastating monogenic diseases. According to the FDA, as of January 2020, there are four approved gene therapies and over 900 Investigational New Drug (IND) in the clinic. With a safe profile, well-developed scientific knowledge, and appropriate genetic tools to deploy it, gene therapies have gained momentum with numerous drug companies focused on developing therapies and delivery vectors for various disease indications with a focus on the central nervous system (CNS).

Successful gene therapies require targeted delivery of genetic material into the right organ and cells followed by sufficient levels of protein expression, or modulation to elicit a therapeutic response. Various parameters determine the outcome of gene therapies, including the type of gene vector, such as exosomes, viruses and lipid nanoparticles, gene promoters, administration routes and dosing. A key factor when conducting gene therapy research is understanding the biodistribution of the vector and more importantly, the subsequent protein regulation in whole animals when administered systemically, or at the organ level when administered locally. Importantly, it is crucial to confirm that the vector has reached the target organ and that it is properly expressing the therapeutic protein, or modulating the target gene as well as understanding off target expression for safety reasons.

Current biochemical methods used to detect gene targets or proteins, such as western blots, polymerase chain reaction (PCR), or immunohistochemistry (IHC) are labor-intensive and require tissue collection followed by homogenization and/or section collection from multiple organs. While these methods are well-established and quantitative, they do not preserve spatial information and provide limited 2D information. In gene therapy discovery programs, green fluorescent protein (GFP) and/or luciferase are the most widely used proteins in exploratory assays. While these fluorescent and chemiluminescent imaging markers may provide temporal and spatial whole animal information, they do so at low resolution and sensitivity. Sections from various organs are also collected and immunostained for GFP or luciferase to offer increased sensitivity and much higher resolution, but lack volumetric information across whole animal or organ. A large gap in resolution and sample volume exists between *in vivo* optical imaging and immunostaining.

In this technical note, we will demonstrate how this gap can be bridged using a novel three-dimensional tissue imaging approach known as cryofluorescence tomography (CFT) (Figure 1A). We include several examples of multimodality imaging studies in which CFT along with *in vivo* imaging modalities were used across a wide range of volumes, resolutions and sensitivities.

OVERVIEW OF CRYOFLUORESCENCE TOMOGRAPHY

CFT, commercialized by Emit Imaging, is a 3D blockface imaging modality in which large samples, such as whole animals or whole organs are embedded in optimal cutting temperature (OCT) compound followed by sequential sectioning and image acquisition throughout the entire sample. In this process, a single image is acquired at each plane of the block in both fluorescent and white light channels. With the aid of fiducial markers, planar images are reconstructed into 3D images along with the co-registration of the fluorescent and white light channels, thus providing complete anatomical context. Standard CFT output includes a 3D reconstructed image of the fluorescent signal and co-registered planar images in all the acquired channels to provide a flythrough of the imaged sample (Figure 1B).

With a high-resolution camera and no signal attenuation, CFT provides higher resolution and sensitivity compared to other fluorescent or chemiluminescent *in vivo* imaging modalities. It is important to emphasize that the physical section and exposure of each plane in the animal increases the ability to excite and detect fluorescent proteins in the visible range, such as GFP or TdTomato, which are less ideal for *in vivo* fluorescence, but are still the most commonly used fluorophores in preclinical gene therapy research.

High resolution 3D imaging coupled with sample sectioning, which in turn enables section collection and microscopy, allows CFT to bridge the gap between *in vivo* modalities and histopathology techniques. CFT imaging, when conducted in a multimodality approach, provides temporal and spatial resolution as well as macro to micro resolution in a single animal. Together, with the ability to embed multiple samples in a single block, CFT enables high throughput and fast turnaround times, thus providing a powerful tool to screen various gene vectors in whole animals or organs to better understand vector biodistribution and subsequent protein expression.

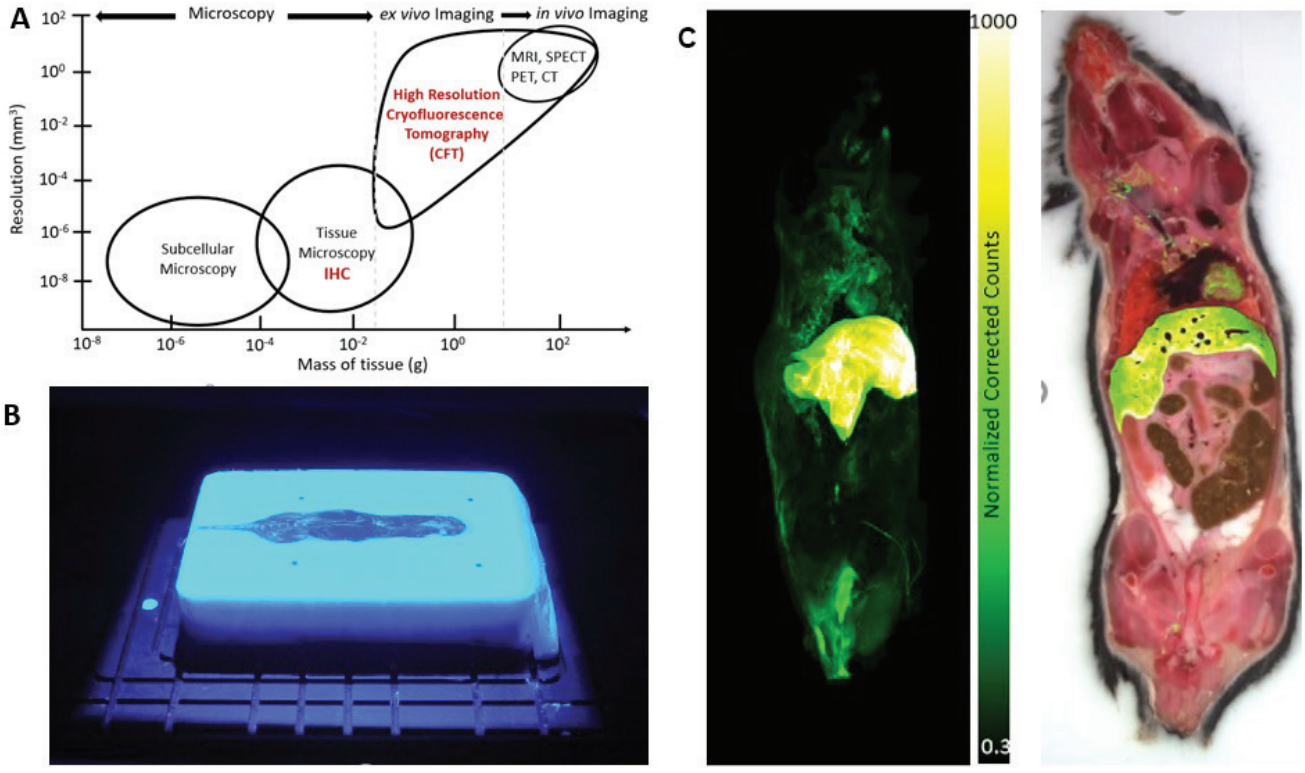

Figure 1. CFT bridges the gap between *in vivo* imaging and microscopy. (A) A tradeoff exists between image resolution and sample volume-CFT bridged the gap between *in vivo* imaging and microscopy by enabling high-resolution 3D imaging of whole animals. (B) A fresh frozen whole mouse is embedded in an OCT block with a camera and lasers located above the block to conduct blockface imaging throughout the entire sample. (C, left) Acquired planar images are reconstructed and viewed as MIPs to visualize the fluorescent marker in 3D. (C, right) Fluorescent planar images are co-registered to the white light channel to create a flythrough, thus providing anatomical information.

APPLICATION EXAMPLE 1: ANTISENSE OLIGONUCLEOTIDE (ASO) PHARMACOLOGY

In recent work conducted by Invicro, Biogen and Ionis Pharmaceuticals, which was published in the *Journal of Clinical Investigation*, ASO pharmacology was investigated in rat CNS.[1] When targeting neuromuscular diseases, and in order to avoid periphery degradation and achieve sufficient concentration at the target site, ASOs can be administered intrathecally (IT). However, the pharmacology of ASOs in the intrathecal space is poorly understood. While various imaging modalities can support pharmacological research applications ranging from biodistribution, pharmacokinetics (PK), pharmacodynamics (PD) and cellular localization, no single imaging modality can support all. To better understand ASO pharmacology in the intrathecal space, a multimodality study was designed. SPECT/CT was used for PK, while CFT was used to visualize ASO biodistribution and immunofluorescence was used to confirm cellular localization.

ASOs targeting the non-coding region of *Malat-1* were iodinated and administered intrathecally in rats. Rapid rostral distribution was observed along the rat neuroaxis, reaching the cranium at 15-minutes post dose (Figure 2A). At 4-hours post dose, ^{125}I signal accumulation was observed in the cranium alongside clearance as demonstrated by signal in the kidneys and cervical lymph nodes. While SPECT/CT provided a better understanding of the kinetics of ^{125}I-ASO distribution in rat CNS, and confirmed its localization to the cranium, it lacked sufficient resolution to determine whether the ASOs penetrated the brain parenchyma. In order to increase resolution and allow higher spatial determination in the brain, the same ASOs were conjugated to Cy7 to enable three-dimensional visualization using CFT. Building off the *in vivo* SPECT/CT data, rats were IT administered with Cy7-ASO and sacrificed at several timepoints. Upon sacrifice, brains were extracted, flash frozen and blocked in OCT. Using CFT, planar images were acquired across the entire brain and 3D reconstructed and visualized as a maximum intensity projection (MIP) (Figure 2B).

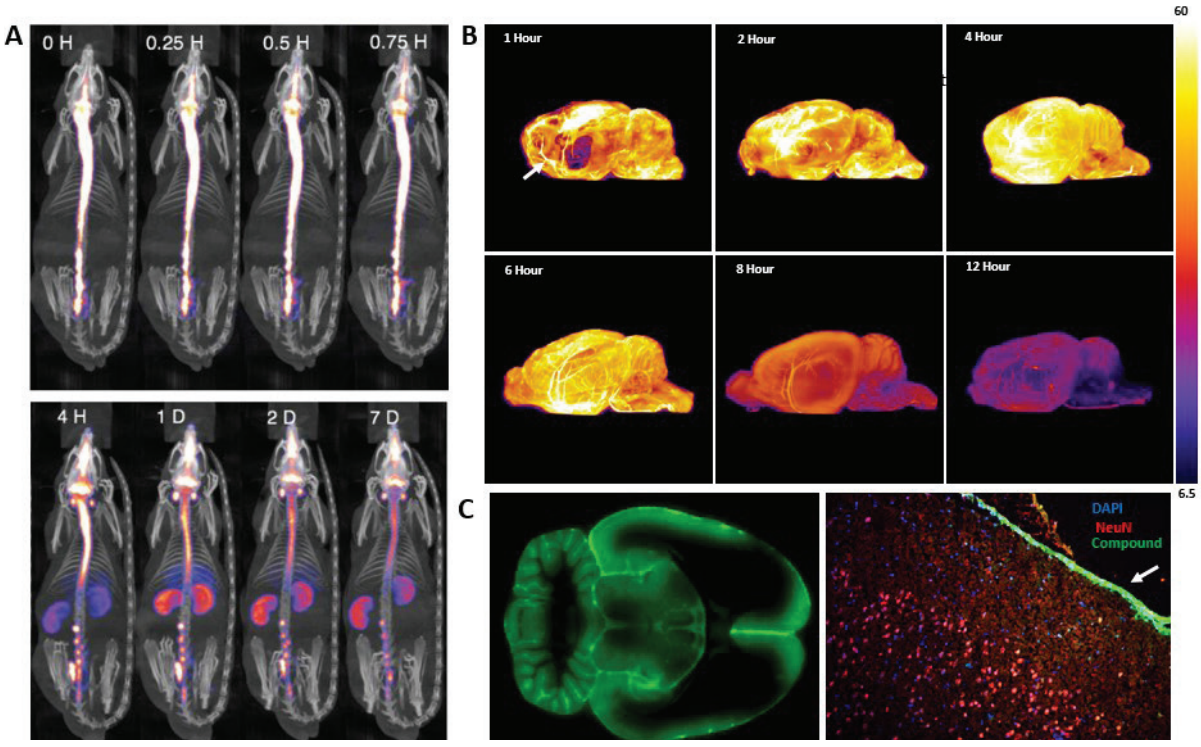

Figure 2. Pharmacokinetics and biodistribution of ASOs in the intrathecal space. (A) SPECT/CT scans of ^{125}I-AAV taken at several time points revealed rapid rostral distribution following lumbar IT administration, reaching the cranium as early as 15-minutes. At 4-hours post dose, clearance was observed in the kidneys and cervical lymph nodes. (B) 3D reconstructed images of Cy7-ASOs in rats brain following IT administration provided high-resolution visualization of ASO biodistribution. At early time points, most ASO signal was localized to the meninges and meningeal blood vessels (arrow). At 8-hours post dose, ASO signal was lower and more diffused indicating clearance and parenchymal penetrance. (C) Tissue sections collected off the face of the block (left) during CFT were stained for several cellular markers. Co-localization of Cy7-ASO with NeuN stains confirmed that ASO has reached their target site of neuronal nuclei (right).

The high-resolution, 3D images not only demonstrated strong signal up to 6-hours post-dose, they also provided sufficient resolution to localize the majority of the signal to the meninges as shown by Cy7-ASO signal in meningeal blood vessels (Figure 2B. white arrow). At the 6 and 8-hour timepoints, lower and more diffuse Cy7 signal was observed. The reduction in Cy7 signal is indicative to ASO clearance and is consistent with the observed ^{125}I-ASO clearance demonstrated as soon as 4-hours post IT administration. The diffused signal, however, suggested that the ASOs are not only cleared from the cerebrospinal fluid (CSF), but also penetrated into the brain parenchyma. To confirm and determine the cellular localization of ASOs in the brain, tissue sections were collected from the same brain samples on which CFT was conducted (Figure 2C). This is possible because CFT is a process in which the sample undergoes physical sectioning, allowing for section collection. Further, since the operator observes the signal from the face of the block in real-time as the sample is being imaged and before sectioned (Figure 2C), this allows for guided collection of sections (Figure 2C). Microscopy images of collected sections confirmed the direct detection of the IT administered ASOs. Once confirmed, the collected brain sections were stained for various markers, such as IBA-1, GFAP, NeuN, SMA and RecA-1 to better understand the cellular localization of ASOs in the brain. Although most of the administered ASOs remained localized to the meninges (Figure 2C, white arrow), consistent with CFT data, some ASOs did reach the brain parenchyma as demonstrated by the co-localization of Cy7-ASO and NeuN staining (Figure 2C).

APPLICATION EXAMPLE 2: ADENO-ASSOCIATED VIRUSES (AAV) BIODISTRIBUTION VERSUS EXPRESSION

AAVs have high tropism to mammalian cells but lack the ability to replicate in them. As a result, AAVs offer a safe and efficient way to deliver genes to target cells with factors, such as AAV serotype, gene promoters and administration route to control specificity and selectivity. Since the biodistribution of AAVs and their subsequent expression are not mutually inclusive, it is important to understand AAV distribution compared to AAV-mediated protein expression in order to fully determine the biodistribution of AAVs. In order to track and image both AAV capsid distribution and AAV-mediated protein expression, we designed a multimodality study in which radiolabeled AAVs provided PK data, while AAV-mediated iRFP expression provided AAV transduction data.[2] When conducted in the same animal, this approach allowed us to directly compare AAV capsid distribution versus AAV transduction.

To enable *in vivo* imaging using SPECT/CT, AAV capsids were directly iodinated with ^{125}I. Following iodination, the labeled AAVs were evaluated to determine functionality, and were shown to have a reduction in transfection, which resulted from the chemical

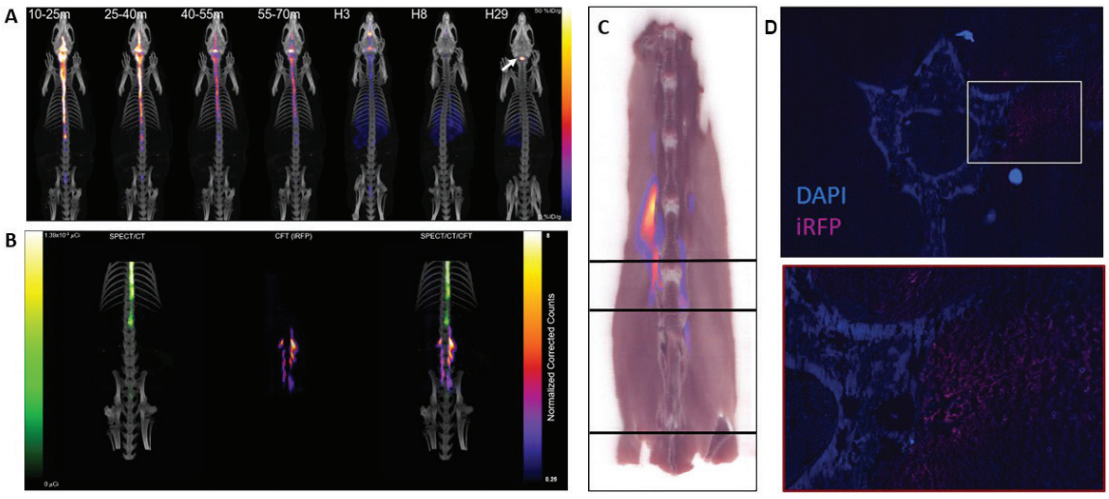

Figure 3. Comparison of AAV biodistribution versus AAV-mediated transduction. (A) SPECT/CT scans of ^{125}I-AAV conducted at several time points following IT administration demonstrated rapid rostral distribution within the first hour of administration. ^{125}I conjugation was stable for up to 8-hours as indicated by signal localization to the thyroid (arrow). (B) Four weeks following SPECT imaging, whole rat vertebrae was dissected and embedded in an OCT block for imaging with CFT. (C) MIP of iRFP (middle) was overlaid onto the SPECT/CT scan (left) to compare AAV capsid biodistribution to AAV-mediated transduction (right). Images indicated high discrepancy between AAV distribution and subsequent transduction. (D) CFT dataset suggested that iRFP expression occurred outside the vertebrae. Sections collected from the same sample were imaged on the tissue level. Results showed iRFP expression in muscle tissue outside the vertebrae.

reaction itself and not the conjugated iodine (not presented). Thus, a treatment regimen was designed to include a mixture of ^{125}I-labeled AAV capsids that were empty together with non-labeled, fully functional, same serotype AAVs that carried the *iRFP* gene. Rapid rostral AAV distribution was observed along the rat neuroaxis, reaching the cranium as soon as 10-minutes post-IT administration. At 8-hours, ^{125}I signal diminished and at 29-hours post-dose, ^{125}I signal was detected in the thyroid, which indicated that the iodination is no longer stable and dissociates from the AAV capsids. Following *in vivo* imaging, animals were housed for a few weeks to allow for AAV-mediated iRFP expression prior to CFT imaging. Upon takedown, the entire vertebrae of the rats were dissected, flash frozen and embedded in an OCT block. CFT 3D imaging was conducted to visualize iRFP expression across the entire vertebrae. Planar fluorescent images were reconstructed and visualized as a MIP, which was then co-registered with the SPECT/CT MIP to allow for a direct head-to-head comparison of the AAV vector in the SPECT dataset and AAV transduction in the CFT datasets. Importantly, since the vertebrae is a rigid structure, and although the SPECT and CFT datasets were acquired several weeks apart, they were co-registered to the same space in order to directly make the comparison. Unlike AAV capsid distribution, AAV-mediated iRFP expression was visible only in the lumbar vertebrae, with no iRFP signal detected in the rostral vertebrae or the brain. Furthermore, a closer evaluation of the spatial distribution of iRFP in the lumbar regions suggested AAV transduction has occurred outside the intrathecal space even though SPECT data indicated a successful IT administration. Because CFT is a sectioning-based method, several sections were collected off the face of the block during the image acquisition. Collected sections scanned at high resolution demonstrated clear expression outside the intrathecal space in muscle tissue surrounding the vertebrae. This unexpected result is attributed to leakage and backflow of AAVs around the IT administration site where the cannula was implanted. The results obtained in this multimodality study conducted on the same animal provided longitudinal, spatial and cellular information to enable direct comparison between AAV capsid distribution and subsequent transduction. Moreover, it demonstrated that the distribution of the AAV did not correlate to its subsequent AAV-mediated expression, confirming that the two are not mutually inclusive.

SUMMARY

Biomarker imaging using fluorescent or chemiluminescent proteins provides scientific value in determining PK, PD and biodistribution of gene therapies in the preclinical phase. However, when using the most common imaging modalities for gene therapy research, a tradeoff exists between sample volume and image resolution. *In vivo* imaging modalities offer the ability to image whole animals at low resolution and sensitivity as opposed to microscopy that provides high-resolution and sensitivity, but is limited to tissue sections. A multimodality imaging approach can be adopted by which the limitations of one imaging modality can be supplemented and overcome by use of another. When conducted on the same animal, this approach delivers increased layers of information and lower inter-animal variability. The ability to conduct multimodality studies requires: 1. access to a wide range of imaging modalities, 2. broad domain knowledge to properly design, execute and analyze studies that utilizes several modalities, 3. proper tracers, biomarkers or conjugates that can support the use of several imaging modalities, and 4. imaging modalities that complement each other. In this technical note, we demonstrated the applicability of multimodality studies with an emphasis on the utilization of CFT for gene therapy research. CFT is a novel high resolution and sensitive 3D imaging modality that detects fluorescent proteins in whole animals and organs. Together with *in vivo* imaging and microscopy, CFT can provide comprehensive understanding of gene therapy kinetics, biodistribution and cellular localization of the vector-carried gene.

References

1. Mazur C *et al* (2019). Brain pharmacology of intrathecal antisense oligonucleotides revealed through multimodal imaging. JCI Insight, 4(20).
2. Dimant H *et al* (2019). Multimodality Imaging Study to Determine AAV Biodistribution Compared to AAV Transduction in Whole Animals. GTND.

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