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Immunolabelling Myofiber Degeneration in Muscle Biopsies

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Corresponding Author:	Maximilien Bencze INSERM U955 Créteil, FRANCE
Corresponding Author's Institution:	INSERM U955
Corresponding Author E-Mail:	m.bencze@ucl.ac.uk
Order of Authors:	Maximilien Bencze
	Baptiste Periou
	Yasmine Baba-Amer
	Francois J Authier
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Cover Letter

Maximilien Bencze, PhD
Research Associate
-Team 10 Biology of the Neuromuscular system
IMRB U955, Faculté de médecine
8 rue du Général Sarrail
94000 Créteil, France
Tel: +33 (0)1 49 81 39 44
-The Dubowitz Neuromuscular Centre
Institute of Child Health, UCL
30 Guilford Street
London WC1N 1EH
email: m.bencze@ucl.ac.uk

Dear Dr. Cao

We wish to submit a revised version of the manuscript JoVE59754 entitled *"Immunolabelling myofibre degeneration in muscle biopsies*". Editorial and reviewers' comments were carefully addressed.

A point-by-point response to the reviewers' comments is attached. We hope that our revised manuscript is now acceptable for publication by JOVE.

Thank you for considering our manuscript.

Yours sincerely,

Maximilien Bencze

TITLE:

Immunolabelling Myofiber Degeneration in Muscle Biopsies

AUTHORS AND AFFILIATIONS:

Maximilien Bencze[1,2], Baptiste Periou[1], Yasmine Baba-Amer[1], Francois J. Authier[1]

[1]Inserm, IMRB U955-E10, Créteil, France

[2]The Dubowitz Neuromuscular Centre, Molecular Neurosciences Section, Developmental Neurosciences Program, UCL Great Ormond Street Institute of Child Health, London, United Kingdom

Corresponding author:

Maximilien Bencze (m.bencze@ucl.ac.uk; maximilien.bencze@inserm.fr)

Email addresses of co-authors:

Baptiste Periou (baptiste.periou@aphp.fr)

Yasmine Baba-Amer (yasmine.baba-amer@inserm.fr)

Francois J. Authier (francois-jerome.authier@inserm.fr)

KEYWORDS:

muscle degeneration, myofibers, sarcolemma, cell death, muscular dystrophies, necrosis, myonecrosis, necroptosis, inflammation, immunoglobulin

SUMMARY:

Described here is a protocol for direct immunolabelling of necrotic myofibers in muscle cryosections. Necrotic cells are permeable to serum proteins, including immunoglobulin G (IgG). Revealing the uptake of IgG by myofibers allows the identification and quantification of myofibers that undergo necrosis regardless of muscle condition.

ABSTRACT:

The necrosis of muscle fibres (myonecrosis) plays a central role in the pathogenesis of several muscle conditions, including muscular dystrophies. Therapeutic options addressing the causes of muscular dystrophy pathogenesis are expected to alleviate muscle degeneration. Therefore, a method to assay and quantify the extent of cell death in muscle biopsies is needed. Conventional methods to observe myofiber degeneration in situ are either poorly quantitative or rely on the injection of vital dyes. In this article, an immunofluorescence protocol is described that stains necrotic myofibers by targeting immunoglobulin G (IgG) uptake by myofibers. The IgG uptake method is based on cell features characterizing the necrotic demise, including 1) the loss of plasma membrane integrity with the release of damage-associated molecular patterns and 2) the uptake of plasmatic proteins. In murine cross-sections, the co-immunolabelling of myofibers, extracellular matrix proteins, and mouse IgG allows clean and straightforward identification of myofibers with necrotic fate. This simple method is suitable for quantitative analysis and applicable to all species, including human samples, and does not require the injection of vital dye.

The staining of necrotic myofibers by IgG uptake can also be paired with other co-immunolabelling.

INTRODUCTION:

Striated skeletal muscle mainly consists of muscle fibres (myofibers), which are responsible for the characteristic voluntary contractile function. These cells are multinucleated, post-mitotic structures that support mechanical stress occurring during contraction. Structural stability of the myofiber membrane (sarcolemma) and its extracellular matrix are crucial for tissue homeostasis. Satellite cells comprise the main muscle progenitor population in mature skeletal muscle and exist in a quiescent state in healthy muscles. Following myofiber death, muscle regeneration is supported by satellite cells following a myogenic program that involves satellite cell activation, proliferation, differentiation, and fusion to ultimately form new multinucleated myofibers.

Myofiber demise can occur in multiple muscle conditions, including mechanical trauma, ischemia-reperfusion injuries, or muscular dystrophies, and it is associated with the necrotic morphology of dead cells[1,2]. Necrotic death is characterized by the rapid permeability of the plasma membrane and release of cell content in the extracellular compartment[3]. It can result from either an unregulated process involving no proper cell signalling (i.e., accidental necrosis), or an orchestrated intracellular pathway (i.e., regulated necrosis). In myofibers, both regulated[4] and unregulated[5] processes can lead to necrosis. A typical consequence of myonecrosis is the release of damage-associated molecule patterns, activating a powerful inflammatory response[6]. The presence of macrophages is observed at around 48 h and 72 h following injury[7]. Besides their role in the clearance of necrotic debris, they are also important in muscle regeneration[8,9].

Muscular dystrophies (MDs) are a heterogeneous group of pathologies which often result from a defect in the sarcolemma structure. Duchenne muscular dystrophy (DMD) is a juvenile X-linked disease affecting approximately 1 out of every 3,500 male births worldwide[10], and it is caused by the absence of dystrophin expression at the sarcolemma. Chronic degeneration of the muscle tissue in DMD boys leads to extreme muscle weakness and early mortality. Inflammation resulting from necrotic death enhances cytotoxicity, and promotes muscle fibrosis and the loss of muscle function[11,12]. Treatments currently in clinical trials targeting the roots of muscle degenerative disorders, such as gene therapy, are expected to alleviate myonecrosis. Simple techniques to accurately quantify muscle degeneration are therefore needed.

Several methods are routinely used to monitor myofiber loss in vivo. The measurement of the enzymatic activity of creatine kinase (CK) in the blood allows reliable quantification of ongoing necrosis in muscle and heart tissues. In situ, the haematoxylin and eosin (H&E) staining is the most popular method currently used in diagnosis to assess degeneration-regeneration remodelling. However, the molecular basis of the H&E labelling of dead cells remains unclear. Furthermore, color modifications suggesting myofiber death in H&E staining are relatively subtle and do not facilitate reliable and reproducible quantification. Methods revealing DNA fragmentation, such as the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL), imperfectly label necrotic death[3]. They are also poorly adapted to monitor the death of syncytial cells such as myofibers. The injection of vital dyes, such as Evan's blue dye (EBD),

represents a useful alternative for assessing myofibers that have lost the integrity of sarcolemma, but is not necessarily convenient in some experimental protocols. For instance, the presence of EBD in blood samples can affect results of CK measurements, a colorimetric assay. Furthermore, intracellular uptake of EBD makes co-immunolabelling challenging. Therefore, an alternative method allowing direct labelling of myofibers undergoing necrosis is of interest.

The mechanism of action of vital dyes relies on the loss of plasma membrane integrity in necrotic myofibers and the passive uptake of the injected dye. Similarly, necrotic myofibers uptake blood proteins such as albumin, for which EBD has a strong affinity[13,14], immunoglobulin G (IgG), and IgM[15]. The abnormal presence of blood proteins within myofibers therefore represents convenient markers for myonecrosis in situ. Staining these proteins can be an alternative for the use of vital dyes.

By using IgG uptake as a marker of myonecrosis in situ, this protocol is used to assess muscle degeneration in the tibialis anterior (TA) of mdx dystrophin-deficient mice. This method presents significant advantages over alternative techniques: 1) it is reproducible and simple in its execution; 2) it does not require any animal treatment prior to muscle collection, such as the injection of circulating vital dyes, and 3) as any conventional immunolabelling, it is compatible with co-labelling.

PROTOCOL:

Experiments were performed in accordance with the French and European Community legislation (license number 11-00010).

1. Tragacanth gum preparation

1.1. In a glass beaker, dissolve 6 g of tragacanth powder in 100 mL of deionized water. Cover with foil and leave for at least 3 h. Occasionally stir manually with a metal spatula as the mixture rapidly becomes viscous.

1.2. Leave the tragacanth mixture at 60 °C overnight in a water bath or in the oven. Carefully seal the beaker to avoid drying. Stir at least once before aliquoting.

1.3. Aliquot and store at 4 °C for up to 2 weeks.

2. Muscle collection

NOTE: For this experiment, a 4-week-old male mdx mouse was sacrificed by cervical dislocation. This procedure does not require anaesthesia and is a humane killing method in accordance with local legislation.

2.1. Dissection of the tibialis anterior (TA) muscle

2.1.1. After shaving the mouse leg, lay the mouse on its back and pin the feet on a corkboard

with needles. Using precision scissors (or a scalpel) and forceps, remove the leg skin from the foot up to the knee to expose the entire length of the tibia and TA.

2.1.2. With scissors, make an incision between the tibia and TA. This will facilitate separation of the TA from the bone and provide an easy grip to the epimysium. Using precision forceps, carefully remove the epimysium layer from the surface of the TA.

NOTE: From this step, the TA may be more susceptible to drying, which should be avoided.

2.1.3. At the ankle region, isolate the TA tendon from other tendons with forceps. Gently pull up the TA tendon to isolate it from the tibia and surrounding muscles.

2.1.4. When the TA belly is entirely separated, hold the tendon up so that only the proximal part of the TA muscle is attached to the knee. Gently sever the proximal tendon as close as possible to the knee bone.

2.1.5. Place the TA in a gauze that is lightly dampened with saline to avoid drying before freezing the muscle.

2.2. Pre-cool 70–100 mL of isopentane in a plastic or polytetrafluoroethylene beaker by partially dipping it into liquid nitrogen. Allow it to cool until the isopentane at the bottom of the beaker becomes a white solid.

2.3. On a circular piece of cork (20 mm x 11 mm x 8 mm), place ~0.5 mL of tragacanth gum. Carefully pre-label the other side of the cork so that the biopsy can be properly identified after the freezing step.

2.4. Embed the TA into the gum with forceps, distal tendon up. To allow appropriate cross sections of the muscle, hold the muscle with its axis perpendicular to the cork surface. The quality of the cryosections will improve if the biopsy is not completely embedded into the gum. Allow for at least half of the TA (tendon side) to be uncovered by the gum.

2.5. Quickly dip the cork with the embedded muscle into the unfrozen, cold isopentane layer. Note that the cork will float in the liquid isopentane. Hold the sample upside down as the muscle needs to be dipped into isopentane. Leave the samples in the isopentane for around 2 min to allow complete freezing.

NOTE: From this stage, the TA must remain frozen until cryosection. Store the muscle in dry ice before long term storage in a -80 °C freezer.

3. Cryosectioning

3.1. Set the cryostat temperature at -25 °C. Keep the object temperature at around -20 °C. Fix the object in the cryostat using optimal cutting temperature (OCT) compound. Trim the muscle until reaching the muscle belly then cut 7–10 µm sections.

NOTE: The stabilization of the object temperature requires at least 10 min.

3.2. Keep glass slides used for collecting cryosections at room temperature (RT). Collect at least two sections on each slide. The muscle sections will automatically stick and thaw at the contact of the warm glass slide. Remove the slide and keep it at RT.

3.3. Allow sections to dry at least 20 min at RT in a ventilated environment.

3.4. Store the cryosections on glass slides at -80 °C until use.

4. Immunolabelling

4.1. Thaw slides at RT for at least 15 min in a ventilated environment.

4.2. Delineate the sections area with a hydrophobic pen. Allow the hydrophobic barrier to dry.

4.3. Fix the tissue with 2% paraformaldehyde for 10 min in a humid chamber.

4.4. Wash the sections with phosphate buffered saline (PBS) 2x for 5 min each.

4.5. Block the muscle sections with 10% goat serum diluted in PBS for 1 h at RT in a humid chamber.

4.6. Incubate the sections for 2 h at RT (or overnight at 4 °C) in a humid chamber with the primary antibodies diluted in 5% goat serum. For simple IgG uptake labelling in myofibers, only incubate sections with rabbit antibody to mouse pan-laminin.

NOTE: Other antigens of the extracellular matrix can be targeted as long as they provide clear labelling of the surrounding extracellular matrix of the myofibers. Markers for muscle regeneration, inflammation, or other parameters can be combined as long as the antibodies are not raised in a mouse host. The microscope used for the analysis should include a supplementary fluorescence color.

4.7. Wash the sections with PBS 3x for 5 min each.

4.8. Incubate the sections with red fluorescent goat polyclonal secondary antibody to rabbit IgG H&L and green fluorescent goat polyclonal secondary antibody to mouse IgG H&L. Incubate at RT for 45 min in a humid chamber.

4.9. Wash with PBS 3x for 10 min each.

4.10. Mount the sections in fluorescent mounting medium containing 100 ng/mL 4',6-diamidino-2-phenylindole (DAPI) and cover each slide with a coverslip.

4.11. Allow drying overnight at 4 °C before imaging.

REPRESENTATIVE RESULTS:

Myofibers are surrounded by a laminin-containing extracellular matrix. Red staining delimits myofibers periphery and allows for their identification. IgG is shown in green. Nuclei are stained with DAPI and are found blue under the microscope. However, nuclei are shown in white here (**Figure 1**). A weak IgG immunoreactivity is expected in the extracellular compartment, which can be increased in case of inflammation. Green staining within the myofibers reflects the presence of IgG.

Mdx mouse TAs are characterized by a transitory phase of acute myonecrosis at 3 weeks of age, followed by asynchronous degeneration-regeneration events. As a consequence, TAs from 4-week-old mdx mice often display heterogeneous profiles including poorly affected areas, and degenerating and regenerating areas together in the same cross-section (**Figure 1a**). Unaffected/mildly affected muscle areas contain myofibers with large and homogenous size, and nuclei are found at a low density and are mainly located at the periphery or in between myofibers (**Figure 1b**). IgG-positive myofibers are generally absent in such healthy or mildly affected areas.

IgG-immunoreactivity within myofibers indicates myonecrosis (**Figure 1c**, lower part). Following cell degeneration, necrotic fibres are cleared out by phagocytes. Newly formed myofibers present small size at early stages, and then progressively enlarge as muscle progenitors fuse and contribute to the syncytial cell. During this process, myonuclei remain at the central position. Clusters of small, centrally nucleated myofibers indicate recently regenerating myofibers (**Figure 1c**, upper part).

FIGURE LEGENDS:

Figure 1: Representative image of IgG uptake staining in a cross-section from degenerating mdx muscle. (**a**) TA from a 4-week-old mdx mouse was cryosectioned and immunolabelled using antibodies to pan-laminin raised in rabbit (red) and mouse IgG (green). Nuclei were labelled using DAPI (white). (**b, c**) Enlarged areas of panel a. Scale bar = 500 µm.

DISCUSSION:

Myofiber necrosis is a common consequence of traumatic exercise in normal muscles. It is well-compensated by a powerful regenerative capacity of the local muscle progenitors. However, in several muscle conditions such as in MDs, the regenerative capacity of satellite cells is compromised by chronic myonecrosis and excessive fibrosis. Recent findings show that muscle fibres can die by necroptosis, a regulated form of necrosis. More specifically, the inhibition of necroptosis may become a new therapeutic strategy for DMD treatment[4]. Investigating cell death pathways in muscle degeneration disorders requires reliable methods of muscle cell death

quantification. This protocol describes the IgG uptake immunofluorescence technique that labels myofibers that underwent necrosis.

The loss of plasma membrane integrity that characterizes necrosis leads to the release of damage-associated molecules patterns and the uptake of plasmatic proteins such as albumin, IgG and IgM[15]. The mechanism of action of the IgG uptake labelling method is similar to that of vital dyes such as EBD. Necrotic myofibers become permeable and trap blood proteins of injected vital dyes, while living cells do not. Proof-of-concept of this technique has been validated by Kevin Campbell's group on MDs[15] but remains insufficiently disseminated.

Figure 1 provides typical results of IgG uptake labelling in dystrophic muscles affected by myonecrosis. With one immunolabelling including only three colors (IgG in green, extracellular matrix in red and nuclei in blue/white). Several important parameters of the muscle histology can be quantified, including the number and the size of myofibers and extent of myonecrosis, expressed either by the percentage of labelling in the cross-sectional area or percentage of necrotic myofibers. A fair estimation of the regeneration can also be determined with this staining. Indeed, the presence of centrally nucleated myofibers (**Figure 1c**, upper part) reflects local regeneration and thus past the degenerating event. For comparison, healthy muscle tissue is presented in **Figure 1b**. Of note, central nucleation in newly formed myofibers lasts for several weeks in the regenerated myofibers of adult mice. However, nuclear repositioning occurs significantly faster before the weaning age in mice[16].

This type of result may be easily enriched by adding another labelling revealed by another color. For instance, the nature and phenotype of the infiltrating cells may be further examined using appropriate markers. Antibodies directed against CD68 will preferentially label macrophage populations, regardless of their inflammatory status[4,9]. If needed, the inflammatory status of these cells can be further investigated[17].

As any conventional fluorescent staining, the quality of the muscle is crucial. Muscle biopsies and cryosections should be kept in dry ice at all times and for long-term storage at -80 °C until use. Storage at -20 °C should be avoided as it could affect the tissue preservation. Freeze-thawing cycles of samples should also be strictly avoided.

This technique has significant advantages over the most popular techniques, such as the EBD and H&E stains. It is simple and flexible and only requires conventional immunolabelling material and a fluorescent microscope. Flexibility is offered regarding the choice of fluorophore color associated with the IgG according to experimental needs, as well as regarding the performance of co-immunolabelling against further antigens. As a comparison, H&E stain and EBD can only be revealed in the same color and cannot be associated with other labelling to assess important parameters such as the myofiber location or the extent and nature of inflammatory cell infiltrate. The EBD method requires dye injection of animals around 24 h before harvesting the muscles, while the IgG uptake method can be performed in any samples, including humans. The whole musculature of EBD-injected animals contains the dye that can possibly affect further analysis such as fluorescent immunolabelling of muscles or the blood CK colorimetric assay.

Determining the precise quantification of myonecrosis preferentially implies a reliable marker of cell type. Here, the nature of the cells can be assessed together with necrotic fate by co-labelling IgG with the extracellular compartment surrounding myofibers. In **Figure 1**, the myofibers were identified using antibodies directed against proteins of the surrounding extracellular matrix such as laminin. Other components such as collagen can also be stained. However, antibodies against proteins belonging to the sarcolemma should be avoided. In our experience, their immunoreactivity promptly vanishes following necrosis of the fibre.

The immunolabelling of IgG uptake within myofibers is a simple and reliable method to specifically stain necrotic muscle fibres. It can be easily and routinely performed and is applicable to samples amenable to classic immunofluorescence staining. Considering its specificity and general lack of counterindications, it is recommended to use as a gold standard for myonecrosis assessment, regardless of the nature of the necrotic injury.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure

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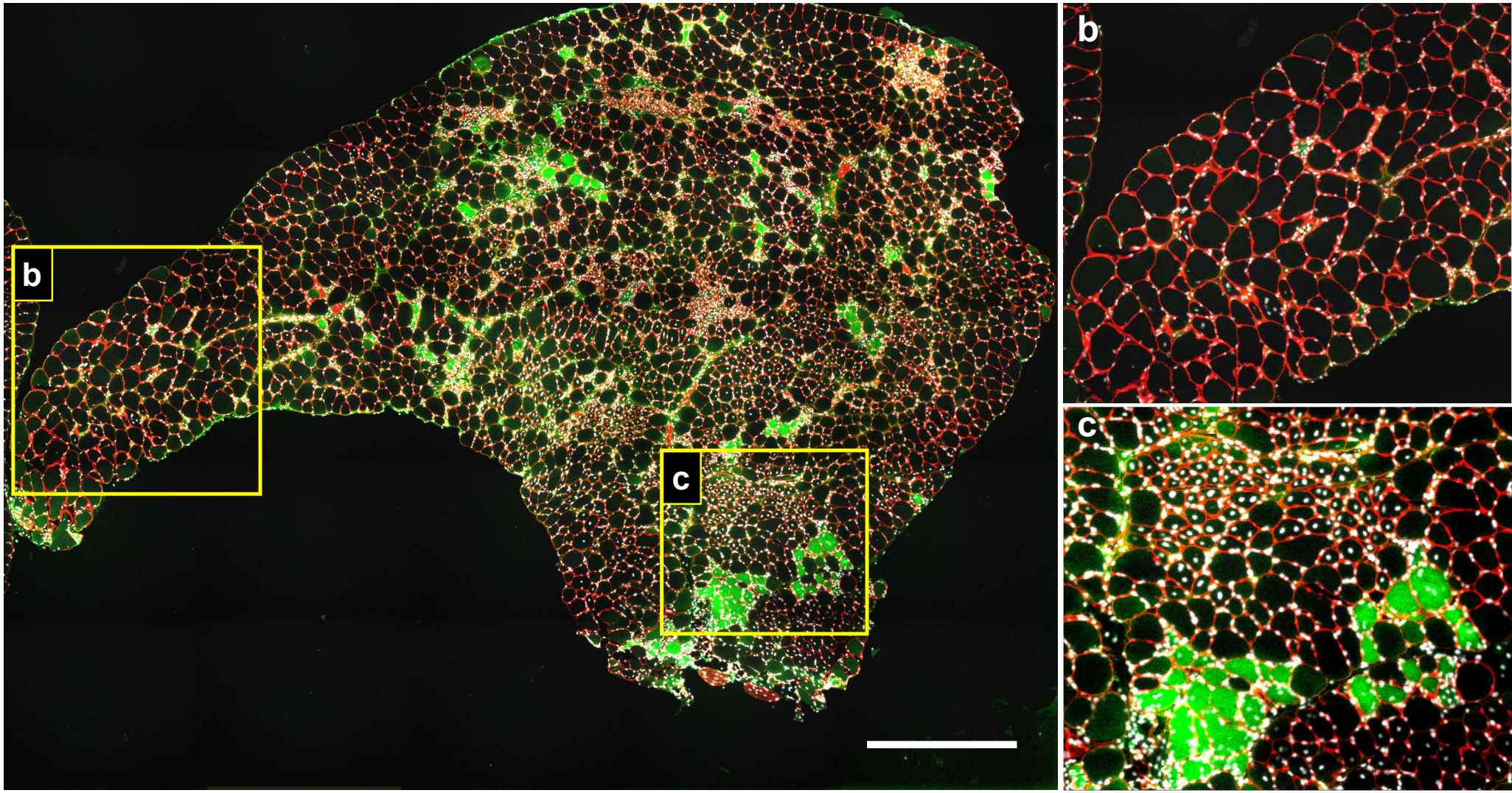

Table of Materials

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Name of Material/Equipment	Company	Catalog Number	Comments/Description
Circular cork disks	Pyramid Innovation	R30001-E	Don't forget to clearly label the cork so that the the ID of the sample can be determined after freezing
Cryostat	Leica	CM3050 sn34	Muscle cryosectionning should be performed between -20 and -25°C. Thickness:7-10 micrometers.
Dakopen	Dako	S2002	A hydrophobic barrier around the muscle sections. It prevents the dispertion of medium during incubation
Forceps	FST	91117-10	/
Goat anti-Mouse IgG (H+L) antibody, Alexa Fluor Plus 488	ThermoFischer Scientific	A-11029	Dilution: 1/500
Goat anti-Rabbit IgG (H+L) antibody Alexa Fluor Plus 594	ThermoFischer Scientific	A32740	Dilution: 1/500
Goat serum	Jackson ImmunoResearch	005-000-121	At the blocking step, use 10% dilution in PBS. For antibodies incubation, use 5% dilution in PBS
Isopentane	Sigma Aldrich	78-78-4	Freezing medium. Should be cooled down in a beaker placed in liquid nitrogen.
mdx mouse	Jackson Laboratory	C57BL/10ScSn-Dmdmdx/J	Mdx mice are mutated for the dystrophin gene. From three weeks of age, muscles are characterized by chronic degeneration
Microscope	Zeiss	Imager.D1	/
OCT	Cellpath	KMA-0100-00A	Embedding matrix
PFA (Paraformaldehyde)	ThermoFischer Scientific	28908	Used for fixing cryosection (2% or 4% PFA can be used)
PBS	Eurobio	CS3PBS00-01	Dilution medium for immunolabeling
Precision scisors	FST	fst 14001-12 and fst 14001-14	Used for the muscle collection
Rabbit antibody to mouse pan-Laminin	Sigma Aldrich	L9393	Dilution: 1/1000
Tragacanth	Sigma Aldrich	G1128	Aliquots to keep at +4°C

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Author(s): Maximilien Bencze, Baptiste Periou, Yasmine Baba-Amer, Francois J Authier

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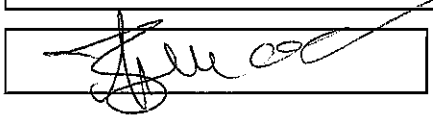
CORRESPONDING AUTHOR

Name:	Maximilien Bencze
Department:	Institut Mondor de Recherche Biomédicale U955
Institution:	Inserm
Title:	PhD
Signature:	
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Editorial comments:

More specifically, we focused on the following editorial requirements:

- Language was improved to reach publication grade.
- Summary was shortened to no more than 50 words.
- The advantages of the presented method are further developed.
- All abbreviations are defined before use, spacing is edited, and an ethics statement is included accordingly.
- More details are provided to the protocol steps, including mouse age, muscle collection, freezing steps, and the nature of surgical tools.
- Discussion was significantly rearranged so that the critical steps within the protocol are highlighted: the significance of the technique, its advantages over the alternative methods, and the extent of application.
- Journal titles are not abbreviated in the References.
- The Table of Materials is updated and items are sorted in alphabetical order.

Reviewers' comments:

We thank the reviewers and the editor for their careful evaluation of the manuscript.
The language in the manuscript is not publication grade. Please employ professional copy-editing services.
The language is improved in the edited version.

Reviewer 1
Major Concerns:
The authors should establish the utility of this technique for labeling of different stages of fiber degeneration/regeneration by inducing acute muscle injury by cardiotoxin injection and then sampling the muscle for staining at different time points after the injury. (Currently, the authors do include some discussion on this topic but provide no data on which this discussion is based.)

We thank the reviewer for their suggestion. However, the relevance of this technique has already been demonstrated by Kevin Campbell's group (Straub et al.1997). Its utility has been confirmed by several independent groups, including ours[1-6].

We indeed made the choice to include in the discussion some relevant precisions on the typical histological features of regenerative muscles. Not all readers are necessarily familiar with the histology of the skeletal muscle tissue in health and disease. We believe that these "reminders" are useful to fully appreciate the phenotype of the muscle cross sections which have been labelled with our technique. As so, we think this part is most appropriate in the discussion.

We agree that the description of the kinetics of IgG uptake in myofibres following an acute injury would be of interest. However, the purpose of this manuscript is to describe a reliable method to assess myofibre demise, not the downstream events. The regenerative events following an experimental injury are already extensively investigated and described in the literature [7-9], while cell death processes are largely overlooked and therefore remain poorly understood. This manuscript aims to present a simple and specific tool for assessing myofibre cell death, and regenerative events are only taken as indirect indications of past myofibre death event.
Therefore, we do not believe that such a characterization is crucial in this manuscript.

Minor Concerns:

The manuscript emphasis on muscular dystrophies is too narrow, as necrotic fibers are encountered in many myopathies and this technique should be applicable to all of them.

We fully agree that this technique applies to a broad range of muscular disorders.

Please note that this article is entitled "Immunolabelling myofibre degeneration in muscle biopsies » and the technique is not restricted to muscular dystrophies. Furthermore, the abstract begins with the following statement (Line 50-51): "… myonecrosis has a central role in the pathogenesis of muscle conditions, including in muscular dystrophies".

A decision was made to apply this method in this manuscript to a broadly used model for muscle degeneration. Duchenne muscular dystrophy is the most investigated and the best known muscle pathology involving chronic myonecrosis. We recently provided the first demonstration of the involvement of a regulated form of cell death in the muscle tissue, using this model.

To fully address the reviewer's concern, we clarified this point in the introduction with the following statements:

> *Line 69: "Myofibre demise can occur in multiple muscle conditions, including mechanical trauma, ischemia-reperfusion injuries or muscular dystrophies, and is associated with the necrotic morphology of dead cells".*
>
> *Line 262: "Myofibre necrosis is a common consequence of traumatic exercise in normal muscles. It is well compensated by a powerful regenerative capacity of the local muscle progenitors. However, in several muscle conditions such as in MDs…"*
>
> *Line 321: "Considering its specificity and its general lack of counter indications, we recommend its use as a gold standard for myonecrosis assessment, regardless of the nature of the necrotic injury."*

How does this technique compare with immunostaining for the membrane attack complex C5b-9, which is routinely used to label degenerating/regenerating fibers in tissue sections?

We wish to emphasize the fact that degeneration and regeneration are two distinct processes. A better understanding of the degeneration mechanism(s) in neuromuscular disorders requires a strict separation of these concepts and the use of distinct and specific tools. Membrane attack complex immunoreactivity can indeed be found on both regenerating and degenerating myofibres[10]. Strong C5b-9 deposition is observed on living muscle fibres in pathological conditions[11,12].

We do not necessarily consider that C5b-9 labelling is a reliable method to accurately identify muscle degeneration. It is clearly more associated with some disorders than others[13], and therefore represents a poor marker for muscle necrosis in some muscle conditions.

Together, this indicates that complement deposition lacks specificity for cell death. In contrast, blood protein uptake by myofibres specifically indicates necrotic myofibres.

Proofreading of manuscript by a native or near-native English speaker is recommended.

The revised version of the article was edited accordingly.

Reviewer #2:

This is a very nice study

We thank the reviewer for his/her very positive evaluation of the manuscript.

I wonder why you chose the tibialis anterior which is not much affected in the MDX mouse and why not also use in the diaphragm to compare.

We fully agree with the comment of the reviewer 2: the diaphragm muscle is much more affected than the hindlimb muscles in dystrophin-deficient mice.
We chose tibialis anterior (TA) muscles over diaphragm for two main reasons:

1. Experimentally, the harvesting and the analysis of TAs are much easier than the diaphragm: TA muscle is an external muscle, with easy access for collection. Its shape is longitudinal and is easier to mount and freeze, whereas the diaphragm is a very thin muscle with an internal location. Diaphragm collection and mounting requires very specific skills. For newcomers to the field of muscle biology, we wanted this part as simple as possible for the manuscript.
2. We aimed to use a muscle for which we would be absolutely sure that ongoing myonecrosis can be observed in young mice. To our knowledge, the kinetics and the extent of myonecrosis is better described in TAs than in the diaphragm. Furthermore, the onset of myonecrosis is very early in the TA compared to the diaphragm. In our hands, one month-old mdx mice offered the advantage of significant cell death, inflammation and recent regenerative events, so that all features associated with myonecrosis and its consequences can be observed within the same cryosection.

There are several things that are not clear, for example, statements that need to be corrected. When it says in the introduction, last paragraph, "however the injection of vital dyes is counter indicated in some experimental procedures such as creatinine kinase", it is not clear and needs to be explained more clearly.
We thank the reviewer 2 for giving us the opportunity to clarify this point.
In one of our previous studies, we found that the reading of blood samples for CK activity was perturbed by the presence of Evan's blue dye in the mouse bloodstream. This observation was made on dysferlin-deficient mice. For this reason, blood CK measurement (reflecting general myofibre membrane permeability) and Evan's blue labelling were each performed at different timepoints in distinct animals. This limitation doesn't exist using the IgG uptake assay and this advantage is now more clearly specified in the edited version of the manuscript.

Line 98: *"The injection of vital dyes, such as Evan's blue dye (EBD), represents a useful alternative for assessing myofibres that have lost the integrity of sarcolemma, but is not necessarily convenient in some experimental protocols. For instance, the presence of EBD in blood samples can affect the results of CK measurement, a colorimetric assay."*

We also included in the discussion a larger part dealing with the advantages of the IgG uptake technique over alternative methods such as the Evan's Blue dye injections.
Line 309: "*The whole musculature of EBD injected animals contains the dye that can possibly affect further analysis such as fluorescent immunolabelling of muscles or the blood CK colorimetric assay."*

I wonder if the description of the freezing is necessary, as this is a very standard method.
We understand this comment. However, editorial requests were made to develop this part.

The English needs to be improved.
The manuscript has been edited accordingly.

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