

Myoblots: dystrophin quantification by in-cell western assay for a streamlined development of DMD treatments

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Aims. New therapies for neuromuscular disorders are often mutation-specific and require to be studied on patient's cell cultures. In Duchenne muscular dystrophy (DMD) dystrophin restoration drugs are being developed but, as muscle cell cultures from DMD patients are scarce and do not grow nor differentiate well, only a limited amount of candidate drugs are tested. Moreover, dystrophin quantification by western blotting requires a large number of cultured cells; so fewer compounds are as thoroughly screened as desirable. We aimed to develop a quantitative assessment tool using fewer cells to contribute in the study of dystrophin and to identify better drug candidates.

Methods. An "in-cell western" assay is a quantitative immunofluorescence assay performed in cell culture microplates that allows protein quantification directly in culture, allowing a higher number of experimental repeats and throughput. We have optimised the assay ("myoblot") to be applied to the study of differentiated myoblast cultures.

Results. After an exhaustive optimization of the technique to adequate it to the growth and differentiation rates of our cultures and the low intrinsic expression of our proteins of interests, our myoblot protocol allows the quantification of dystrophin and other muscle associated proteins in muscle cell cultures. We are able to accurately distinguish between the different sets of patients based on their dystrophin expression and detect dystrophin restoration after treatment.

Conclusions. We expect that this new tool to quantify muscle proteins in DMD and other muscle disorders will aid in their diagnosis and in the development of new therapies.

INTRODUCTION

Duchenne muscular dystrophy (DMD), a fatal, rare disease that affects one in 4.000 male children, is associated with total or nearly total lack of dystrophin protein at the muscle sarcolemma [1].

Dystrophin connects the cytoskeleton to the extracellular matrix in association with other proteins, forming the dystrophin associated glycoprotein complex (DGC) [2] and is notoriously difficult to quantify due to its very large

Myoblots: dystrophin quantification by in-cell western assay.

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size (427 KDa) and low natural expression [3]. With the development of several drugs aiming to restore dystrophin expression [4-6], new methods to quantify it in biopsies from patients have been developed [7-9], and dystrophin quantification has been given much consideration: the FDA hosted in 2015 a workshop exclusively on this topic [10], and granted accelerated approval to the drug eteplirsen in 2016 on the basis of minimal dystrophin restoration [11, 12]. Currently two drugs that aim to restore dystrophin expression have been marketed, one in Europe (ataluren) and one in the US (eteplirsen), none of them sufficiently effective and each of them indicated to a different 13% of all DMD patients, due to their mutation-specific therapeutic mechanism [13, 14]. The development of better drugs for these and other mutations is undergoing, but the preclinical development of these drugs face several hurdles, one of which is the lack of adequate dystrophin quantification methods in cell culture.

Cell cultures derived from muscle or skin biopsies from patients (myoblasts or fibroblasts respectively) are essential to test new DMD treatments. The need for cultures with specific mutations makes those samples, already difficult

to find as they are obtained from paediatric patients with a rare disease, even more rare. Moreover, using standard methodology, dystrophin is usually only detectable in differentiated muscle cells (myotubes)[15], and patients' cultures are often difficult to expand [16] and do not differentiate well [17]. All these facts limit the number of candidate compounds and the number of replicates that can be evaluated in cell cultures with conventional methods.

An in-cell western assay (ICW) is a quantitative immunofluorescence assay performed in microplates that permits the quantification of proteins directly in cell culture, combining the specificity of western blotting with the reproducibility and throughput of ELISA [18, 19]. Signals from fluorophores conjugated to specific antibodies are quantified by a two-channel near-infrared scanner (Odyssey®, Li-Cor Biosciences), allowing for signal normalisation to cell number or specific endogenous proteins. We have worked in the optimisation of this assay, which we refer to as "myoblot", to be applied to the study of differentiated myoblast cultures with the main aim of quantifying dystrophin and other sarcolemmal proteins (**Figure 1**).

Myoblots: dystrophin quantification by in-cell western assay.

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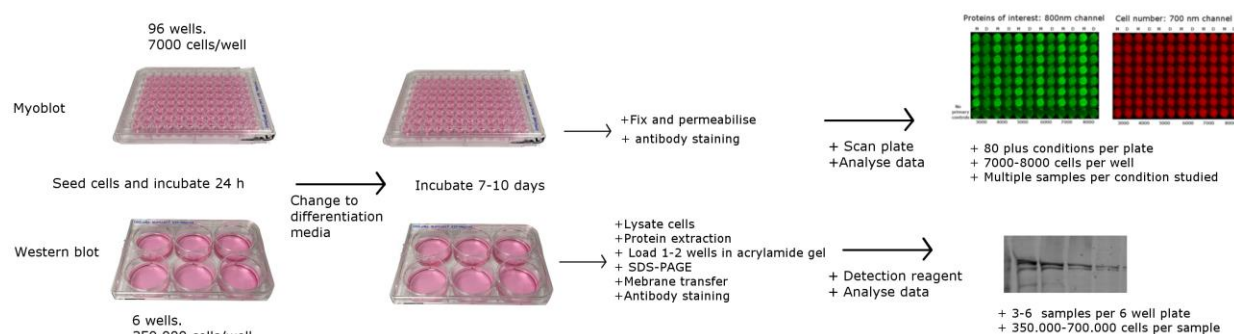


Figure 1 Illustration of Myoblot technique vs dystrophin western blotting from cultured cells

MATERIALS AND METHODS

Samples

Immortalised myoblasts [20] were used to set up the protocol and these and additional primary myoblast cultures were used to test it. They were requested from the Queen Square Centre for Neuromuscular Disorders BioBank (CNMD Biobank, London, UK) and the Institut de Myologie (Paris, France, immortalised cultures), and had been derived from muscle biopsies from healthy controls (3), and DMD (5) and BMD (2) patients after informed consent (Table 1).

Cell culture

Myoblast were expanded using skeletal muscle medium (SMM, Promocell,) supplemented as described before [21]. Doubling times were calculated following the formula: $\text{doubling time} = \frac{\text{days} \times \log(2)}{\log(\text{final concentration}) - \log(\text{initial concentration})}$, as described [22].

To express proteins of the DGC, cultures had to undergo differentiation: after being seeded on matrigel-coated wells (0.1 mg/ml) and once over 80%

confluent, cultures were switched to differentiation medium [DMEM plus 2% Horse Serum and Penicillin-Streptomycin]], and incubated for further 7-10 days.

Preliminary characterisation of the cultures used to optimise the in cell western (ICW) protocol included differentiation assays as described in [23]. Briefly, cells were seeded in matrigel coated 8-well chamberslides at a density of 5×10^4 cells per well and cultured in SMM for 2 days, when they were switched to differentiation media for 3 days, after which they were fixed and immunostained with a desmin antibody to determine whether they were capable of fusion into multinucleated myotubes. Nuclei inside and outside desmin positive myotubes (with 3 or more nuclei) were counted and the percentage of those in myotubes was calculated (differentiation rate, table1).

Myoblots: dystrophin quantification by in-cell western assay.

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	Sample culture	DMD exon deletion	Culture	Doubling time[22] (days)	Differentiation rate %[23]	Differentiation (visual)
1*	Control 1	None	Immortalised	1.3	54	Good
	DMD 1	△48-50	Immortalised	1.5	35	Average
	DMD 2	△48-50	Immortalised	2.0	24	Average
	DMD 3	△52	Immortalised	1.7	61	Good
2*	Control 2	None	Immortalised			Very good
	Control 3	None	Immortalised			Good
	DMD 4	△52	Primary			Very good
	DMD 5	△52	Primary			Very good
	BMD 1	△48	Primary			Bad
	BMD 2	△48-49	Primary			Good

1* Cultures used in the method optimization 1* and 2*: Cultures used in the myoblot evaluation

Immunocytochemistry procedure

During differentiation assays, chamberslides were fixed with 4% PFA for 15 min at RT, washed, permeabilized with 0.5% Triton at RT for 5 min, washed and blocked (10% NGS, 0.3% Triton in PBS) at RT for 30 min. After washing, incubation with primary antibody at 1/100 dilution (AbCam ab15277, Thermo Fisher PA532388) was performed at RT for 1 hour, washed and secondary antibody (1/500, Alexa Fluor 488 goat-anti rabbit & goat-anti mouse) incubated for 1 hat RT in the dark. Slides were studied under a Nikon Eclipse TE2000-E microscope at 20x magnification.

Dystrophin restoration experiments

Cultures were treated with a 2'O-Me phosphorotioate antisense

oligonucleotide aiming to skip DMD exon 51 (5'-[UCAAGGAAGAUGGCAUUUCU]-3', Eurogentec, Belgium) by transfection with Lipofectamine as described in [21, 24]. Transfected cultures were studied by either western blotting or our myoblot method 7-10 days after treatment, and RNA was collected from duplicate experiments in 6-well plates 2 days after treatment. For these experiments, RNA was extracted and a standard nested RT-PCR protocol [24] was performed to confirm the effect of the treatment at RNA level.

Western blotting

The protocol, based on that of Anthony et al [3], was as follows: samples were solubilised in lysis/loading buffer[24] and loaded in 3-8% gradient

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polyacrylamide gels in tris-acetate buffer (Life Technologies) before being subjected to electrophoresis for 45 min at 80 V + 120 min at 150V. After an overnight wet transfer at 4 °C, membranes were blocked and incubated overnight at 4 °C with the anti-dystrophin antibody AbCam ab15277 (dilution 1/200), while the α -actinin antibody (Sigma-Aldrich A7732, dilution 1/5000) was added for the last hour of incubation at room temperature. After washing, secondary antibodies (IRDye 800CW goat anti-rabbit 926-32211, IRDye 680RD goat anti-mouse 926-68070, LiCor Biosciences, dilution 1/15000) were incubated for 1 h and images were acquired with an Odyssey Clx imager after careful washing of the membranes.

In-Cell Western Assay (myoblot)

Myoblasts were seeded in matrigel coated 96-well plates at the required concentration (6.500-7.500 cells/well) and incubated for 24 hours in SMM. After 24 hours, SMM was switched to differentiation media. 7-10 days after differentiation started, plates were collected for ICW analysis: after fixing the cultures with ice cold methanol (99.8% Sigma 32213, for 10 min), plates were washed with PBS, permeabilised with PBS+0.1%Triton and blocked with blocking buffer (Li-Cor)) for 2 hours. All wells were treated with the cell number control stain CellTag 700 Stain (Li-Cor),

while a minimum of 4 wells were incubated with antibodies against the proteins of interest: anti-dystrophin mix (Dys1: NCL-DYS1 Leica Biosystems, Mandys1, Mandys106, kindly provided by Prof. G Morris, The MDA Monoclonal Antibody Resource) at a dilution 1:100 and Ab anti-utrophin mix (NCL-DRP2 Leica Biosystems, 1/10) [25, 26] (**Figure 1**). When biotin/streptavidin amplification was required, the following antibodies were used: AbCam ab6788 goat anti-mouse IgG H&L biotin and ab97049 goat anti-rabbit IgG H&L biotin (both 1/2000) followed by IRDye 800CW Streptavidin 925-32230 (1/2000). When no such amplification was needed, secondary antibodies IRDye 800CW goat anti rabbit 626-32211 or IRDye 800CW goat anti mouse 926-32210 were used. Every plate set-up included non-primary background controls for each of the primary antibodies used. As expression of DCG proteins depends on the differentiation status of each culture, every condition studied included replicate wells incubated with an anti-myosin heavy chain antibody (MF20, dilution 1/100, DSHB), whose expression is correlated with differentiation. When necessary, results were presented in a double graph, with a line showing the MF20 expression indicating the differentiation of the culture, and bars showing the expression of the different proteins of interest.

Myoblots: dystrophin quantification by in-cell western assay.

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Statistical Analysis

Data was analysed using GraphPad Prism 7 (GraphPad Software Inc.). Student's t-tests were applied. Data are presented as mean \pm SEM. Differences

were reported as significant at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***)

Name	Dilution	Provider	Dystrophin epitope	Myoblot signal	
				Primary plus IRDye® fluorescent secondary antibody	Primary plus biotin/streptavidin and IRDye® fluorescent secondary antibody
NCL-Dys1	1/100	Novacastra Leica Biosystems	Rod domain exons 26-29 Amino acids 1181-1388.	++	+++
NCL-Dys2	1/10	Novacastra Leica Biosystems	C-terminus Exons 77-79. Amino acids 3669- 3685.	-	+++
Mandys 1	1/100	MDA Monoclonal Antibody Resource	Exons 31-32. 1431-1505 Amino acids 816-1749. [26, 27]	+	+++
Mandys 106	1/100	MDA Monoclonal Antibody Resource	Exon 43. amino acids 1749-2248[25, 26]	++	+++
Mandys 108	1/10	MDA Monoclonal Antibody Resource	Exon 39-46. Amino acids 1749-2248.[25, 26]	-	+
Mandys 125	1/10	MDA Monoclonal Antibody Resource	Exons 38-39. amino acids 1749-2248 [25, 26]	-	++
Dystrophin antibody ab15277	1/100	AbCam	Polyclonal. Amino acids 3661-3667	++	++
Dystrophin antibody PA532388	1/100	Thermo Fisher Scientific Pierce Biotechnology	Polyclonal C-terminus	++	NT

Table 2 Anti-dystrophin primary antibodies tested in this project

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RESULTS

Optimization prior to myoblot development:

A detailed **characterization of the cell cultures** is necessary when setting up any ICW technique and particularly important in myoblots, as proteins of interests are mainly expressed in differentiated cultures[15], and primary cultures have different doubling times and differentiation rates. A summary of doubling times [22] and differentiation rates [23] of the cultures used in this project is presented in **Table 1**. The differences in growth rates (as seen in the differences in doubling times) need to be accounted for to either seeding different amounts of cells per well when using several cultures in an experiment, or incubating slow growing cultures for longer before switching to differentiation media. The second key element in the optimisation of an ICW technique is the **selection of the primary antibody** to use. We tested a panel of anti-dystrophin primary antibodies (**Table 2**), including monoclonal and polyclonal antibodies. Although the two polyclonal antibodies tested also showed a good response, we decided to continue our tests with a combination of the best performing monoclonal antibodies, to avoid future specificity problems derived from the use of polyclonal antibodies. After titration of these antibodies, the sensitivity was increased with the use of a streptavidin/biotin system (1/2000)

conjugated to the antibodies, and this was further improved with the use of a combination of three of those antibodies, Dys1, Mandys1 and Mandys106 (**Figure 2A and B**, referred in methods as "dystrophin mix").

As our cultures are incubated for a long period before being processed, the final number of cells per well and choice of normalisation marker to use, are also two factors that need to be considered and are intrinsically related. We performed several cell dilution tests to find the cell number that would offer a good differentiation rate in the experiment's time course. We also considered the possibility of using a muscle differentiation marker (myosin heavy chain antibody, MF20) as a muscle specific normalising agent, and indeed our first experiments with control cultures showed a good correlation between a validated cell number marker (CellTag 700 Stain) and MF20 antibody signal. However, that correlation was not present in patient's cultures (**Figure 2C**) and it was decided to use the cell number marker CellTag 700 Stain to normalise the signal from the antibody of interest, while use MF20 in replicate wells (normalised as well for cell number) and include it as an experiment quality control to confirm that our cultures are differentiated, a pre-requisite to measure our proteins of interest (**Figure 2D**).

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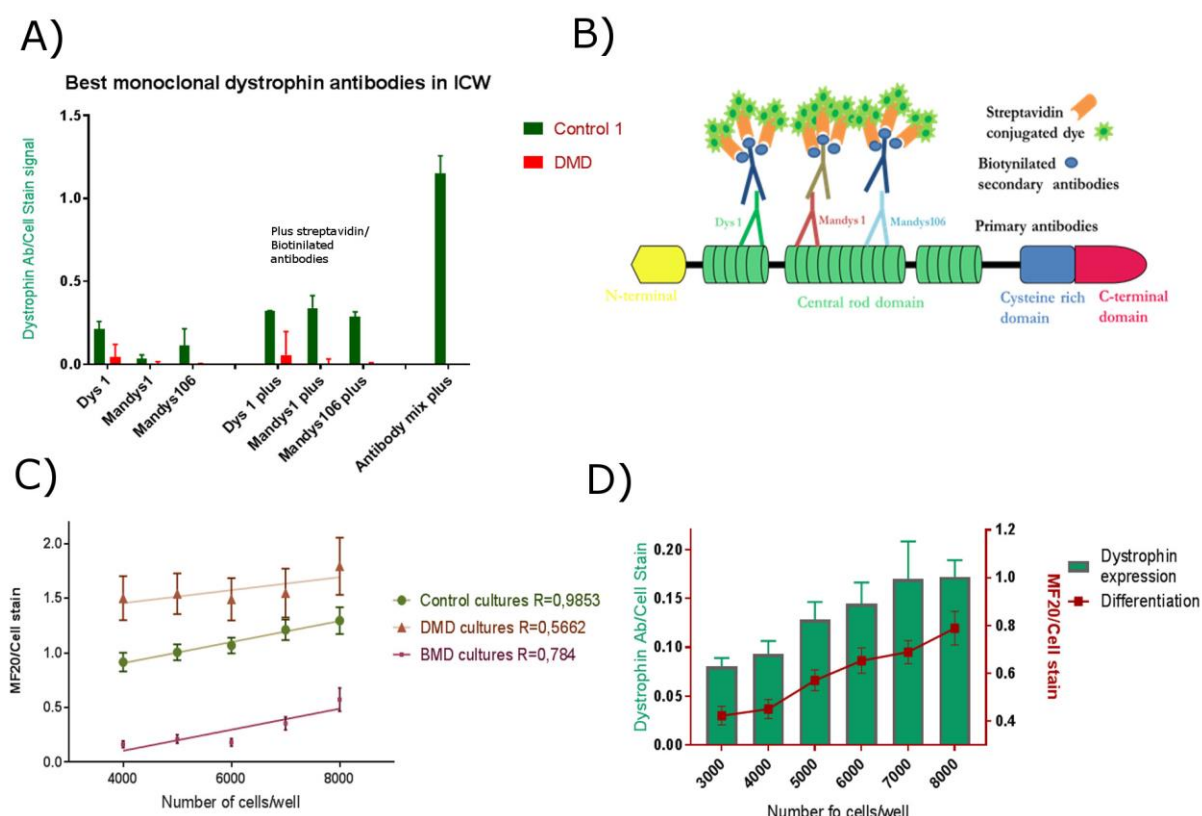


Figure 2 Optimisation A) Anti-dystrophin antibody intensity signal on differentiated cultures of control 1 and DMD1, DMD2 and DMD3. B) The optimised antibody mix used included Dys1, Mandys 1 and Mandys 106 plus streptavidin/biotin detection. C) Anti-myosin heavy chain (MF20) antibody myoblot signal correlated closely with a Cell Stain 700 cell number stain in control cultures, but this was not replicated in DMD or BMD cultures. D) Example of the use of a double graph: bars represent the intensity of proteins of interest while the line represents the intensity of myosin heavy chain staining, as a quality control for the culture differentiation.

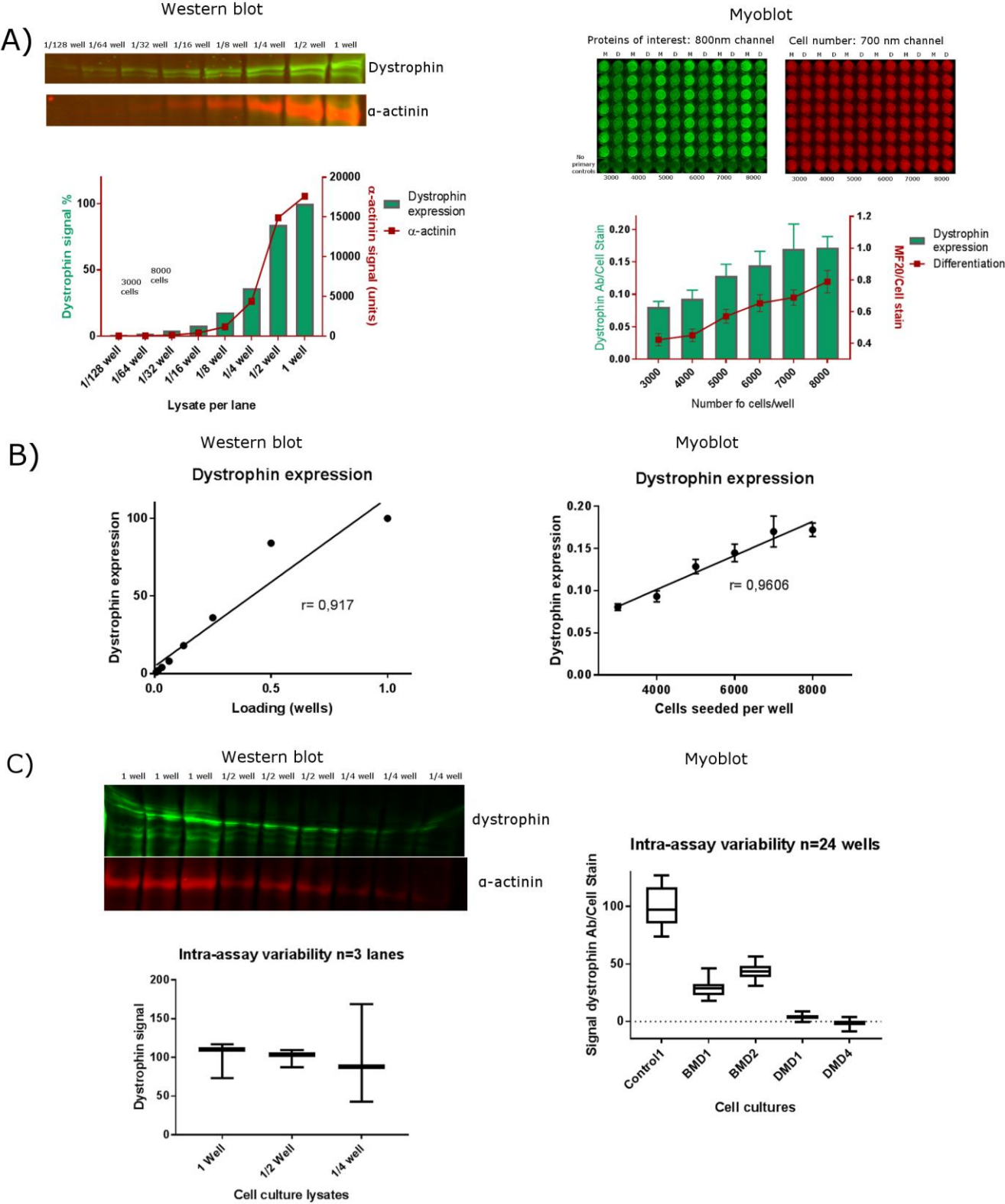
Assay sensitivity and precision.

To test the limit of detection of our technique we seeded an increasing number of control myoblasts in a 96 plate to measure dystrophin. This has the caveat that dystrophin is only expressed in differentiated cultures and there is a minimum concentration of cells needed in the culture to be able to differentiate. With that consideration, a range between 3000 and 8000 myoblasts per well were seeded and

compared with a western blot analysis of a standard differentiated culture lysate, loaded in serial dilutions. As shown in **figure 3A**, dystrophin expression was detectable by both methods at the lowest dilutions tested (the 3000-8000 range would correspond approximately to the lowest 3 conditions tested in the western blot). Both western blots and myoblots showed a good linear correlation when dystrophin was quantified. (**Figure 3B**), However, it has to be considered that if

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Figure 3 Assay precision / sensitivity. **Left: western blot-Right: myoblot A)** **Left:** Western blotting of serial dilutions of differentiated control myotube lysates: α -actinin is used as a loading control. Cultures were differentiated in 6 well plates (1 well=350.000 cells, 1/128 well= 2734 cells) before lysis. **Right:** Serial dilutions (3000-8000 cells per well) of control 1 myotubes analysed in a myoblot: the red channel shows the cell number marker and the green channel the proteins of interest. M= myosin heavy chain; D= dystrophin. **B)** Linear regression of data from western blots and myoblots in A) **C)** Intra-assay variability. **Right:** triplicate samples loaded in same western blot gel, presenting a CV of: 23,62% (1 well), 11,32% (1/2 well) and 63.56% (1/4 well) **Left:** CV 24 replicate wells corresponding to control, two BMD and two DMD patients, with the corresponding CV values: Controls- 15,69%, BMD1- 22,21%, DMD 59,24% DMD2 202%

treated patient's cells had to be used, the standard western blot protocols suggest loading the equivalent 2-3 wells from a 6 well plate per western blot lane, the equivalent to 700.000- 1 million cells, albeit to detect very low dystrophin expression [24, 28]. This makes dystrophin quantification by western blots from treated cells difficult due to overexpression of loading controls and control samples. To assess the intra-assay variability in western blotting and myoblot, we loaded triplicate samples of different concentrations in the same gel and western blot analysis showed variable results in this experiment, not being able to accurately discriminate between samples of different concentrations. On the other hand, myoblot analysis of replicate samples from different wells showed a clear discrimination between samples and lower variation coefficients (Figure 3C).

Dystrophin quantification in control and patients showing varied amounts of dystrophin protein.

Cultures derived from control and patients' biopsies were studied with our optimised method. We studied myotubes from controls (expressing normal amounts of dystrophin), from Becker muscular dystrophin patients (BMD, expressing an internally truncated but partially functional dystrophin) [29] and DMD (expressing no dystrophin or traces of dystrophin). In all cases, control and patients were clearly identified according to their protein expression level (Figure 4). While western blotting techniques required large amounts of cell lysate loaded in a single lane to be able to detect dystrophin expression, myoblots reliably provided easily quantifiable signals.

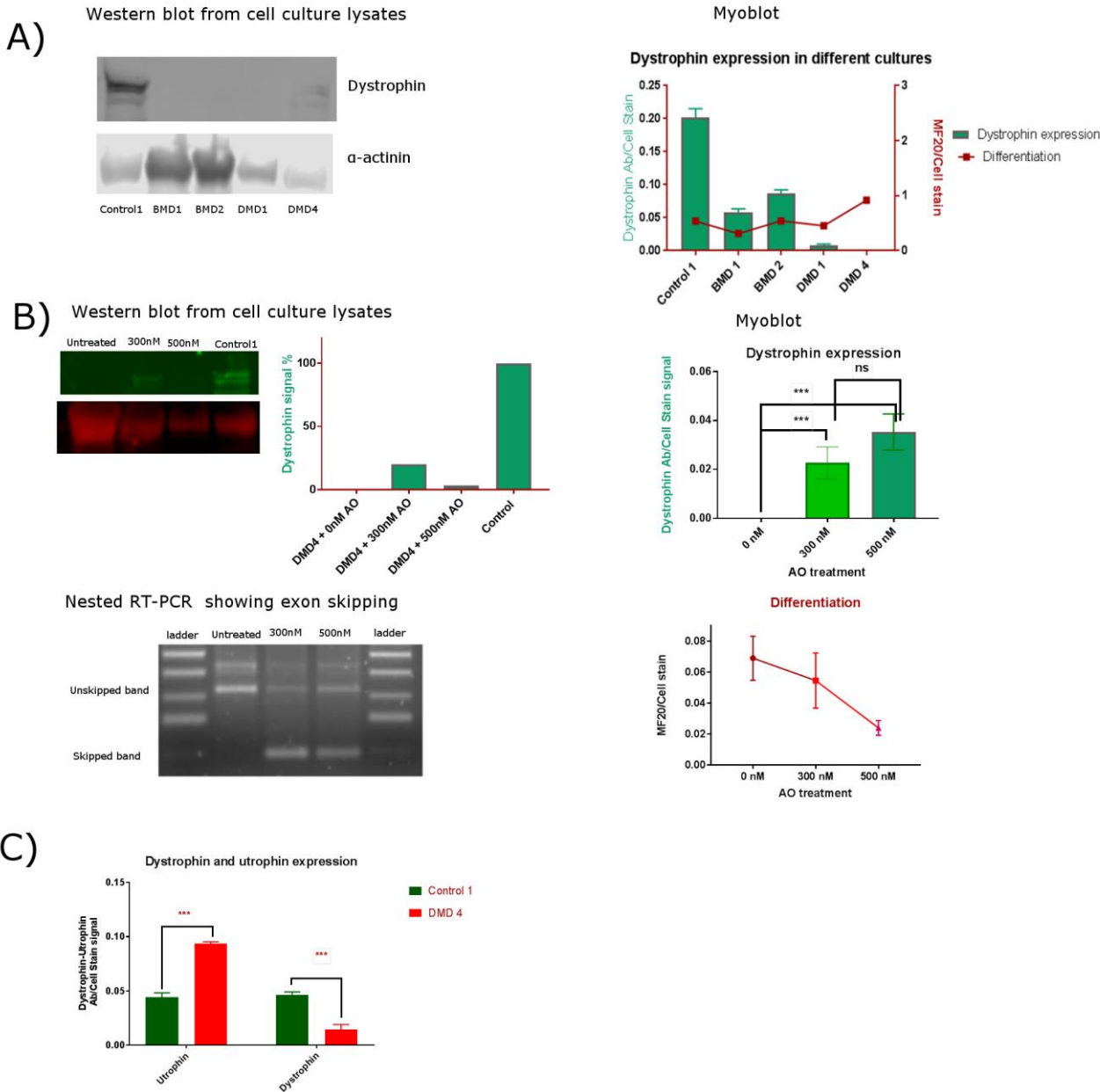
Dystrophin quantification after treatment with dystrophin restoration drugs

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We studied patients' cultures treated with antisense oligonucleotides that aim to restore the open reading frame and expression of dystrophin protein (**Figure 4B**) [6]. We tested this on myoblots and on replicate 6 well plates that we used to extract RNA and protein for nested RT-PCR and western blotting analysis, the standard methods used for the

selection of antisense sequences. Myoblots were able to show protein expression better than western blots but, in many cases, it was possible to detect exon skipping at RNA level after treatment at lower doses than those needed to detect dystrophin restoration to using our myoblot method or western blotting method.



Myoblots: dystrophin quantification by in-cell western assay.
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Figure 4 Study of protein expression in cultures, both naturally and in response to treatment. A) Left: Standard western blot assay using lysates from differentiated muscle cell cultures shows a band for dystrophin in the control and a faint one in one of the DMD samples. α -actinin is used as a loading control. Right: Myoblot quantification of the same cultures (n= 24 wells). Bars show dystrophin expression while the line shows myosin heavy chain expression, as a marker of differentiation. Measurements are normalised to cell number. B) Patient DMD4's response to AO treatment shown by RT-PCR, western blotting and myoblot: RT-PCR analysis of treated cells clearly shows exon skipping at RNA level. Western blot of cell lysates (lysate corresponding to one well of a 6-well plate of control lysate and two wells for DMD cells) after treatment show dystrophin expression and ICW analysis of cultures also shows dystrophin expression with significant differences ($p < 0.0001$) between untreated and treated samples, but not significant amongst the different AO concentrations tested (one way ANOVA). C) Myoblots analysis of control and DMD cultures showing dystrophin and utrophin expression being significantly different ($p = 0.0007$, Mann-Whitney test).

Quantification of other muscle and muscle differentiation proteins

We used our myoblot technique to study in the same plates dystrophin and its orthologue protein utrophin. Whereas dystrophin is naturally expressed in controls, reduced in BMD patients and almost missing from DMD patients, utrophin is not expressed postnatally in controls, but it is overexpressed in DMD and BMD patients. We were able to confirm this inverse correlation also using our myoblot technique. (Figure 4 C)

DISCUSSION

Quantifying sarcolemma-associated proteins has been for years a matter of academic investigation with little interest outside academia. However, with the recent development of new therapies aiming to restore the expression of many of such proteins, it is vital to develop accurate quantification methods for these proteins. We devised in the past one of

such methods to be applied to muscle biopsy sections [7] and it has been used since in the characterisation of patient [3, 30] and animal samples [31], and in the evaluation of the efficacy of several clinical trials [4, 32, 33]. Indeed, the recently approved antisense oligonucleotide drug eteplirsen was granted accelerated approval on the basis of the quantification of dystrophin expression [12].

We have now developed a method that we think may contribute to a faster development of new treatments for neuromuscular disorders, as it allows the accurate quantification of many proteins implicated in such disorders with the use of a limited number of cells (figure 1). We could accurately distinguish between controls and patients and detect the response to treatment. This system has several advantages over western blotting: it does not only use less cells, it also shows more precise and reproducible

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quantification data. The plate format allows for a robust quantification over larger number of replicates and for the study of more targets and/or samples.

It should be noted that the method we describe should always be used with either a control or an untreated sample as a reference, and that quality controls should be considered before analysing the data of the proteins of interest. We have chosen to normalise our data with a cell number marker (CellTag 700 Stain) and use the myosin heavy chain antibody MF20, a marker for differentiation, as a quality control to aid in the evaluation of the results. As the differentiation status of the cell cultures was vital for the development of our technique, we have also in our hands a valuable tool to study muscle differentiation, and we are currently exploring its use in muscle cell biology studies. Also, whereas this technique is superior to western blotting when testing protein expression restoration in cultured cells, studies at RNA level would still be necessary as they can provide efficacy data in a shorter period of time at sometimes lower doses. It should be noted, however, that results at RNA level should be corroborated with results at protein level, and this is where our myoblots could be of use.

The first targets described in our myoblots are dystrophin and utrophin, both proteins whose expression is the goal of several new therapies. The

method we describe would allow several candidate therapies to be thoroughly tested and it might, in a future of truly personalised medicine, allow the selection of personalised treatments for specific patients. We hope this would be a useful tool for the muscle community.

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AUTHOR CONTRIBUTIONS

ERdY performed the majority of the experiments and analysed the data, IGJ performed some experiments and analysed the data, KM performed the immortalisation experiments, VAG

Myoblots: dystrophin quantification by in-cell western assay.

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designed the study, analysed the data, wrote the first draft and revisions. All authors critically read the manuscript.

AUTHOR DISCLOSURE STATEMENT

The authors declare no competing financial interests.

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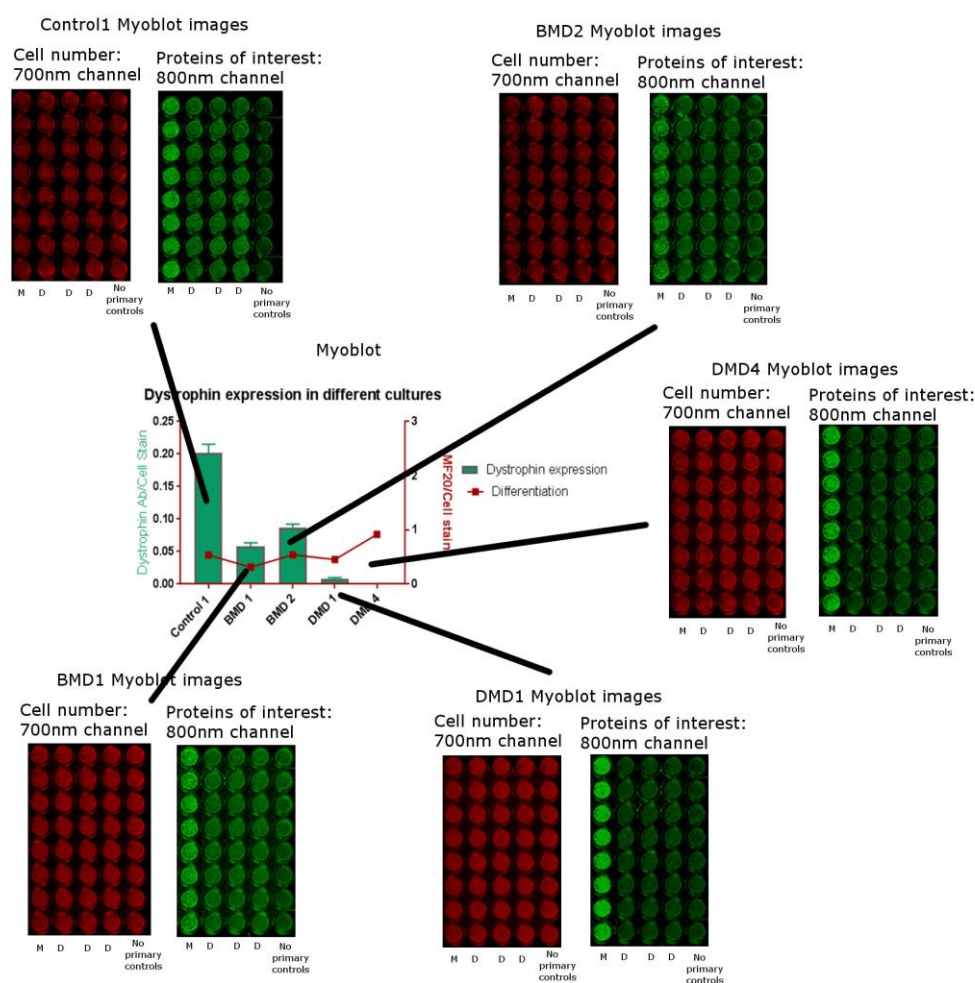
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SUPPLEMENTARY FIGURES



Source data for figure 4 A)

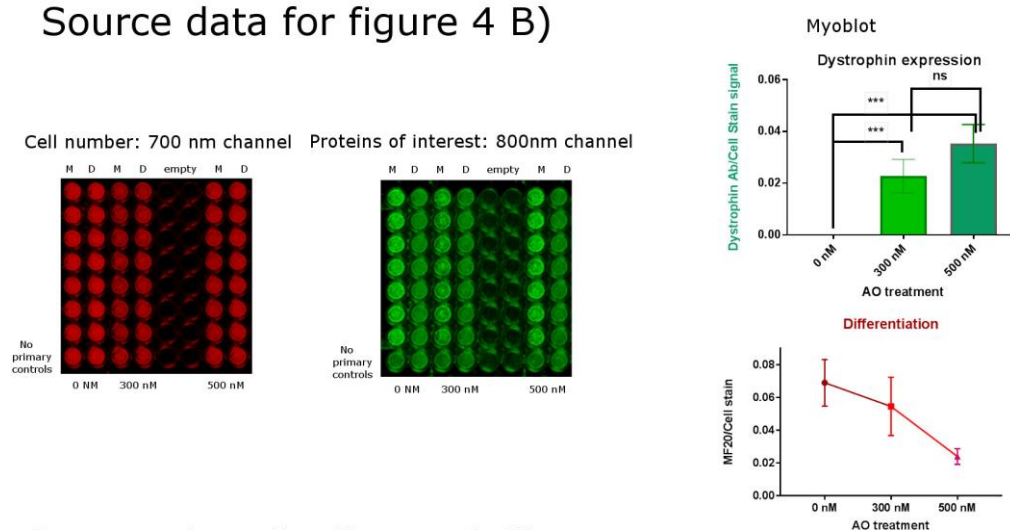
Supplementary figure 1. Source data for figure 4 A

Images of the culture plates used to generate each of the bars and data points in the myoblot graph in figure 4A are shown. Dystrophin expression bars (green) were generated with the intensity data in wells labelled D, for dystrophin, while the differentiation line (red in the graph) was generated with the intensity data from wells labelled M, for the MF20 antibody. The intensity signal from each individual well measured at 800 nm (green, specific antibodies) was normalised for cell number variations using the corresponding signal captured in the 700nm channel (Red, Cell Stain). After subtracting the average signal of background controls in the last column of each plate (labelled "No primary" controls), the resulting data was summarised in the myoblot graph.

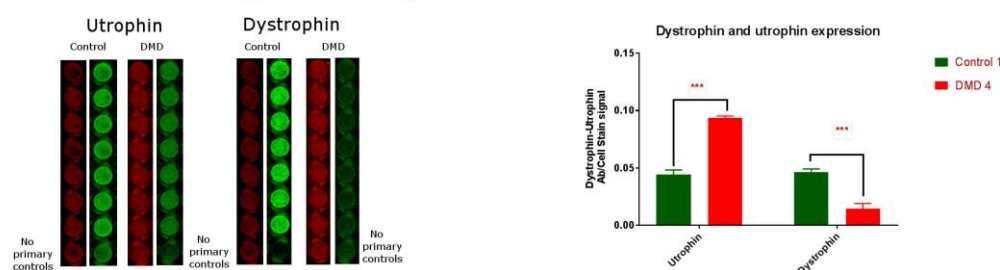
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Source data for figure 4 B)



Source data for figure 4 C)



Supplementary figure 2. Source data for figures 4 B and C

Source data for figure 4 B: Images of the culture plate used to generate the myoblot graphs in figure 4B are shown. In this case, dystrophin expression and differentiation were shown in two graphs to make it easier to read. Dystrophin expression bars (green) were generated with the intensity data in wells labelled D, for dystrophin, while the differentiation graph (red line) was generated with the intensity data from wells labelled M, for the MF20 antibody. The intensity signal from each individual well measured at 800 nm (green, specific antibodies) was normalised for cell number variations using the corresponding signal captured in the 700nm channel (Red, Cell Stain). After subtracting the average signal of background controls in the lower row (labelled "No primary" controls), the resulting data was summarised in the graphs.

Source data for figure 4 C: Images of the culture plate used to generate the myoblot graph in figure 4C are shown. Utrophin and dystrophin were studied neighbouring wells in plates seeded with Control and DMD cultures, but they have been separated in the image for clarity. Dystrophin and utrophin signal was measured in wells at 800 nm (green) and normalised for cell number with the signal measured at 700 nm (red). After subtracting the average signal of background controls in the lower row (labelled "No primary" controls), the resulting data was summarised in the graphs.

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