Challenges and variability in dystrophin detection: immunofluorescence, antibody staining and imaging methodologies

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Agenda

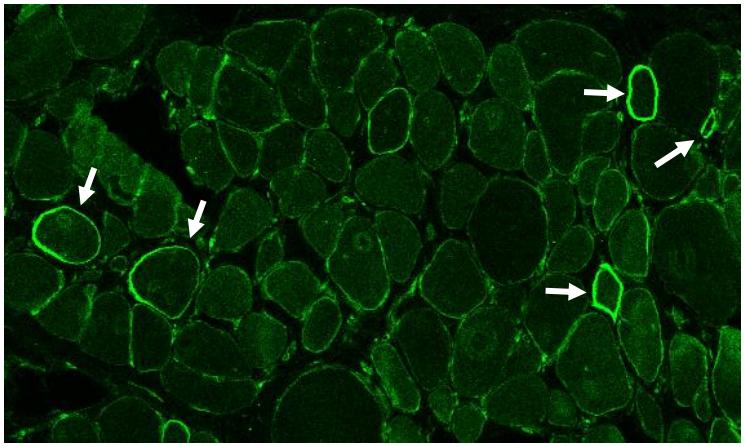
- Existence and variability of trace dystrophin & revertants
- Dystrophin intensity by a reproducible automated image analysis method
- Limitations: variability, linearity, control, biopsy quality
- Clinical study perspective on biopsy dystrophin analysis, next steps

Counting positive fibers that look like revertants is complicated

Revertant fibers: bright muscle fibers with high levels of dystrophin

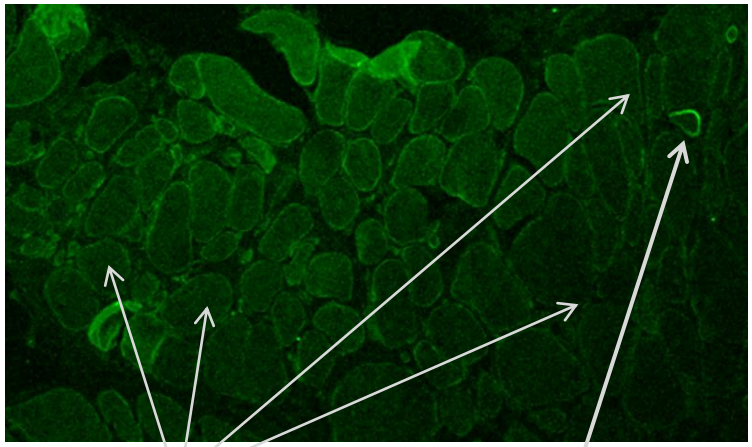
DMD Exon 45-50

Ab15277 (C-terminal)



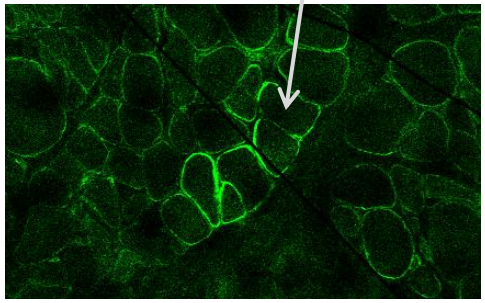
detection of revertant fibers

MANDYS106 (exon 43)



Revertants without exon 43

Revertants with exon 43



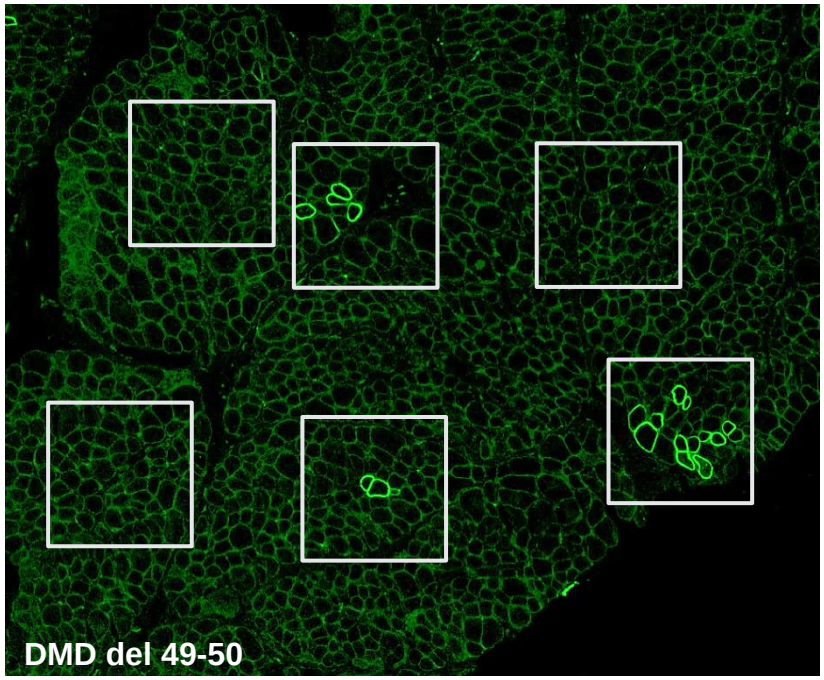
Multiple types of revertant fibers present in a biopsy

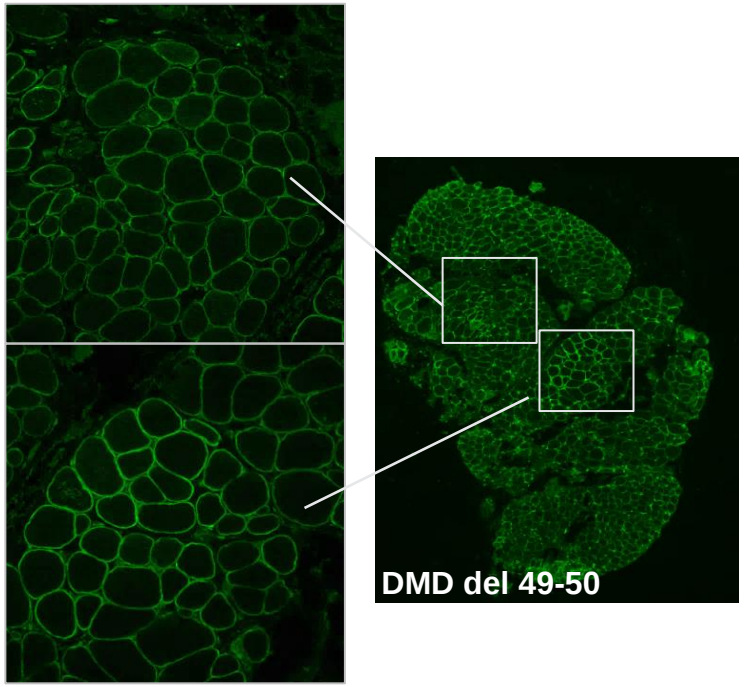
Counting fibers that look like revertant fibers is difficult to interpret

Variability in trace dystrophin and revertant fibers in DMD

Dystrophin traces: low levels of expression in many fibers throughout a biopsy

- Patches of low dystrophin-positive areas at the sarcolemma (Nicholson *et al.*, 1990, 1993)



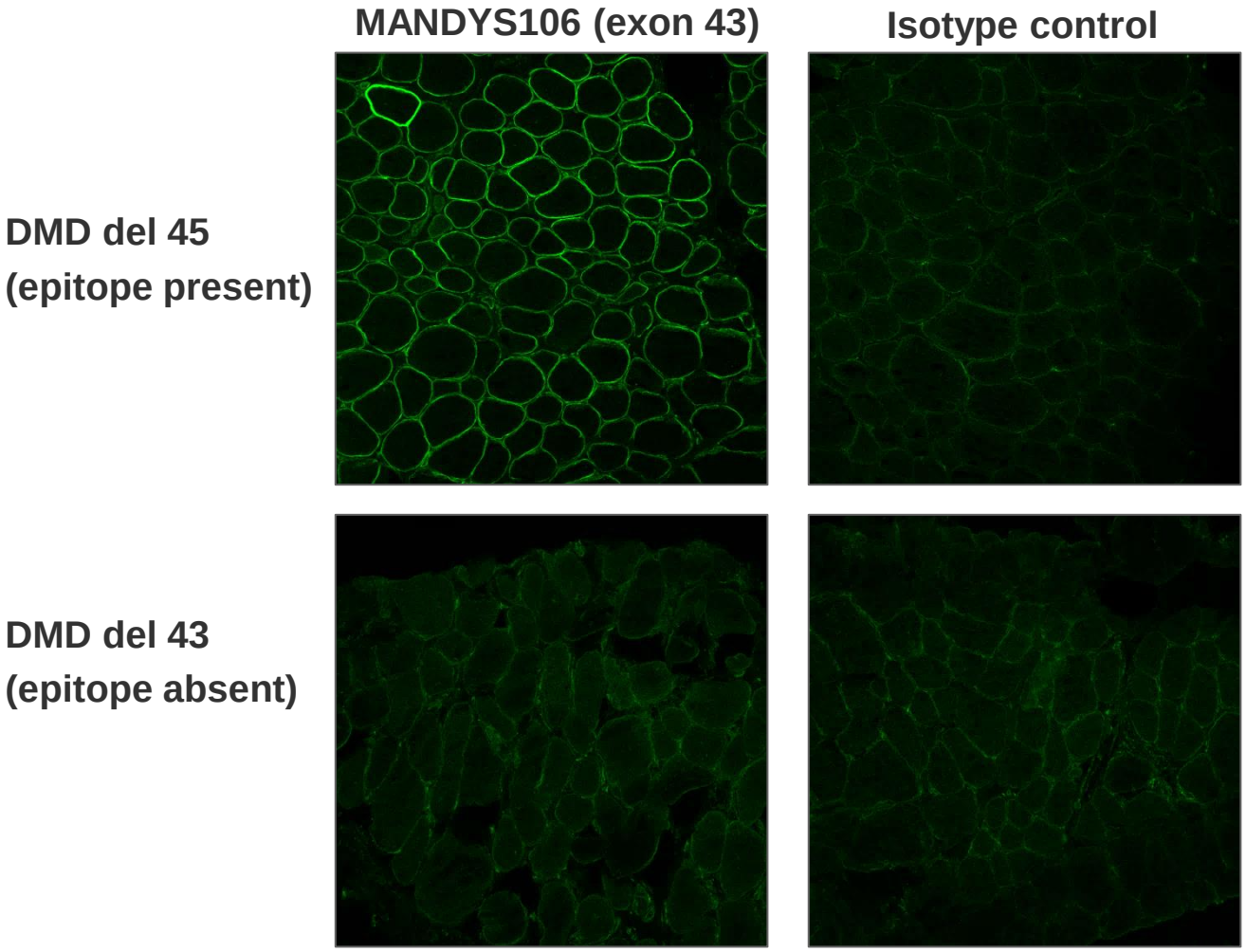


- Trace dystrophin observed in all patients
  (> 500 biopsies; 16 deletion mutations)

Essential

- **representative images capturing many fibers**
- **measure dystrophin intensity in the entire membrane**

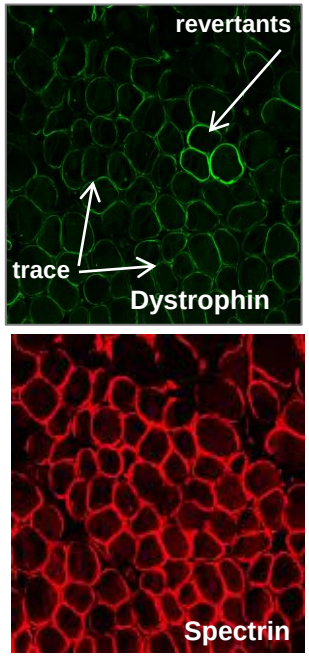
The low trace signal detected in DMD is dystrophin specific a genetic control analysis



Beekman *et al* 2014

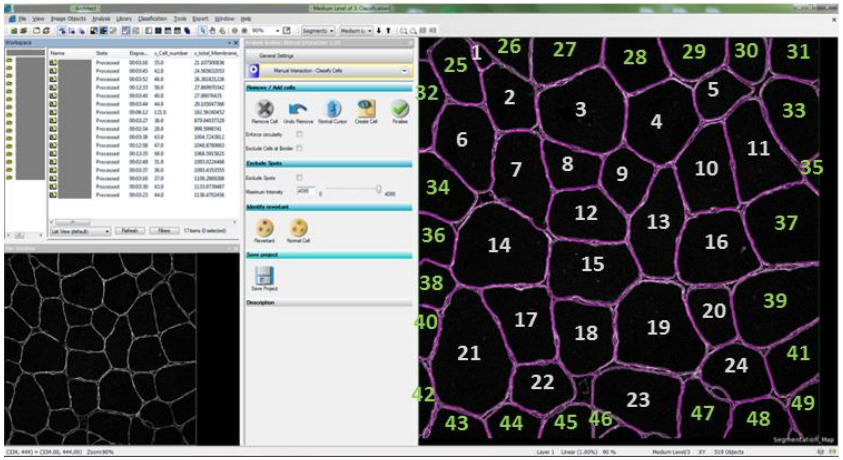
Method captures the variation in dystrophin intensity between fibers in DMD

Staining & imaging



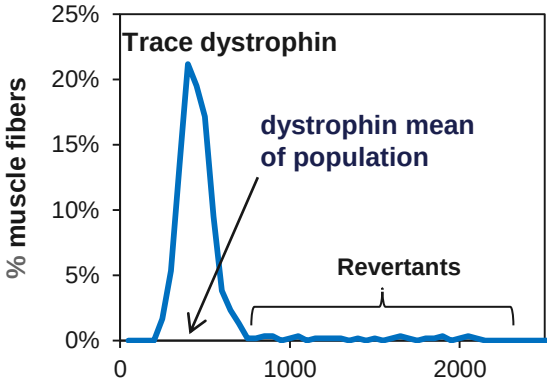
Automated image analysis

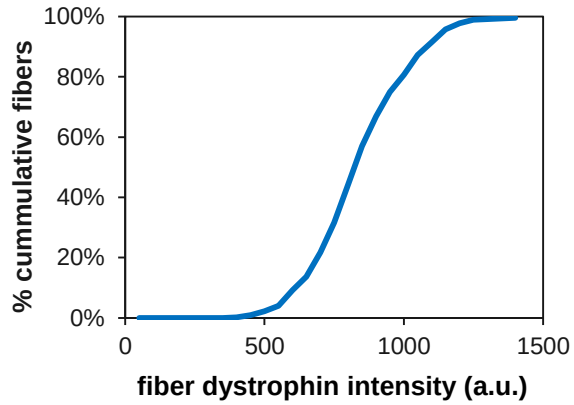
Definiens customised software



- confocal microscopy (25x magnification)
- identification of individual fibers
  - minimum 90% fibers
  - generally 250 - 600 fibers / section
- measure per fiber dystrophin intensity
  - entire membrane for every fiber
  - operator independent, objective
  - high reproducibility

Dystrophin measurement





Beekman *et al* 2014 (PLoS One)

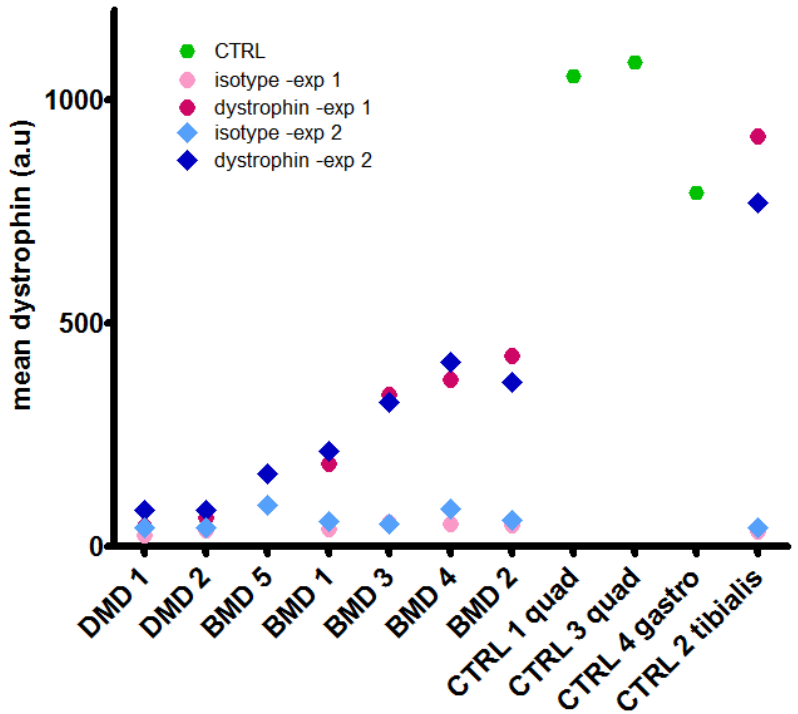
Reproducibility in dystrophin measurement in different DMD biopsies

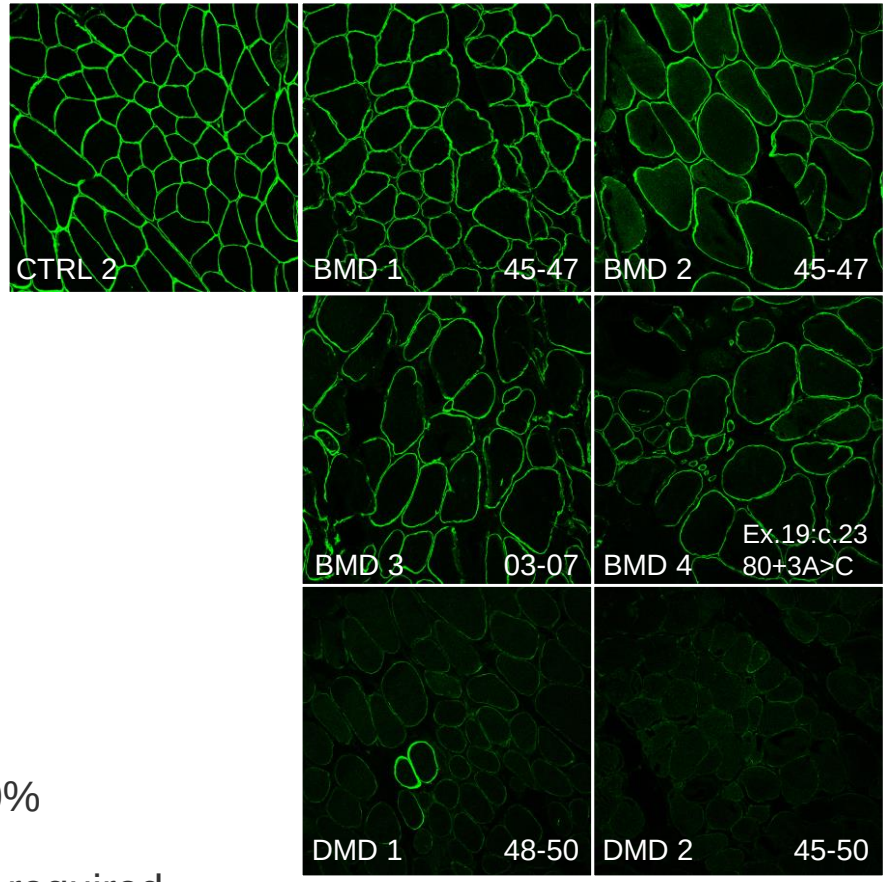
- 4 DMD biopsy samples
- 2-3 experiments (over a 1.5 year period) providing inter-assay precision
- 2-4 sections analysed providing intra-assay precision
- 8 operators randomly involved in staining, imaging and image processing

Sample	Dystrophin membrane intensity MANDYS106 (a.u)			Ranking	Precision	
	Exp 1	Exp 2	Exp 3		Intra assay; CV% sections	Inter assay; CV% experiments
DMD 3	255	241	208	4	3-10%	10%
DMD 1	286	298	228	3	2-8%	14%
DMD 4	306	427	354	2	2-5%	17%
DMD 5	406	-	397	1	4-6%	2%

Beekman *et al* 2014

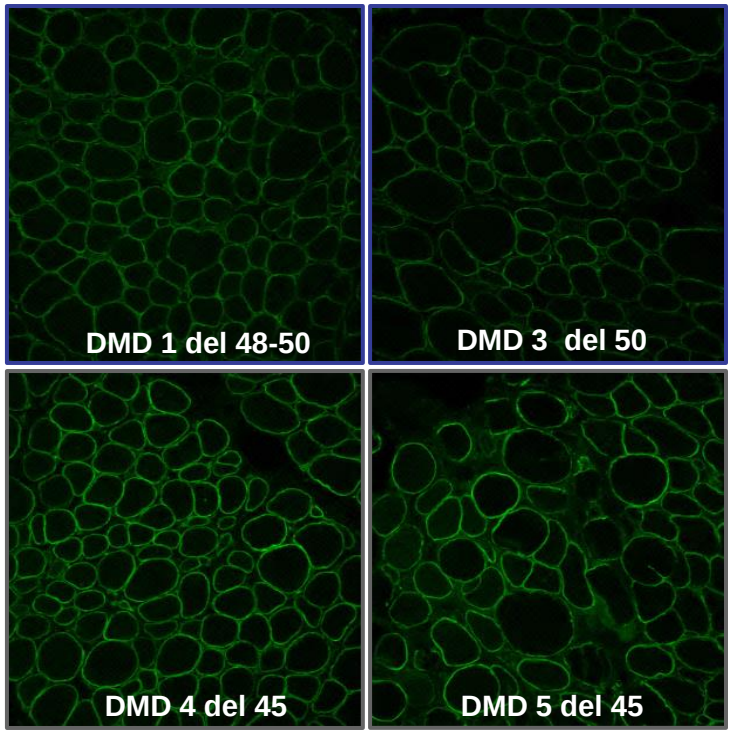
Immunofluorescence can detect differences but is not quantitative

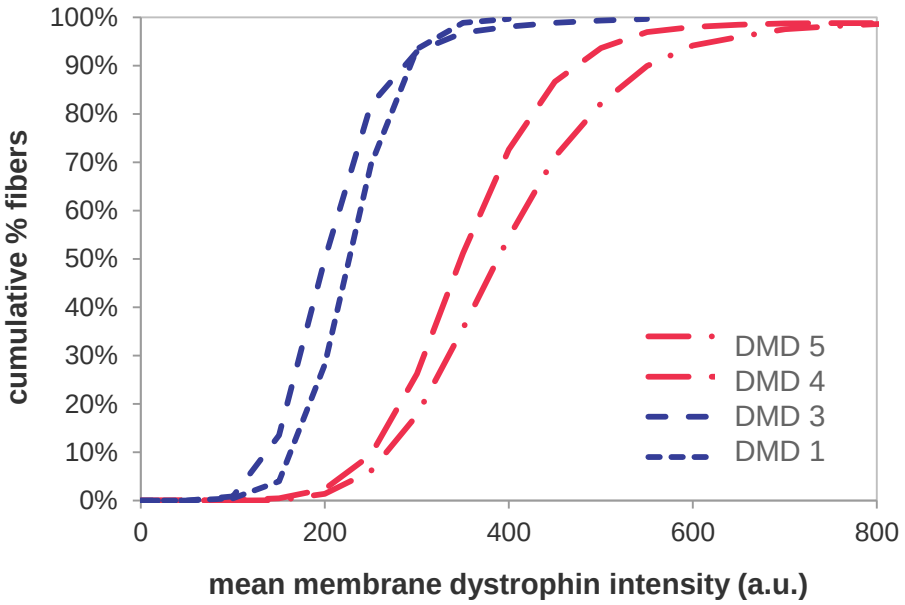




- Different control muscles and donors vary by 30%
- Negative control differentiates from DMD and is required
- Reproducible differences over a wide range of dystrophin intensity
- Linearity between intensity signal and dystrophin concentration cannot be established
- Relative comparison of dystrophin differences between biopsies is informative

Assay detects differences accurately in DMD

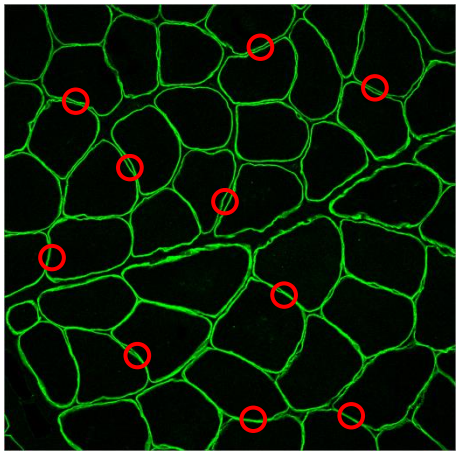




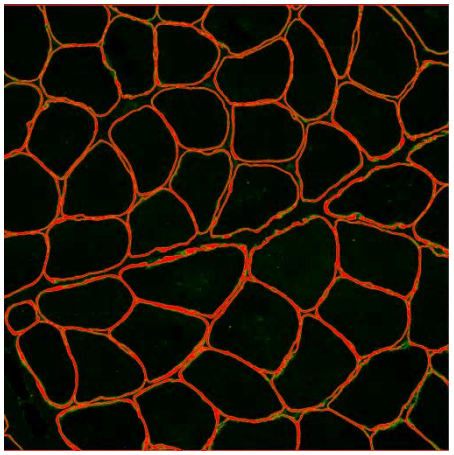
Beekman *et al* 2014

Dystrophin varies between DMD patients
thus important to compare with pre-treatment biopsy per patient

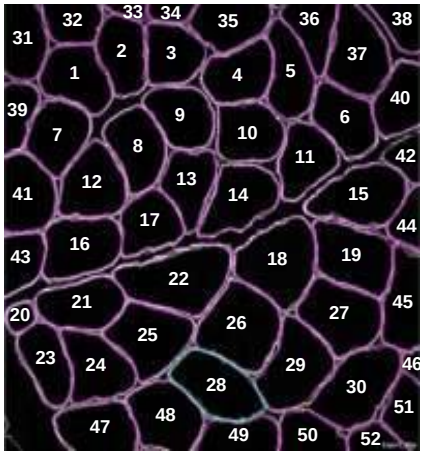
Image Method comparison: Biological Outcome Measures 2013



Arechavala-Gomeza



Taylor



Beekman

Arechavala-Gomeza *et al.*

10 circles across membranes; measure min & max; divide by spectrin

Taylor *et al.*

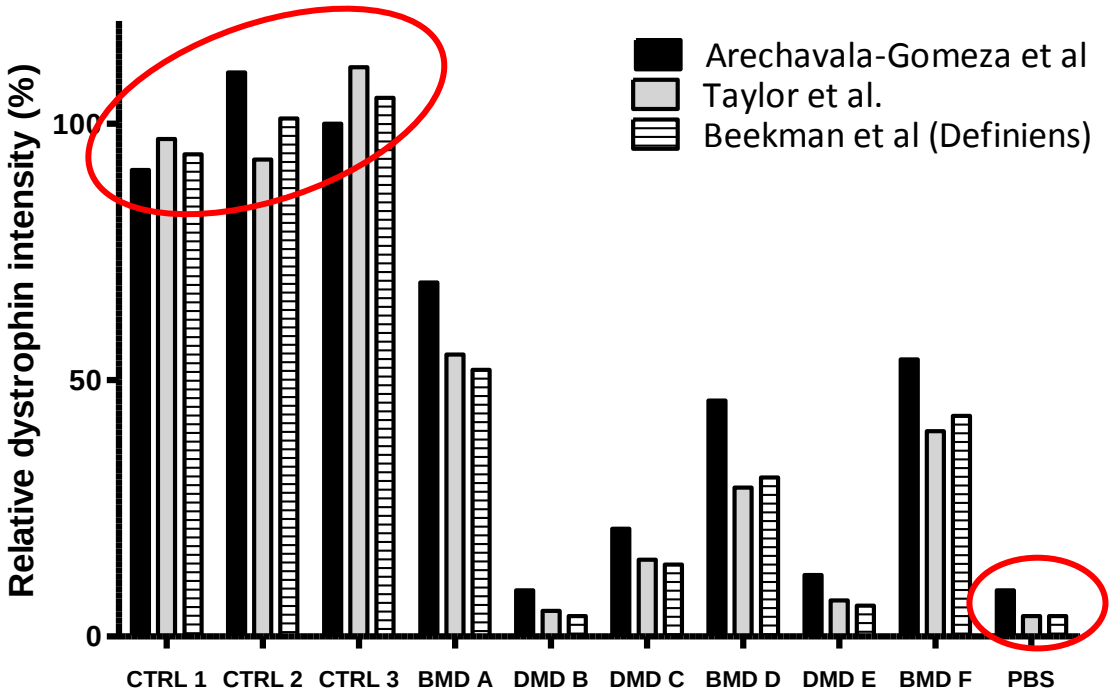
mean membrane intensity per image; threshold approach based on spectrin; divide by spectrin

Beekman *et al.*

mean membrane intensity per fiber, not divided by spectrin

Anthony *et al*. 2014 (Neurology)

Image Analysis Method comparison by Prosensa



- Analysis Methods are in agreement on the relative comparison between biopsies
- Negative control (PBS) can be 50% of DMD trace values
- Positive control dystrophin intensity vary for different muscles
- Linearity cannot be established due to lack of dystrophin standards

Relative comparison between biopsies is most informative

Biopsies and DMD biomarkers in clinical programmes

Biopsies are valuable for assessment of disease progression and drug effect

- Dystrophin protein detection
- Exon skip
- Drug concentration in muscle
- Explore other biomarkers of disease

~500 biopsies from multicenter studies (80 sites) covering 16 different DMD deletions

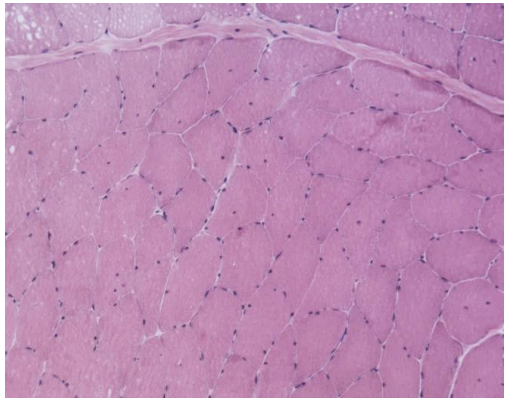
- analysis of biopsies under Good Clinical Laboratory Practices for reliability and traceability

Potential for surrogate endpoints, but issues to consider:

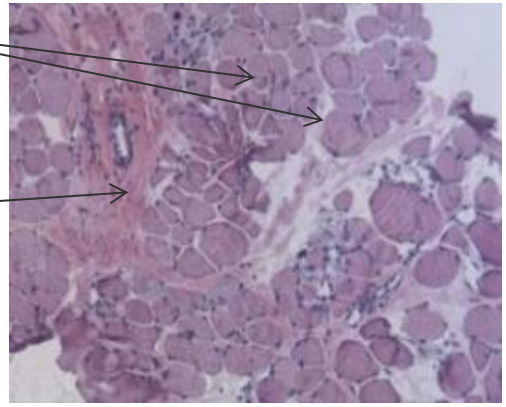
- **Biopsy sampling: which muscle, biopsy size, disease progression**
- **Quality of handling, shipping and storage (procedures and training)**

Disease progression and heterogeneous sampling can affect analysis

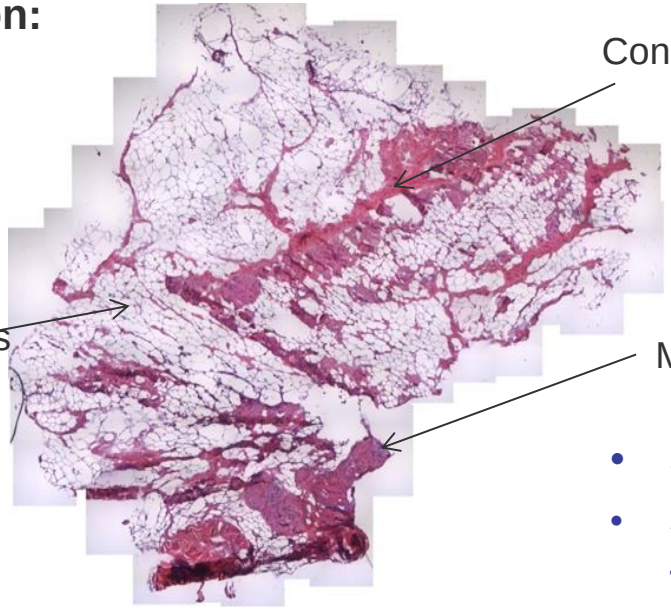
Healthy control

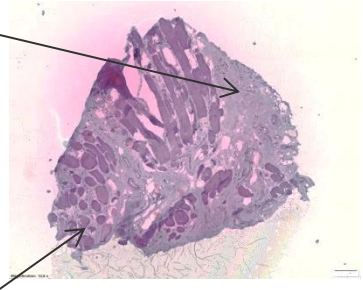


DMD



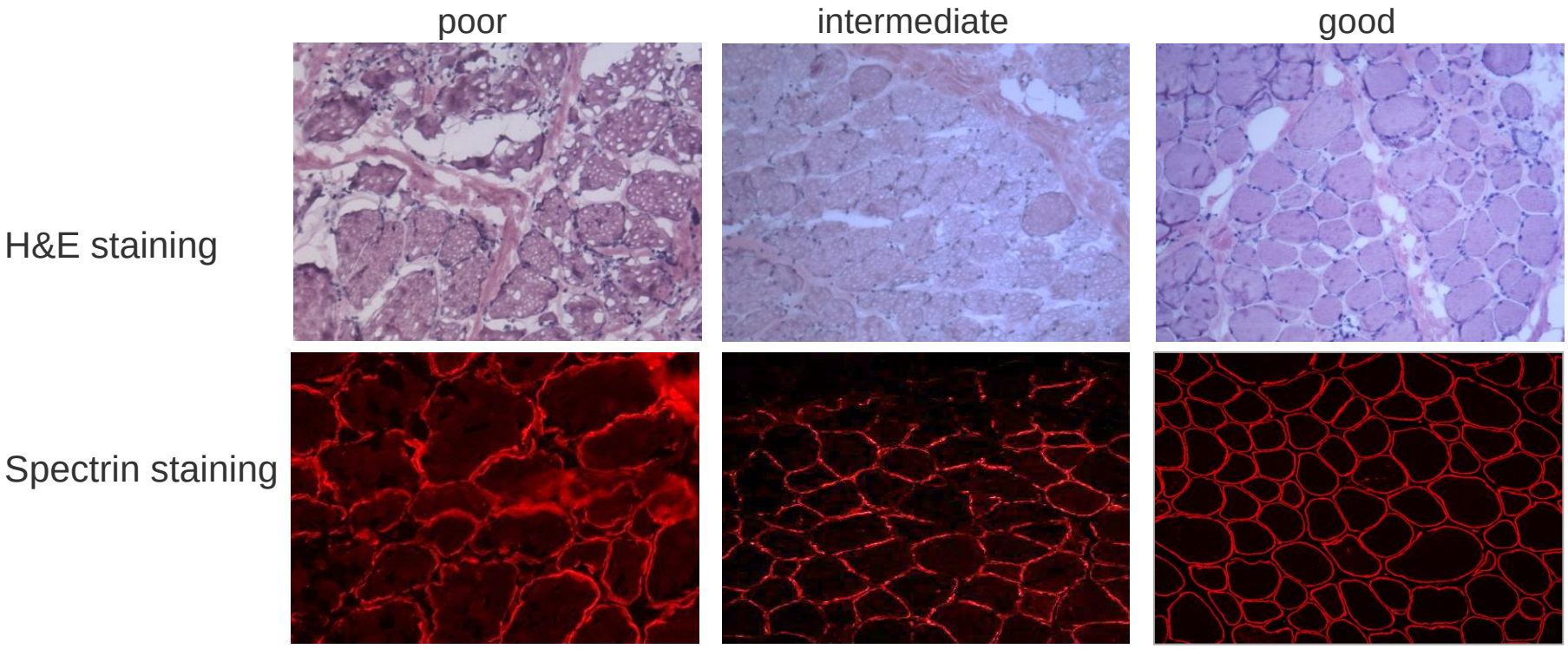
DMD -Extensive disease progression:





- **Sufficient number of muscle fibers required**
- **Small biopsies and low muscle content are prone to experimental artifacts**

Freeze artefacts influence staining and ability to compare biopsies



No reliable comparison can be made between biopsies that differ in quality

% of subjects with evaluable biopsies

phase II DMD114117 (13 sites) 88%
phase II DMD114876 (13 sites) 66%
phase III DMD114044 (46 sites) 50%

CONCLUSIONS

- Dystrophin immunofluorescence analysis using intensity measurements
  - dystrophin signal localisation at entire membrane
  - variable presence of trace dystrophin and revertant fibers
  - requires good quality biopsies
  - reproducibly and accurately detects differences between DMD biopsies
    - intra-assay precision typically <10%; inter-assay precision <25%

- Advancing immunofluorescence methods for quantification requires development and purified dystrophin protein control with a signal dilution relevant to DMD patients

- Further effort and development to correlate dystrophin to clinical outcome in randomized placebo controlled clinical trials is required

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- **DMD patients and families**