# *Detailed methodology for dystrophin quantification*

**FDA-NIH Dystrophin Methodology Workshop**
FDA White Oak Campus
**20th of March 2015**

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***<u>Disclosure of Conflict of Interest</u>***

***I am working on the SKIP-NMD project funded by the EU within the FP-7 framework.***
***Sarepta Therapeutics is one of the participants and co-sponsor.***

SEVENTH FRAMEWORK PROGRAMME

SKIP-NMD

SAREPTA THERAPEUTICS

# **Muscle samples preparation:** fibers orientation and freezing procedure

To get appropriate specimens for performing IHC samples MUST **be handled** and **frozen down** properly

We follow a strict **Standard Operating Procedure** (SOP) and all the people involved have been **trained** properly → successful method

1) SKIP-NMD muscles coming from other centres

2) < 1% of muscle specimens present freezing artefacts

**Stereomicroscope**
(Leica MZ75 with a separate light source Leica CLS 100X)

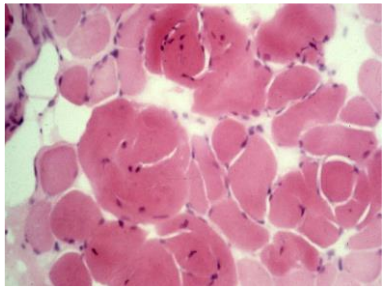
**Frozen isopentane 30''in LN2**

**OCT Compound**
(AGR1180 Agar Scientific)

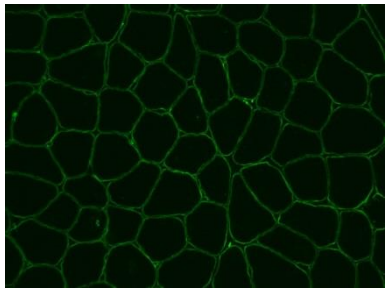
**7 µm sections**
(Leica Cryostat CM1850UV)

Quality control check: H&E and spectrin staining

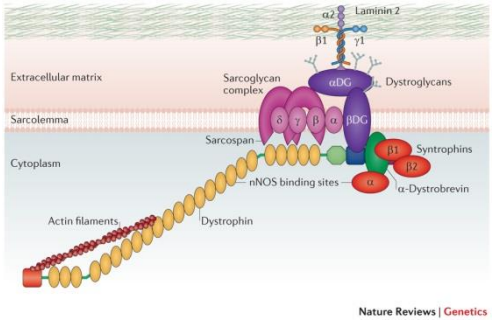
H&E



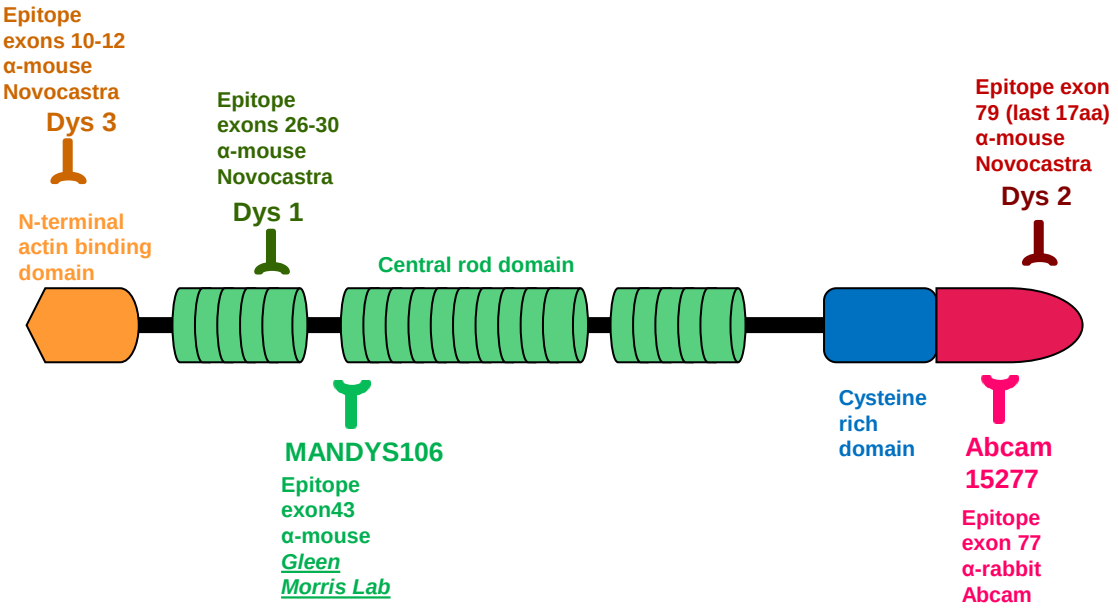
Spectrin (NCL-SPEC1, Novocastra, monoclonal, anti mouse)



# Dystrophin antibodies




Fairclough et al., 2013 Nature Reviews Genetics 14, 373–378




Over the years, selection and validation of antibodies taking account that each antibody:

i. recognizes different protein epitope (validated in patients deleted for the specific epitope)

ii. has its own epitope affinity

iii. gives different intensity values

# Dystrophin Quantification

List of issues to be addressed in order to estimate if dystrophin quantification is **meaningful**

1) Can it be measured reliably?

2) Is it relevant to quantify the different numbers of positive dystrophin fibers?

**In order to answer these questions we have to step back to FUNDAMENTAL BIOLOGY and DMD animal models**

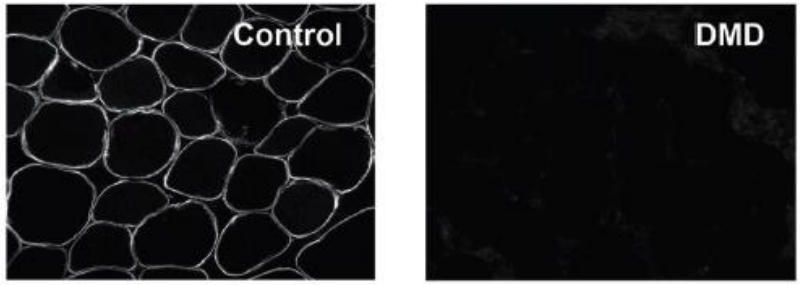
# Can it be measured reliably?

1

*Neuropathology and Applied Neurobiology* (2010), **36**, 265–274

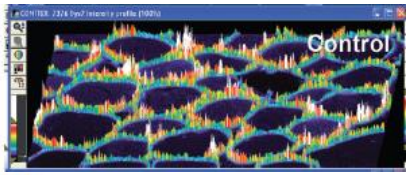
**Immunohistological intensity measurements as a tool to assess sarcolemma-associated protein expression**

V. Arechavala-Gomeza*, M. Kinali*, L. Feng*, S. C. Brownt, C. Sewryt‡, J. E. Morgan* and F. Muntoni*

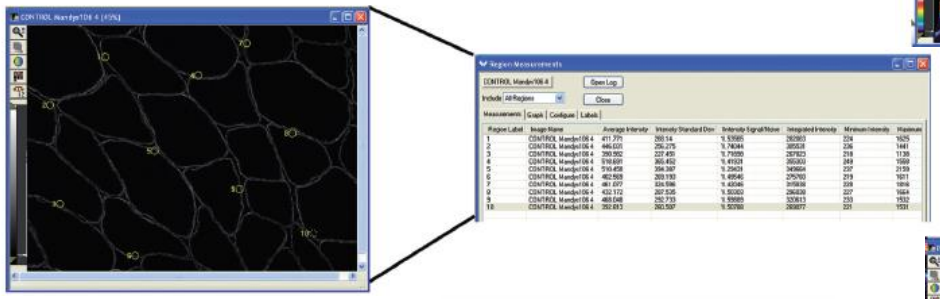



**Acquistion**

Leica DMR Fluorescence microscope
Serial sections:
1 section single labelling Dys2
1 section single labelling P7
1 section single labelling spectrin
Each section → 4 pictures

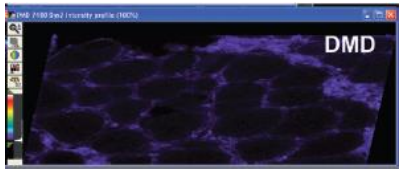



A



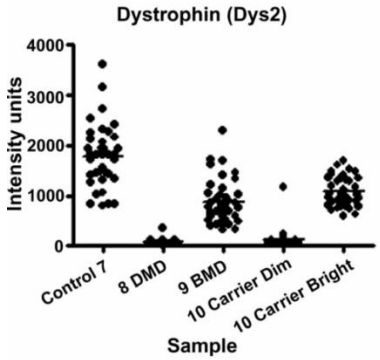
**Software**

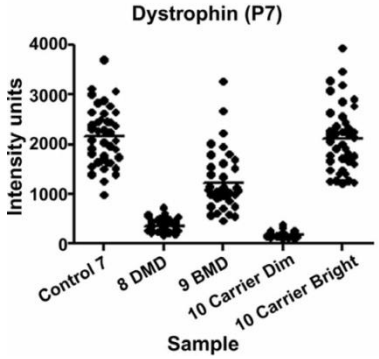
Metamorph (Molecular Devices)
40 different Region of Interest for each pic
Min = cytoplasm
Max = sarcolemma membrane




**Analysis**

Normalizing factor= average (Max-min) Spectrin EACH SAMPLE/ average (Max-min) Spectrin ALL CONTROLS



# Can it be measured reliably?

2

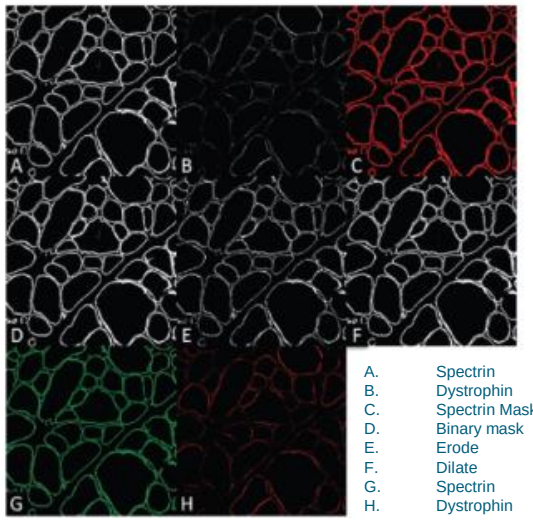
Taylor *et al*., 2012

*Neuropathology and Applied Neurobiology* (2012), **38**, 591–601

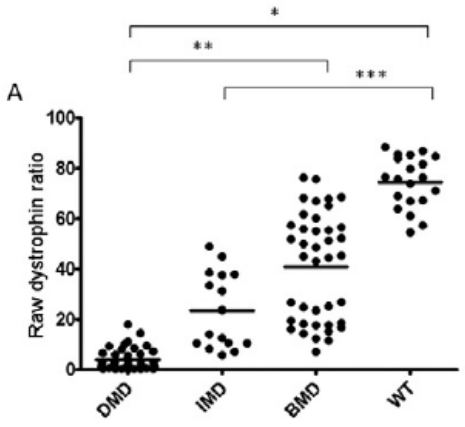
## Quantification of dystrophin immunofluorescence in dystrophinopathy muscle specimens

L. E. Taylor*, Y. J. Kaminoh*, C. K. Rodesch‡ and K. M. Flanigan*†

*Center for Gene Therapy, Nationwide Children's Hospital, †Departments of Pediatrics and Neurology, Ohio State University, Columbus, OH, and ‡The University of Utah Imaging Core Facility, Salt Lake City, UT, USA




A. Spectrin
B. Dystrophin
C. Spectrin Mask
D. Binary mask
E. Erode
F. Dilate
G. Spectrin
H. Dystrophin




3

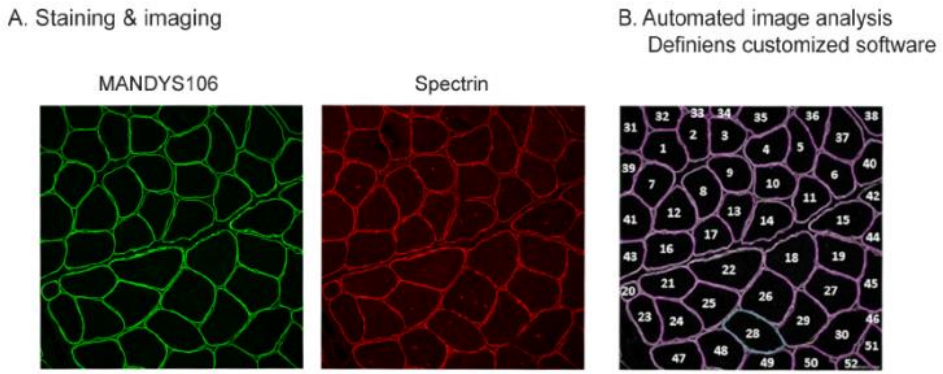
Beekman *et al*., 2014

PLOS ONE

## A Sensitive, Reproducible and Objective Immunofluorescence Analysis Method of Dystrophin in Individual Fibers in Samples from Patients with Duchenne Muscular Dystrophy

Chantal Beekman, Jessica A. Sipkens, Janwillem Testerink, Stavros Giannakopoulos, Dyonne Kreuger, Judith C. van Deutekom, Giles V. Campion, Sjef J. de Kimpe*, Afrodite Lourbakos

Prosensa Therapeutics BV, Leiden, the Netherlands



# International Benchmarking of methods for dystrophin quantification

## Institution of the Biochemical Outcome Measures Study Group (BOM-SG)

The BOM-SG was formed with a goal to provide data-driven, international standard dystrophin quantification for DMD clinical trials

Neurology® 2014;83:1-8

**Dystrophin quantification**

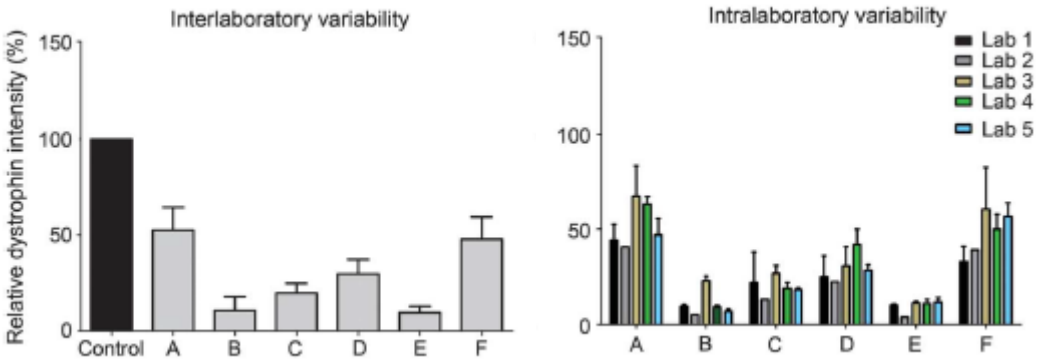
**Biological and translational research implications**

Karen Anthony, PhD*, Virginia Arechavala-Gomeza, PhD*, Laura E. Taylor, BS, Adeline Vulin, PhD, Yuuki Kaminoh, BS, Silvia Torelli, PhD, Lucy Feng, PhD, Narinder Janghra, BSc, Gisèle Bonne, PhD, Maud Beuvin, MS, Rita Barresi, PhD, Matt Henderson, MSc, Steven Laval, PhD, Afrodite Lourbakos, PhD, Giles Campion, MD, Volker Straub, MD, Thomas Voit, MD, Caroline A. Sewry, PhD, Jennifer E. Morgan, PhD, Kevin M. Flanigan, MD‡ and Francesco Muntoni, MD‡

| BOM Partners | Arechavala method | Taylor method | Beekman method |
|---|---|---|---|
| Muntoni Lab, London, UK | ✓ | ✓ | |
| Flanigan Lab, Columbus, USA | ✓ | ✓ | |
| Straub Lab, Newcastle, UK | ✓ | | |
| Voit Lab, Paris, France | ✓ | | |
| Prosensa Therapeutics, Leiden, | ✓ | ✓ | ✓ |

### Immunohistochemistry technique

***Results from different labs using Arechavala method***

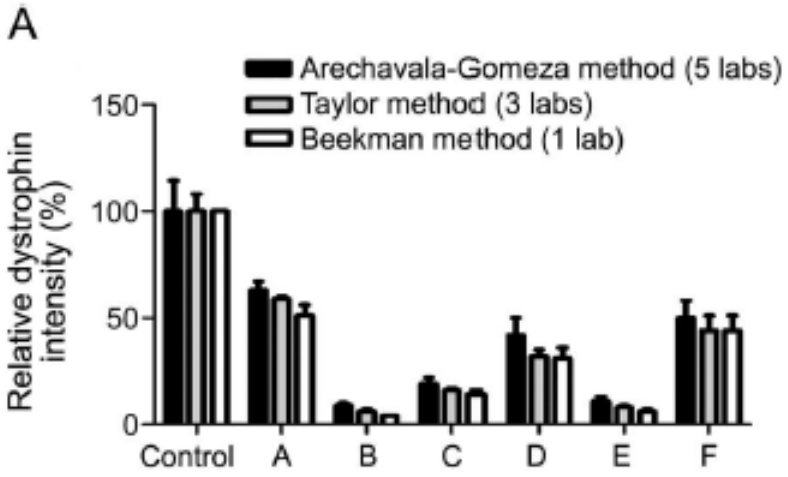


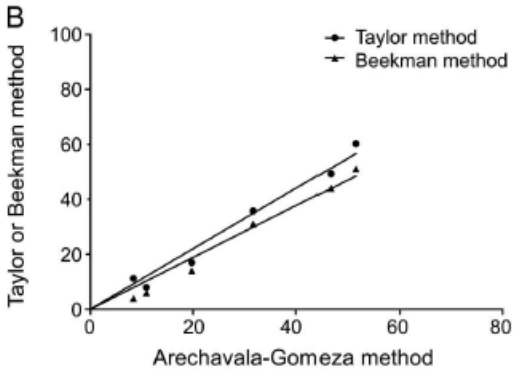
## Comparison of different dystrophin quantification methods

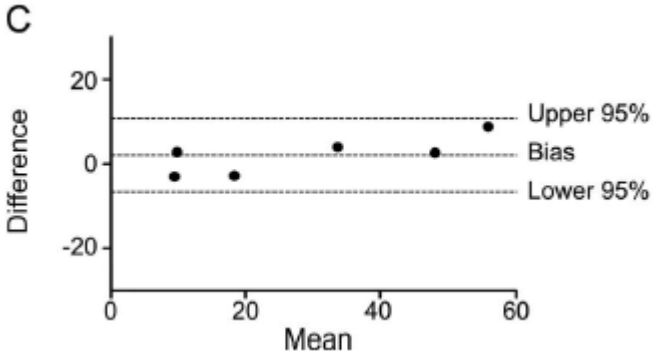
***Results using Arechavala et al., 2010a method (5 Labs)***

***Taylor et al., 2012 method (3 Labs)***

***Beekman et al., 2014 method (1 Lab)***



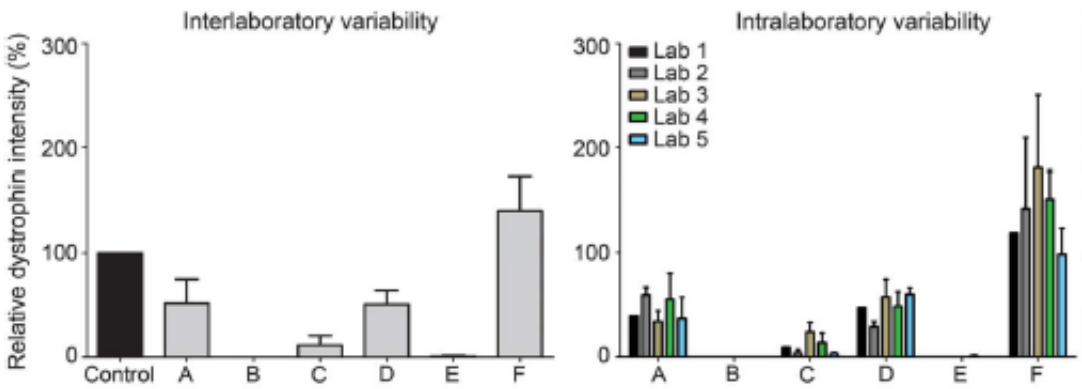



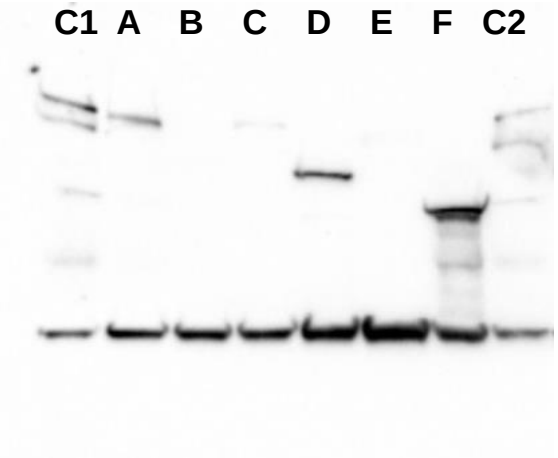



# Western Blot assay

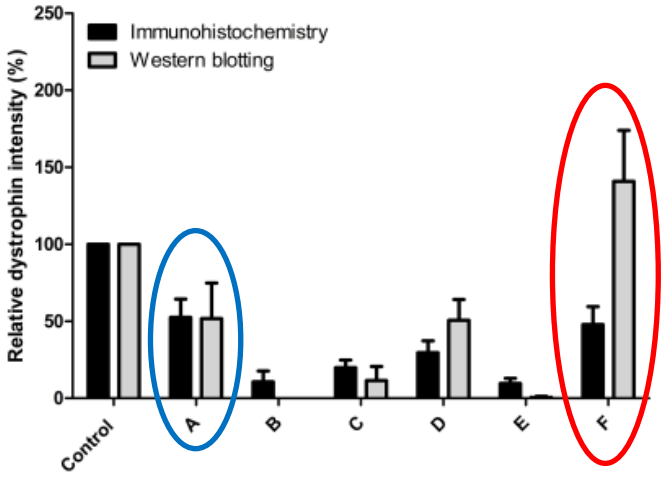
Protocol based on **Taylor *et al.*, 2012** published method

- Muscle samples lysed in protein extraction buffer
- 25ug of protein loaded on a 3-8 % Tris-acetate gel
- Wet transfer
- 1st Antibodies abcam 15277 1:400 O/N
  α-actinin 1:3000 1hr
- ECL detection with 2nd antibodies (1:15000 α-rabbit, 1:500000 α-mouse)
- Bands quantification performed by ImageJ or Odyssey
- Data normalized on α-actinin and presented relative to the average of the 2 ctr used



# Immunohistochemistry versus Western Blot




In several samples (e.g., BMD sample A, **c.40_41delGA**), the level of dystrophin determined by both techniques was highly comparable

In others (e.g. BMD sample F, **del ex 10-44**) the level of dystrophin quantified by western blotting was significantly higher than that determined by immunohistochemistry.

**IHC and Western blot give different information**

© 1995 Oxford University Press — Human Molecular Genetics, 1995, Vol. 4, No. 8 1245–1250

**Expression of human full-length and minidystrophin in transgenic *mdx* mice: implications for gene therapy of Duchenne muscular dystrophy**

Dominic J.Wells[1,*], Kim E.Wells[1,2], Emmanuel A.Asante[1,+], Gaynor Turner[2,3], Yoshihide Sunada[4], Kevin P.Campbell[4], Frank S.Walsh[2] and George Dickson[2,3]

The amount of dystrophin on blot varies in different subcellular fractions between full length and shorter isoforms → minidystrophins are less associated with the sarcolemmal fraction compared to wild type

**IHC and Western blot are different techniques with different range of sensitivity**

**IHC is more sensitive in detecting low levels of protein and allows high level of inter lab agreement:**

- **Takes into account dystrophin distribution, that in DMD and BMD patients is PATCHY**
- **Provides confirmation of subcellular protein localization (Western Blot is based on a homogenous protein extraction)**
- ***Anthony et al., 2014 proved reproducibility of this method***

# Is it important to quantify the different numbers of positive dystrophin fibers?


Human Molecular Genetics, 1995, Vol. 4, No. 8 1251–1258

**Expression of full-length and truncated dystrophin mini-genes in transgenic *mdx* mice**

Stephanie F.Phelps[1], Michael A.Hauser[1], Neil M.Cole[2], Jill A.Rafael[1], Richard T.Hinkle[2], John A.Faulkner[2] and Jeffrey S.Chamberlain[1,3,*]

February 2012 PLoS one

**The Effects of Low Levels of Dystrophin on Mouse Muscle Function and Pathology**

Maaike van Putten, Margriet Hulsker, Vishna Devi Nadarajah, Sandra H. van Heiningen, Ella van Huizen, Maarten van Iterson, Peter Admiraal, Tobias Messemaker, Johan T. den Dunnen, Peter A. C. 't Hoen, Annemieke Aartsma-Rus*

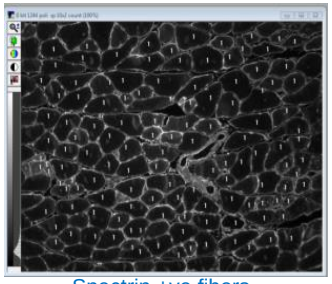
The same level of protein (detected on a blot) had a different effect in different transgenic lines based on the **uniformity of expression**:

- Mice with non-uniform expression had more pathology and a less favourable functional outcome
- Mice with same overall levels but more uniform dystrophin distribution had less pathology and better functional outcome

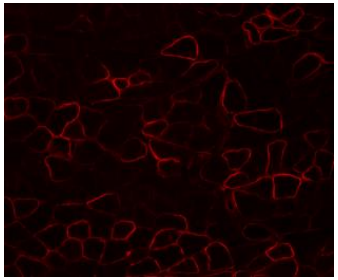
# Additional important information from IHC:

- Numbers of positive dystrophin fibers per section
- Percentage of the fiber expressing dystrophin

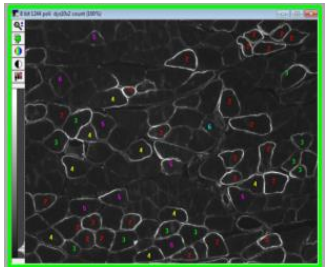
## 1st METHOD : Operator Dependent counting from images of sections



Spectrin +ve fibers
Excluding fibers at the edges



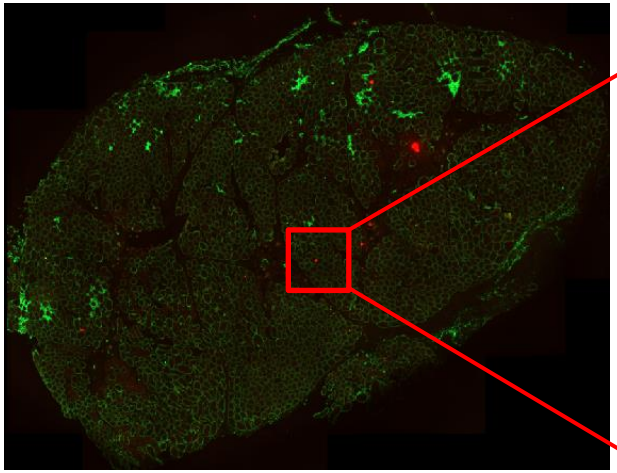
Dystrophin staining



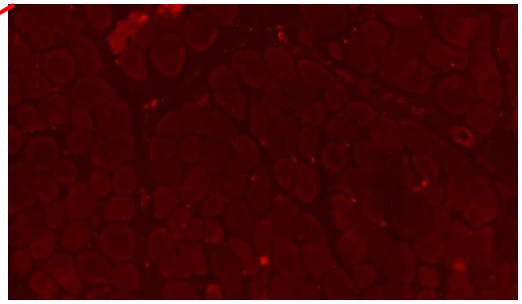
Scoring dystrophin +ve fibers considering the % of the fiber expressing dystrophin

Establishment of a common method across the BOM-SG is in progress

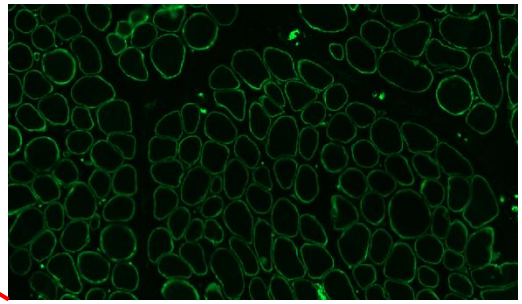
## 2nd METHOD : Algorithm based counting from scan of entire section



DMD



Dystrophin



Spectrin

# Take home message

## Dystrophin quantification

1) Can it be measured reliably? ✓

2) Is it relevant to quantify the different numbers of positive dystrophin fibers? ✓

# Acknowledgements

## Patients and their families











MRC Centre for Neuromuscular Diseases

***Thanks for your attention***