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Optimal Immunofluorescent Staining for Human Factor IX and Infiltrating T Cells following Gene Therapy for Hemophilia B

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Abstract

Immunofluorescent imaging is a valuable tool for investigating the outcome of gene therapy within the transduced tissue. With a multi-labeling technique, it is possible to both characterize local expression of the transgene and to evaluate the severity of the adaptive immune response through cytotoxic T cell infiltration. It is critical that the experimental parameters are optimal in order to prevent misinterpretation of important pathological events. To optimize this staining protocol, murine liver and skeletal muscle was transduced using recombinant adeno-associated virus encoding human factor IX. After testing several common cryo-preservative and fixative techniques, we found that optimal tissue integrity and antigen (factor IX and CD8) detection was achieved by freezing muscle tissue on liquid nitrogen-cooled isopentane (also called methylbutane or 2-methylbutane), followed by fixation with acetone at room temperature. The staining protocol described herein requires only about two hours, yet maintains exquisite specificity even at high magnification under confocal microscopy.

Keywords: AAV; Viral vectors; Gene therapy; FIX; T cell; Hemophilia; Immunofluorescent

Introduction

In clinical trials for the treatment of hemophilia B, the liver has become the preferred target tissue for gene therapy due to its tolerogenic microenvironment and ability to induce antigen-specific regulatory T cells following gene transfer [1,2]. For preclinical studies, injection of adeno-associated virus (AAV) into skeletal muscle enables researchers to investigate the immune response to vector administration in a non-tolerogenic target tissue; this is relevant for diseases which will not be corrected by hepatic gene transfer, such as muscular dystrophies and Pompe disease. When investigating the immune response following viral gene therapy, many studies focus on the inhibitory or neutralizing antibody response. From a technical perspective, the detection of vector and transgene-specific antibodies are easily measured in circulating blood by enzyme-linked immunosorbent assay (ELISA). However, this approach only examines one arm of the adaptive immune response and, by itself, does not provide a comprehensive assessment of the immunology of gene therapy. We have previously demonstrated that in peripheral tissues such as skeletal muscle, successful gene therapy treatment has been hampered by both the humoral and cellular immune responses [3,4]. Circulating antibodies against transgene can bind to the protein, rendering it functionally inactive and clearing it from circulation, or they may simply interfere with binding by the ELISA antibodies. On the surface, either scenario can lead to the conclusion that transgene is not being produced. In order to determine if the transduced cells have indeed been eliminated by a cytotoxic T lymphocyte (CTL) response, the tissue itself must be histologically evaluated for transgene expression and the presence of lymphocytic infiltrates.

Histological analyses of target tissue following gene therapy using hematoxylin and eosin (H&E) staining can disclose inflammation, but does not distinguish between a short-lived innate immune response and adaptive immunity. Immunohistochemistry (IHC) or immunofluorescence (IF) combines anatomical, immunological and biochemical techniques to identify discrete tissue components by the interaction of target antigens with specific antibodies or ligands tagged with a label. Colorimetric IHC (e.g. using HRP-conjugated antibodies) allows for examination of transgene expression in tissue with a high degree of sensitivity, but tends to lose information regarding the local immune response due to the poor resolution of co-localization by colorimetric labeling. IF labeling, on the other hand, makes it possible to visualize the distribution and co-localization of specific cellular components or proteins within cells and in the proper tissue context, but at the cost of sensitivity relative to IHC. While there are multiple approaches and permutations to either labeling methodology, the overall processes involved can be separated into two groups: sample preparation and labeling.

Herein, we describe an optimal protocol for the simultaneous immunofluorescent analysis of tissue sections for both transgene expression (hF.IX) and the CTL response ($CD8^+$ infiltration) following AAV-mediated gene transfer. Compared to traditional techniques, this procedure is rapid (~2 hours), yet maintains exquisite specificity even at high magnification under confocal microscopy. The data presented also compares and contrasts the effects of commonly used cryopreservation and fixation techniques and discuss their significance in the staining procedure on freshly isolated tissues. It is critical that the experimental parameters are optimal in order to prevent misinterpretation of important pathological events.

Materials and Methods

Viral vectors

Expression cassettes for AAV1-CMV-hF.IX, AAV2-CMV-hF.IX, and AAV2-ApoE/hAAT-hF.IX vectors were used [5,6]. The cytomegalovirus (CMV) immediate early enhancer/promoter was used

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for expression of hF.IX in muscle; whereas the human α1-antitrypsin promoter combined with the hepatocyte control region and enhancer of the apolipoprotein A were used for liver gene transfer. Both cassettes contain a 1.4-kb portion of intron I of the human F9 gene and SV40 or bovine growth hormone polyadenylation signals. AAV vectors were produced by triple transfection of human embryonic kidney-293 cells in roller bottles, and were purified from lysate by polyethylene glycol precipitation followed by cesium chloride density gradient centrifugation [5-7]. Vectors were filter-sterilized, and slot-blot hybridization and silver staining determined titers and purity.

Mice

All mice were purchased from The Jackson Laboratory or acquired in-house and were housed in a specific pathogen-free (SPF) facility. The University of Florida's Institutional Animal Care and Use Committee approved all procedures.

Mice were injected intramuscularly into the hind leg with 1×10^{11} vg AAV1-CMV-hF.IX. Briefly, mice were anesthetized with Isoflurane and a 1-cm longitudinal incision was made in the lower extremity. Vector was injected into the tibialis anterior (25 µl) and the quadriceps muscle (50 µl) of the same leg using a 33 ga Hamilton syringe. Incisions were closed with Vicryl suture. Some mice received vector via tail vein or splenic capsule injection of 10^{11} vg in order to transduce hepatic tissue, as previously described [8,9].

Tissue harvest, embedding, and sectioning

For immunofluorescent staining, animals were sacrificed at selected time points by CO_2 inhalation, and hind leg skeletal muscle (quadriceps and tibialis anterior) and/or liver were harvested. It should be noted that for the purpose of this study it was not necessary to perfuse the liver. However, each investigator should determine if the presence of peripheral blood in the liver would adversely affect their immunohistochemical analysis. Liver or muscles to be embedded were initially placed in phosphate buffered saline (PBS) on ice for transport.

Liver sections were cut into ~5 mm slices, blotted dry, and placed in a cryo-mold on a thin layer of Optimal Cutting Temperature (OCT) embedding media (Sakura), then covered completely with OCT. In order to demonstrate the significance of the freezing process, OCT-embedded samples were either snap-frozen by floating on liquid nitrogen, placed in a dry-ice/isopropanol slurry, or placed directly on dry-ice. Frozen blocks were further stored at -80°C until sectioned.

Excised muscle tissue was blotted dry on an absorbent cloth and mounted on a ~3 inch × 5/8 inch dowel using a small amount of 10% (w/v) gum tragacanth (Sigma) in PBS. A 15 mL conical tube was filled with 5 mL of 2-methylbutane (isopentane), and submerged into liquid nitrogen (LqN_2). The isopentane was cooled until it became viscous but before crystals began to form. The dowel was inverted and gently submerged in the LqN_2 cooled 2-methylbutane. The diameter of the dowel prevented the tissue from hitting the bottom of the tube (Figure 1). Samples were stored at -80°C in 2-methylbutane until sectioned. Some tissue samples were OCT-embedded and placed in a dry-ice/isopropanol slurry, or placed directly on dry-ice. Frozen blocks were further stored at -80°C until sectioned.

Frozen tissues were cut into ~10 µm sections using a Leica cryostat and mounted on poly-lysine coated slides. Sections were allowed to air-dry overnight at room temperature prior to staining.

Immunofluorescent staining protocol

1. Briefly thaw frozen tissue section to RT.
2. Fixation:
   a. Place in fresh Acetone 10min at RT, remove and air dry.
   b. Place in fresh Acetone 10min at -20°C, remove and air dry.
   c. Place in 4%PFA 10 min at RT, Wash 2 min in PBS x 2, place in MeOH 10 min at RT
   3. Wash all slides in PBS 2 min × 3 times.
4. Block with 5% Donkey serum/PBS (Jackson ImmunoResearch Laboratories, Inc.) for 15 min at RT in humidified chamber.
5. Drain off blocking solution, and replace with ~200 µL of 1° Ab diluted in 2-5% donkey serum/PBS, incubate 30 min at RT*.
   a. Goat α-FIX (1:400) (Affinity Biologicals, Ancaster, ON, Canada)
   b. Rat α-CD8a (1:100) (BD Bioscience clone:53-6.7)
   6. Wash in PBS 2 min x 3 times.
7. Add ~200 µL of 2° Ab diluted in PBS, incubate 30 min at RT* in a humidified chamber protected from light.
   a. Donkey α-Goat Alexa Fluor 568 (1:400) (Invitrogen, Carlsbad, CA)

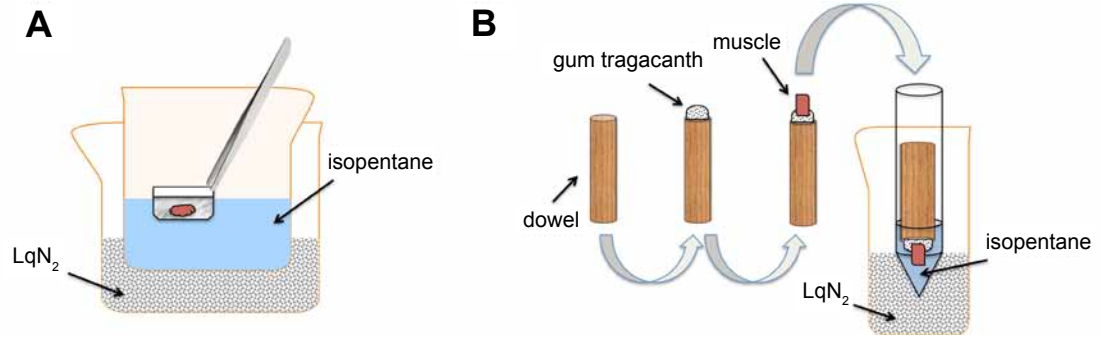


Figure 1: Schematic of freezing techniques utilized. **(A)** Tissue sections were embedded in OCT within a cryo-mold, which was then floated on LqN_2-cooled isopentane. The isopentane should touch the bottom and sides of the cryo-mold, but should not come directly in contact with the OCT, as this will result in the formation of bubbles within the block. **(B)** To optimally freeze skeletal muscle, a wooden dowel is topped with 10% (w/v) gum tragacanth in PBS. The extracted tissue is then partially embedded in this adhesive, with the part to be cut extending above it. The dowel is inverted and submerged in LqN_2-cooled 2-methylbutane to snap-freeze the tissue.

b. Donkey α-Rat Alexa Fluor 488 (1:400) (Invitrogen, Carlsbad, CA)

8. Wash in PBS 2 min x 3 times.

9. Rinse with dH_20.

10. Mount with ProFade Gold with DAPI (Invitrogen, Carlsbad, CA).

11. Store at RT for first 24 hrs, then store at -20 to 4°C.

*TIP: Perform incubations on top of low speed centrifuge to add vibrational motion

Images were captured using a Nikon Eclipse 80i fluorescence microscope and Retiga 2000R digital camera (QImaging, Surrey, BC, Canada) and analyzed with NIS-Elements BR software (Nikon Instruments Inc., Melville, N.Y.)

Results and Discussion

Effects of cryopreservation

Historically, frozen tissue sections have been the gold standard for immunofluorescence and immunohistochemical analysis [10]. Optimal antigen detection and identification is critically dependent on the quality of the tissue being probed. Ideally the freezing process preserves the available proteins in a near-native state for their identification by antibodies raised against naturally folded proteins. Additionally, using frozen tissue can help eliminate many of the problems associated with standard practices of chemical fixation and paraffin or resin embedding. The process of transforming water into ice, however, can dramatically alter the physical and chemical structure of cells and tissue [11].

The goal when freezing the tissue sample is to limit or control ice crystal formation. For example, slowly freezing the tissue on dry ice can result in the formation of large, hexagonal ice crystals that rupture cell membranes and results in a loss of intracellular water and subsequent shrinkage of the cell. On the other hand, when the sample is subjected to a high rate of cooling, smaller cubic crystals are formed and produce less distortion. If the rate of cooling is increased further as with LqN_2,

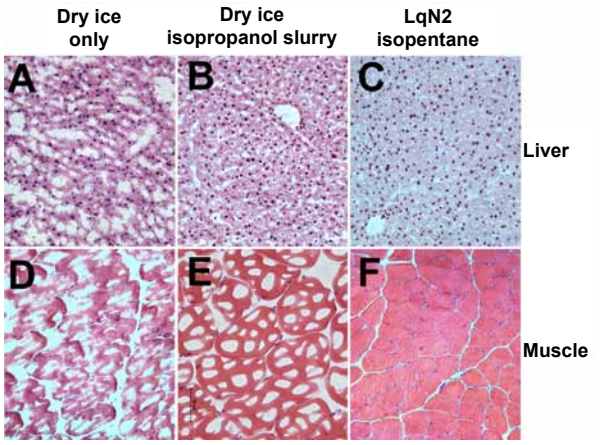


Figure 2: Morphology of skeletal muscle and liver following common freezing techniques.Liver and muscle (tibialis anterior or quadriceps) tissue from C57BL/6 mice was frozen as described and sectioned at 10 μm. **(A-C)** Hematoxylin and eosin staining of liver sections embedded in OCT and frozen with dry ice alone (A), a slurry of dry ice and isopropanol (B), or LqN_2-cooled isopentane (C). **(D-F)** Hematoxylin and eosin stains of skeletal muscle frozen with in OCT dry ice alone (D), a slurry of dry ice and isopropanol (E), or mounted on a wooden dowel and snap-frozen in LqN_2-cooled isopentane (F).

small volumes of water can be solidified without the formation of ice crystals at all [11,12]. This solid form of ice, known as vitreous ice, exists in a temperature dependent, irreversible phase transition with cubic ice, hexagonal ice and water [13]. Collectively, ice crystal formations within the cell/tissue usually results in poor tissue morphology and is referred to as ice crystal or freeze artifacts.

Three different protocols were performed to test and compare the effects of freezing on the tissue integrity and morphological structure of skeletal muscle and liver tissue following gene transfer (Figure 2). In the first procedure, macro-dissected tissue specimens were frozen in a cryo-mold containing OCT freezing media using solid carbon dioxide (dry ice) quick-freeze method (freezing temperature ~ -78°C). This procedure is one of the most common and routinely used in general medical pathology laboratories. Unfortunately, this technique yielded incredibly poor results. When placing the cryo-mold on a bed of dry ice there is minimal surface contact area (usually only one surface). As a consequence, the freezing rate is quite slow, which results in extreme tissue damage. Sections of either muscle or liver cut from these blocks were stained with hematoxylin and eosin to examine gross tissue integrity. Skeletal muscle showed overwhelming tissue damage (referred to as "Swiss cheese" appearance) with large holes appearing within the cells caused by the formation of large ice crystals during the slow freezing on dry ice (Figure 2D). The liver tissue was also visibly damaged (albeit to a lesser extent), showing moderate to severe cell shrinkage (Figure 2A).

The second method evaluated is a modification of the first, dry ice only technique. In this procedure the tissue pieces were processed and placed into a cryo-mold as before. However, to increase the freezing rate the samples were snap frozen by placing the cryo-mold into a dry ice/isopropanol slurry until solid (Figure 1A). The slurry in this situation provides a relative freezing temperature similar to dry ice alone (-78°C) [14]; however, the cryo-mold now has complete contact on five surfaces, which increases the freezing rate substantially. In spite of the increased rate, the muscle tissue continued to exhibit a significant degree of freeze artifact and unacceptable distortion of the tissue architecture due to ice crystal formation (Figure 2E). In contrast, this approach yielded moderately better results for the liver tissue (Figure 2B). Depending on the morphological detail needed, these cryo-sectionsmay be suitable for some immunohistochemical and pathological evaluations.

The third technique for freezing of excised skeletal muscle or liver tissue clearly produced the best results by preserving the cytoskeletal architecture and gross morphology (Figures 2C and 2F). As has been demonstrated, there is significant potential for the formation of damaging ice crystals in muscle samples. In order to eliminate this artifact it is critical to snap freeze the tissue as rapidly as possible. The established method of freezing tissues by direct immersion in LqN_2 is inappropriate for muscle because it creates a layer of nitrogen gas around the tissue, which delays the freezing rate and therefore subjects the specimen to ice crystal artifact [15,16]. In this procedure the muscle tissue samples are directly mounted onto a wooden dowel that is subsequently used in the cryostat in lieu of the mounting disk. To snap freeze the tissue the mounted muscle specimen is inverted and submerged into LqN_2-cooled isopentane (2-methylbutane) (Figure 1B). In contrast to direct immersion in liquid nitrogen, isopentane directly transmits the temperature to the specimen, resulting in a freezing temperature of -160°C [14]. Once the specimen is frozen it is essential that it is handled only with cooled instruments since gripping the specimen with room temperature instruments will cause partial thawing which again induces artifact.

Similarly, significantly enhanced morphology was also seen when thin slices (<5 mm) of liver tissue embedded in OCT were snap frozen by "floating" the cryo-mold on top of the LqN_2-cooled isopentane (Figure 2F).

Effects of fixation

Even before the tissue is excised, cell death and autolysis begins. The fundamental component of all histological and cytological techniques is the preservation, or fixation, of tissue and cells as they naturally occur. This can be accomplished by either physical or chemical methods.

As for physical methods, the act of freezing the tissue and air-drying cryo-sections are the most simplistic and commonly used techniques [17]. Freezing should be performed as promptly as possible after cessation of circulation to avoid morphological distortions and damage due to drying artifact, destruction of tissues or cells by the action of endogenous enzymes, or putrefaction by microorganisms.

Chemical fixation is intended to stabilize the proteins and preserve the tissue as close to its natural state as possible during the process of preparing the sample for histological examination. Common chemical methods of fixation include cross-linking agents such as formaldehyde, glutaraldehyde and succinimide esters. However, aldehyde fixation inhibits subsequent immunoreactivity by both excluding primary antibodies from their targets and chemically modifying the target epitope in a manner that reduces immunoreactivity [18]. Coagulate fixatives such as acetone and alcohols dehydrate proteins, making them insoluble and causing them to precipitate [17,19]. Both classes of chemical fixatives can prevent autolysis by inactivating lysosomal enzymes and maintain the fine structure, both inside and between cells, by making macromolecules resistant to dissolution by water and other liquids. Ideally, chemical fixation would preserve the natural cellular structure and chemical composition without the loss and/or migration of antigens [20]. Additionally, fixation will also stabilize the sample and protect it from the deleterious effects of the immunostaining process and helps to prevent diffusion of cellular components.

Unfortunately, fixation methods that best preserve morphology are usually the most disruptive to antigenic sites. Conversely, fixatives that exert minimal affect on antigenic epitopes are typically not ideal for preserving morphology. As a general rule, the loss of antigenicity increases with the fixative concentration and the time of fixation [20]. Nevertheless, the time needed to fix a 10 μm thick section is considerably less than that required for a large tissue block, regardless of the fixative used.

Unlike paraffin-embedded tissue, cryopreserved samples are not typically fixed prior to snap freezing. Therefore, it is usually necessary to perform this step prior to the staining procedure to ensure that antigen localization and cellular morphology is maintained. Unfortunately, the precise methodology to properly fix a tissue specimen can vary greatly and may need to be empirically determined. Optimization of the fixation process will result in better morphological preservation while at the same time preserving antigenicity and reducing background staining or auto-fluorescent signal. Additionally, the effect of fixation fluctuates by antigen and antibody clone; the same fixative technique is not appropriate for all targets [18,21]. In this study we examined several common methods to determine the optimal procedure for immuno-fluorescent staining of murine tissue sections for the expression of human factor IX (hF.IX), delivered by AAV gene transfer, and infiltrating $CD8^+$ T cells. The presence of infiltrating lymphocytes 2-4 weeks after vector delivery is indicative of an antigen-specific CTL response [4]. However, the procedure presented herein will detect $CD8^+$ infiltrates regardless of their TCR specificity. In other words, this technique will identify $CD8^+$ T cells directed against the transgene product and those against the viral capsid.

To ensure that any differences observed between slides is indeed a function of the tissue fixation process/technique and not a difference of cellular structures actually in the field of view, slides were carefully prepared as 10 μm serial sections. This enabled us to observe the same relative area of the tissue and evaluate the effects of the different fixation procedures independently of tissue pathology. In the first study, three commonly used chemical fixation methods were compared. The slides were treated as follows: 1) left unfixed, 2) fixed with acetone for 3 minutes at room temperature, 3) fixed with acetone for 3 minutes at -20°C, or 4) fixed with 4% PFA for 10 minutes followed by methanol for 10 minutes at room temperature. Following fixation, all slides were allowed to air dry for 5 minutes before washing them in PBS twice. Staining for $CD8^+$ T cells and vector induced expression of hF.IX was performed as detailed in the methods section.

Fixed exposure times

Based on visual observation, chemically unfixed tissue was used to establish baseline exposure times and identify a reference point for the level of hF.IX expression and $CD8^+$ T cell infiltration in the visualized area. Notably, the staining, especially of CD8, was somewhat diffuse and weakly fluorescent (Figure 3A). In contrast, fixation with acetone at room temperature resulted in a crisp, bright signal for both CD8 and hF.IX without any appreciable background in either of the red or green fluorescent channels (Figure 3B). Surprisingly, fixation of the tissue with acetone at -20°C had an extremely deleterious effect on staining (Figure 3C). Muscle cells that should be brightly expressing hF.IX were significantly muted and presented with a stippled or punctate pattern. Furthermore, considerable auto-fluorescent or nonspecific staining of membranes or areas presumed to be extracellular matrix were also observed in the red channel. Similar to Figure 3B, the $CD8^+$ cells

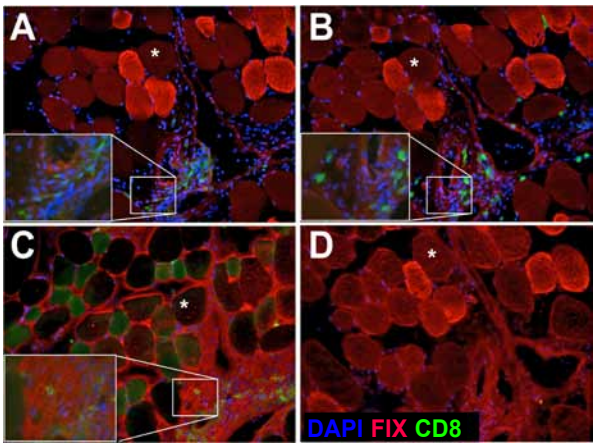
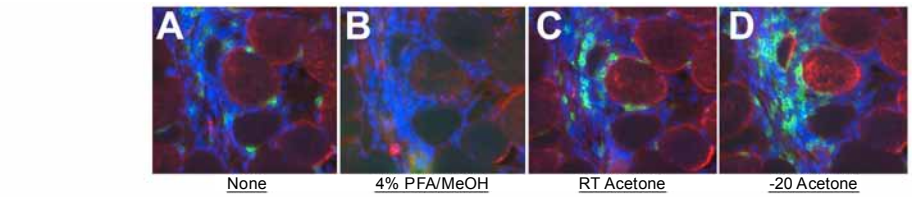
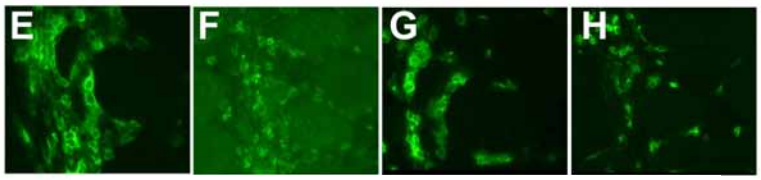


Figure 3: Effects of fixative techniques on immunofluorescent staining for hF.IX and CD8 with a fixed exposure time. Skeletal muscle from various C57BL/6 mice expressing hF.IXwere optimally cryopreserved in LqN_2-cooled isopentane, serial sectioned (10 μm) and subsequently fixed as indicated.(A) Unfixed tissue, (B) fixed with acetone for 3 minutes at room temperature, (C) fixed with acetone for 3 minutes at -20° C, or (D) fixed with 4% PFA for 10 minutes followed by methanol for 10 minutes at room temperature. Following fixation, sections were stained for hF.IX (red) and CD8 (green), and mounted with a media containing DAPI (blue). Exposure times for all slides were optimized using the unfixed slide. Asterisk (*) identifies the same cell in each serial section.



	None	4% PFA/MeOH	RT Acetone	-20 Acetone
Alexa488 (Green)	8.08	30.00	8.34	7.8
Alexa568 (Red)	0.81	2.69	1.07	1.09
DAPI (Blue)	1.07	1.63	1.46	0.84



	None	4% PFA/MeOH	RT Acetone	-20 Acetone
Alexa488 (Green)	1.09	14.74	1.21	1.02

Figure 4: Effects of fixative techniques on immunofluorescent staining for hF.IX and CD8 with variable exposure times. Serial sections (10 μm) of skeletal muscle from various C57BL/6 mice expressing hF.IX were optimally cryopreserved in LqN_2-cooled isopentane were fixed as indicated. (A,E) Unfixed (B,F) PFA/MeOH fixed, (C,G) acetone at room temperature fixed, or (D,H) acetone at -20° C. Tissues were stained for hF.IX (red), CD8 (green), and DAPI (blue). The software automatically optimized exposure time for each slide, and the times used are displayed below each image in seconds. (A-D) Images were taken for all 3 channels using a 10x objective. (E-H) Images were taken only for the green channel (CD8) using a 20x objective.

revealed in the green channel were brightly labeled with considerable specificity and demonstrated characteristic lymphocyte staining with circumferential surface labeling and large $DAPI^+$ nuclei. However there was also an appreciable level of green auto-fluorescence. Lastly, fixation with 4% PFA/methanol did not severely affect the specificity of hF.IX staining (Figure 3D). This fixation process did however increase the overall background signal as compared to no fixation or fixation with RT acetone. More importantly, $CD8^+$ staining in the PFA fixed muscle tissue was virtually undetectable at this magnification and exposure time.

Self-optimized exposure times

In the previous results, the wide field fluorescent exposure times were initially determined and set using the software's algorithm to calculate an auto-exposure time using the non-fixed tissue. Hence, it is possible that the short exposure time was responsible for the lack of $CD8^+$ detection in PFA/methanol fixed cryosections. Using 10 μm serial sections we once again compared the immunofluorescence between the fixation processes while visualizing an area of abundant $CD8^+$ infiltration. However, in this series of images, the exposure times were individually calculated and optimized by the software for each slide (resulting in significant variations in time between each sample) (Figure 4).

As expected, the auto-exposure times based on the fluorescent intensity from the non-fixed and acetone-fixed samples were all similar, only varying due to the original optical magnification (Figure 4, A-D 10x, E-H 20x). At a 10x magnification, CD8 fluorescence was not visible on the PFA-fixed tissue even with an exposure of 30 seconds. Surprisingly, the exposure time was increased nearly 14-fold relative to the other samples at 20x magnification before it was possible to detect a relatively weak fluorescent signal of CD8 following PFA treatment. This also resulted in a green haze of increased background over the image (Figure 4F).

The versatility and specificity of the staining protocol is not limited to muscle tissue. Using optimally cryo-preserved liver tissue, 10 μm liver sections were fixed in RT acetone and immunofluorescently labeled for hF.IX and $CD8^+$ T cells (Figure 5). Image acquisition using the software's auto-exposure function clearly reveals infiltrating $CD8^+$ T cells (green) and hF.IX expressing hepatocytes (red) within the liver parenchyma following liver directed gene therapy.

Confocal microscopy

Immunohistochemical staining has traditionally been performed with relatively long incubation times. It is common practice to incubate the tissue section with the primary antibody overnight at 4°C. Conceptually, it is thought is that performing the incubation at lower temperatures increases specificity but requires long times. Thus, there were concerns that the rapid protocol presented here (~2 hours from start to finish) may have compromised the accuracy of staining. To investigate this possibility, we examined skeletal muscle samples stained as before using confocal microscopy. Confocal microscopy permits greater magnification and finer detail than could be effectively achieved on a traditional fluorescent microscope. Using fluorescently labeled 10 μm cryo-sections of muscle tissue that had been fixed using RT acetone clearly demonstrates that the accuracy of staining is maintained despite the reduced incubation times (Figure 6). At high magnification it is easy to distinguish the surface staining "rings" of CD8 around large DAPI+ nuclei, a distribution consistent with T cell morphology (Figure 6B). The image depicts a positive hF.IX-expressing myocyte surrounded by numerous $CD8^+$ T cells, suggesting that it may be in the process of being eliminated by an antigen-specific CTL response.

Using confocal fluorescent microscopy in combination with differential interface contrast (DIC) microscopy further allowed us to address one of the common limitations of fluorescent microscopy: the inability to visualize cells not expressing the protein of interest. With a sufficiently transduced tissue, this is not a significant problem, as weak auto-fluorescence of membranes and surrounding fluorescent cells

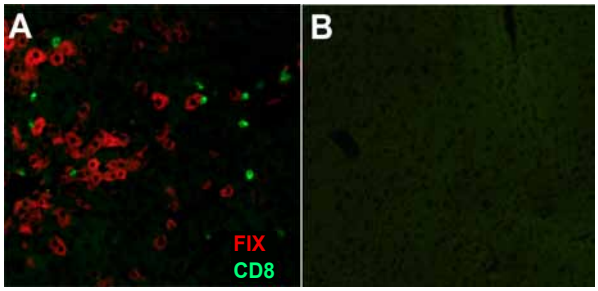


Figure 5: Immunofluorescent staining for hF.IX expressing hepatocytes and infiltrating CD8+ lymphocytes. (A) 10 μm section of liver expression hF.IX that was optimally cryopreserved and fixed with acetone at RT. Infiltrating $CD8^+$ T cells (green) and hF.IX expressing hepatocytes (red) are clearly visible within the liver parenchyma following liver directed gene therapy. (B) Negative control. Original magnification 200x.

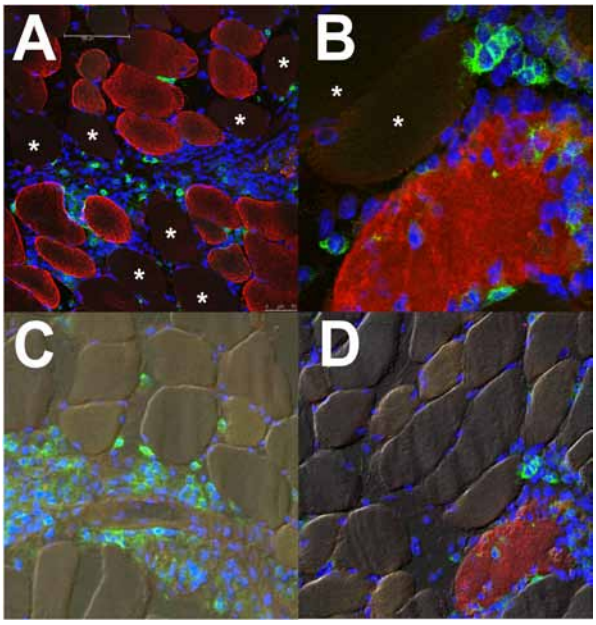


Figure 6: Accuracy of immunofluorescent staining validated via confocal microscopy. LqN_2/isopentane cryosections of skeletal muscle were fixed in acetone at room temperature and stained as before and visualized by scanning confocal microscopy. Images were acquired using sequential scans for hF.IX (red), CD8 (green), and DAPI (blue). (A) Low and (B) high magnification views showing both hF.IX expressing cells (red) and non-hF.IX expressing cells (*) with infiltrating CD8+ (green) lymphocytes. (C) Region of non-hF.IX expressing muscle tissue with DIC overlay inorder to visualize negatively stained cells. (D) Lower magnification of (B) with DIC overlay in order to visualized the non-expressing cells.

give a sufficient overview of the distribution of positive and negative cells. However, if a more accurate comparison of these populations is required, DIC imaging can effectively be overlaid or added to the fluorescent layers. This technique uses visible light to enhance the contrast in unstained or otherwise transparent samples and provides a visualization of depth in the tissue. When combined with fluorescent microscopy it is possible to visualize non-labeled (or in our samples, non-transduced) cells of the tissue (Figures 6C and 6D). The effect is clearly demonstrated in Figure 6D. Notice that the resident cells of the muscle can be easily observed due to the DIC layer, despite the lack of hF.IX staining in this image.

Conclusion

Following AAV-mediated hF.IX gene transfer, circulating antibodies against the transgene can bind to the de novo protein, rendering it functionally inactive and interfering with detection of systemic expression by ELISA antibodies. While transgene levels in the tissue can be evaluated by digesting the tissue and quantitating protein in the lysate by ELISA or western blot, this method does not assess the local CTL response. To completely characterize the outcome of gene therapy, it is valuable to evaluate the transduced tissue directly using immunofluorescent microscopy. In this study, we have compared several commonly used cryopreservation techniques and fixation protocols required to prepare muscle and liver tissue specimens for immunohistochemical labeling. Based on the results presented, using the modified mounting procedure in conjunction with cryo-preservation using LqN_2-cooled isopentane is far superior for maintaining tissue integrity and cellular morphology of muscle tissue. Similar high quality slides exhibiting a more homogeneous and representative cellular morphology were also produced when embedding thin samples (<5 mm thick) of liver tissue in commercially available media such as OCT and floating them on LqN_2/isopentane until frozen.

Considering that the principal objective when freezing the tissue sample is to limit or control ice crystal formation, a significant, but often neglected consideration when manipulating frozen slide sections is the occurrence of freeze-thaw-freeze cycles. When retrieving pre-cut slides from the freezer it is important that the slide box is allowed to equilibrate to room temperature before opening. More importantly, the slides that are being returned to the freezer should be allowed to air dry again. Repetitive opening/closing and refreezing the slide box will eventually lead to a deterioration of the tissue sections as a result of ice crystal formation.

Unfortunately, there is no universal fixative and therefore it is often necessary to try multiple methods. Fixation methods that best preserve morphology are usually the most disruptive to antigenic sites. Any given fixative may preserve the immunoreactivity of one epitope, but it may destroy other epitopes on the same antigen [22]. In this study we assessed several different fixation methods and reagents. Overall, acetone fixation at room temperature was best at preserving the antigenicity of target proteins and was superior for simultaneous detection of CD8 and hF.IX. Specifically, this method enhanced CD8 staining relative to unfixed tissue without having an adverse effect on hF.IX staining. In contrast, our data clearly demonstrates that PFA fixation is undesirable for visualization of $CD8^+$. The use of 4% PFA as a fixative had inconsistent and generally deleterious consequences on the detection of $CD8^+$ T cells. The effect was variable and ranged from a loss of signal, to high background (decrease in signal-to-noise ratio) and increased auto-fluorescence.

Successful gene therapy for hemophilia would result in long-term transgene expression in the absence of a destructive immune response. Immunofluorescent imaging is a valuable and often necessary tool for investigating and determining the outcome of treatment within the transduced tissue. While the freezing methodology presented here clearly produces superior quality results, the fixation and antibody labeling protocol outlined provides an excellent starting point for others investigating the immune response to vector administration. It is essential that each investigator empirically determine the optimal conditions for staining; it is not sufficient to simply use the same assay parameters from one experiment to another. The data presented in

this study demonstrates the need re-evaluate the standard protocol of formalin fixation of tissue cryo-sections. Correctly optimized immunohistochemical techniques can prevent misinterpretation of important pathological events.

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