Supplementary Tables and Figures to

# MyoScreen, a High-Throughput Phenotypic Screening Platform Enabling Muscle Drug Discovery

Joanne Young[1,*], Yoran Margaron[1], Mathieu Fernandes[1], Eve Duchemin-Pelletier[1], Joris Michaud[1], Mélanie Flaender[1], Oana Lorintiu[1], Sébastien Degot[1], Pauline Poydenot[1]

CYTOO SA, Minatec – BHT – Bât 52, 7 parvis Louis Néel, 38030 Grenoble cedex 9

*Corresponding author: jyoung@cytoo.com

| Primary antibody | Raised in | Dilution | Reference/Source |
|---|---|---|---|
| Monoclonal fast myosin heavy chain (clone MY-32) | mouse | 1:400 | M4276-Sigma-Aldrich, St-Quentin-Fallavier, France |
| Polyclonal cardiac troponin T | rabbit | 1:4000 | ab45932-abcam, Cambridge, UK |
| Monoclonal sarcomeric α-actinin (clone: EA-53) | mouse | 1:1000 | ab9465-abcam, Cambridge, UK |
| Polyclonal dystrophin | rabbit | 1:800 | ab15277-abcam, Cambridge, UK |
| Monoclonal nicotinic acetylcholine receptor (AChR) | rat | 1:370 | mAb35-Development Studies Hybridoma Bank (DSHB), Iowa, US |

**Supplementary Table S1A. Primary antibodies used in the study.** The antibodies developed by J. Lindstrom (anti-AChR) were obtained from the DSHB developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology, Iowa City, IA 52242, USA.

| Donor Description | Tissue origin | Race | % desmin positive myoblasts | Myoblasts doubling time | Myoblast mean area |
|---|---|---|---|---|---|
| Female, 4 years | Quadriceps | White | 83% | 24h | 1 289 µm$^2$ |
| Female, 16 years | Quadriceps | White | >60%‡ | - - | - - |
| Female, 20 years | Quadriceps | White | 99% | 44h | 2 847 µm$^2$ |
| Male, 19 years | Quadriceps | Black | >60% ‡ | - - | - - |
| Male, 21 years | Quadriceps | White | 97% | 48h | 2 735 µm$^2$ |
| Male, 37 years | Quadriceps | White | 99% | 136h | 2 709 µm$^2$ |
| Male T2D*, 68 years | Gracilis | White | 99% | 476h | 3 562 µm$^2$ |

**Supplementary Table S2. Donor age, sex, race, tissue origin and resulting myoblast characteristics.** Plated myoblasts were fixed after 24h and stained with anti-desmin antibody. The percent desmin-positive cells and mean myoblast area was determined by image analysis. Only preparations with myogenic purity of >80% desmin-positive cells were used in this study to allow reliable statistical comparison between donors. For calculation of doubling time, 1 million myoblasts were plated in T75 cell culture flasks in growth medium and trypsinized and counted when the culture reached approximately 80% confluence. T2D*: cells derived from a donor with type 2 diabetes. ‡ according to commercial supplier's quality control criteria and not tested in-house; - - indicates these donors were not characterized for myoblast doubling time or mean area in-house.

**S1A**

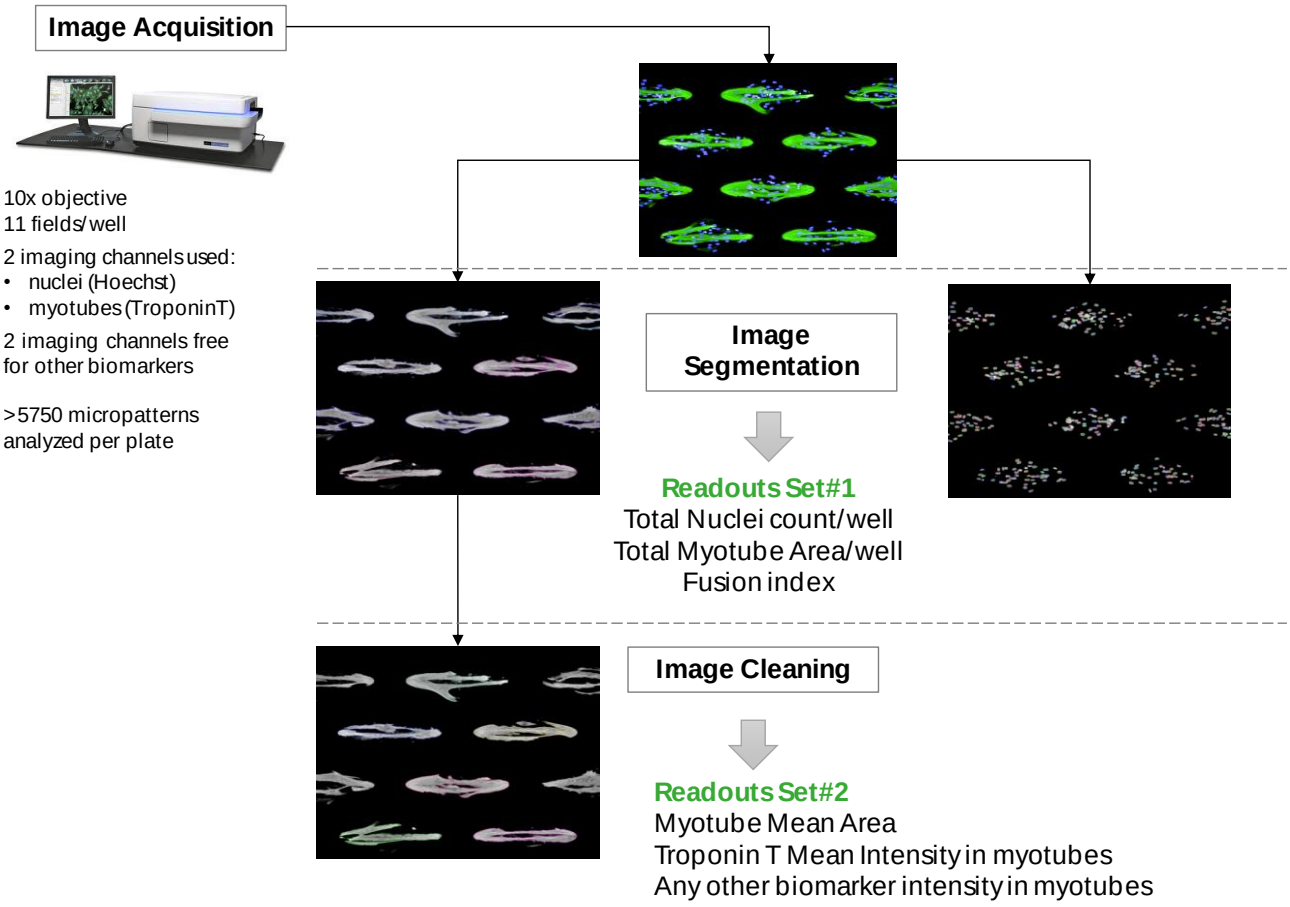
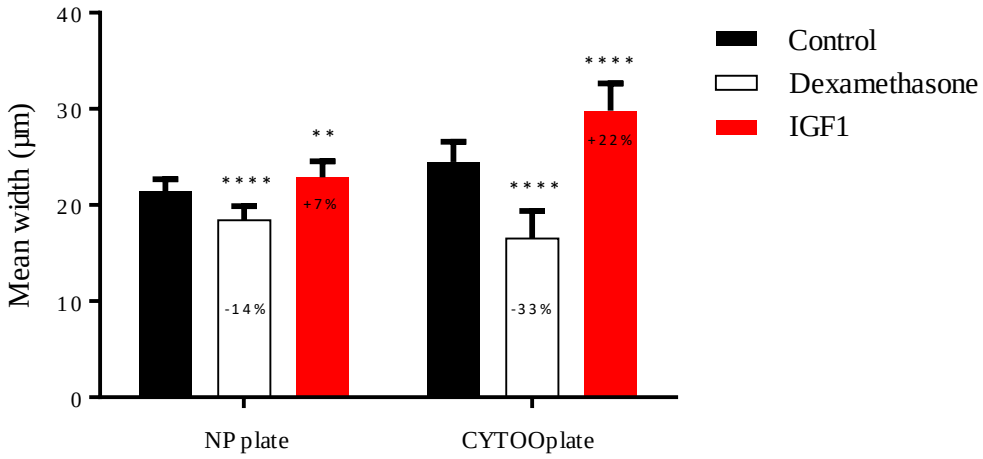


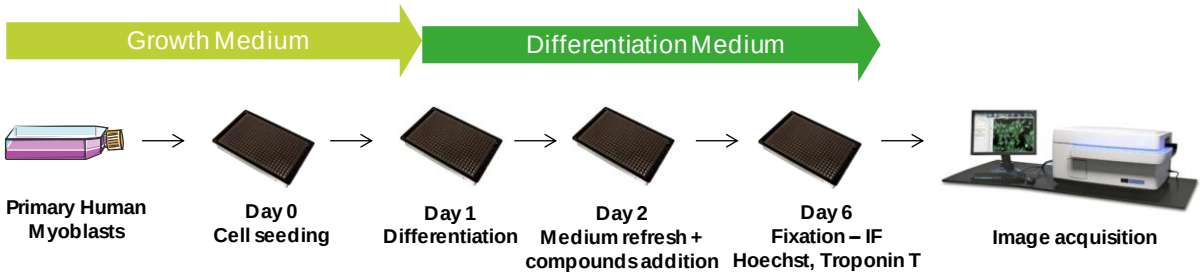
Image Acquisition
10x objective
11 fields/well
2 imaging channels used:
• nuclei (Hoechst)
• myotubes (TroponinT)
2 imaging channels free for other biomarkers
>5750 micropatterns analyzed per plate
Image Segmentation
Readouts Set#1
Total Nuclei count/well
Total Myotube Area/well
Fusion index
Image Cleaning
Readouts Set#2
Myotube Mean Area
Troponin T Mean Intensity in myotubes
Any other biomarker intensity in myotubes

**S1B**


Mean width (µm)
40
30
20
10
0
****
**
+7%
-14%
****
****
+22%
-33%
NP plate
CYTOOplate
Control
Dexamethasone
IGF1

**Supplementary Figure S1A and S1B. Image processing and analysis for phenotypic screening.** (**A**) A dedicated algorithm was developed in Acapella. First, detection and segmentation of myotubes and nuclei is performed using the Troponin T and Hoechst staining respectively. The segmentation is set-up to avoid detecting background noise and eliminate aberrant small myotube structures. Next, specific whole-well readouts are calculated such as total nuclei count, total myotube area and the fusion index (percentage of nuclei included in the myotube staining/total number of nuclei x 100). Finally, an image clean-up step is performed to remove myotubes that touch the borders of the image. The remaining valid myotubes are analyzed to extract parameters concerning myotube size including myotube average width and area, and the number of nuclei per myotube. On average, >5750 micropatterns per 96-well plate are analyzed in total, with each micropattern containing several myotubes. Two other imaging channels are free to assess other biomarkers such as MHC and AChRs. (**B**) Confirmation of atrophy and hypertrophy detection in non-patterned and micropatterned myotubes (donor female, 16-years old) with an increased assay window apparent for micropatterned myotubes. Myotubes were fixed, imaged and analyzed after 5 days of differentiation with 100 µM dexamethasone or 15 nM IGF1 addition at 24 h after the beginning of differentiation. Average myotube width is shown in µm. n = 16 wells. $^{**}p \leq 0.01$, $^{***}p \leq 0.0001$ as analyzed by Mann-Whitney nonparametric test.

S2




**Supplementary Figure S2. Global workflow for drug screening.** Myoblasts are seeded onto micropatterns in growth medium (Day 0). The next day medium is changed for differentiation-inducing medium (Day 1). Differentiation medium is refreshed and compounds added (Day 2). Myotubes are incubated for a further 96 hours in the presence of compounds. On Day 6 of the protocol, equivalent to 5 days of differentiation and 96h of drug treatment, plates are fixed and stained for at least two markers, nuclei and myotubes using Hoechst and Troponin T antibody. Images are acquired at 10x magnification with an automated Operetta microscope system for all the phenotypic assays except the AChR clustering assay that relies on use of the 20x objective.

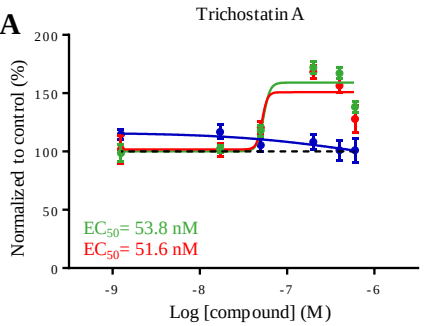
| Hypertophy inducer | % Max activity | $EC_{50}$ |
|---|---|---|
| IGF1 | 156% | 95.5 pM |
| Trichostatin A | 172% | 53.8 nM |

| Atrophy inducer | % Min activity | $IC_{50}$ |
|---|---|---|
| TNFα | 45% | 15.9 pM |
| IL1β | 65% | -- |
| TGFβ | 5% | 15.8 pM |
| Myostatin | 70% | -- |
| Dexamethasone | 40% | -- |

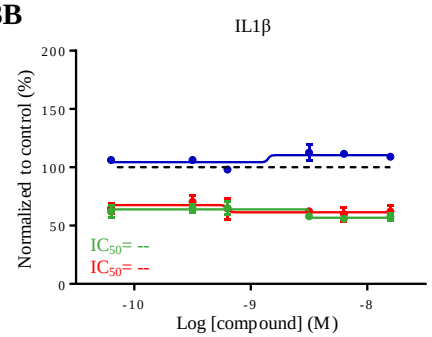
| Statin | $IC_{50}$ Nuclei count | % Min Nuclei count at 100 µM | $IC_{50}$ Fusion index | % Min Fusion index at 100 µM |
|---|---|---|---|---|
| Cerivastatin | 16.8 µM | 1.4% | 23.6 µM | 0% |
| Simvastatin | 12.8 µM | 3.3% | 12.4 µM | 0% |
| Fluvastatin | 6.5 µM | 68.3% | 4.9 µM | 12.9% |
| Atorvastatin | 1.3 µM | 72.9% | 3.3 µM | 25.9% |
| Lovastatin | 939 nM | 54.2% | 559 nM | 8.7% |
| Pravastatin | -- | 83% | -- | 89.8% |

**Supplementary Table S3. Table showing for each of the test compounds: % maximum or minimum activity and $EC_{50}$ or $IC_{50}$ values.** Values were calculated using the fusion index parameter or nuclei count from dose-response curves and GraphPad Prism v6. Control non-treated myotubes were fixed at 100 %. (--): $IC_{50}$ not calculable because response does not achieve a clear minimum even at the lowest concentration due to the limited range of applied concentrations. Differentiated primary myotube cultures from a male 21-year old donor were used for the study.

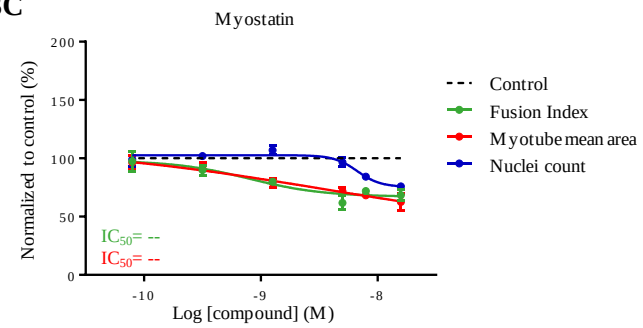
**S3A**




**S3B**




**S3C**

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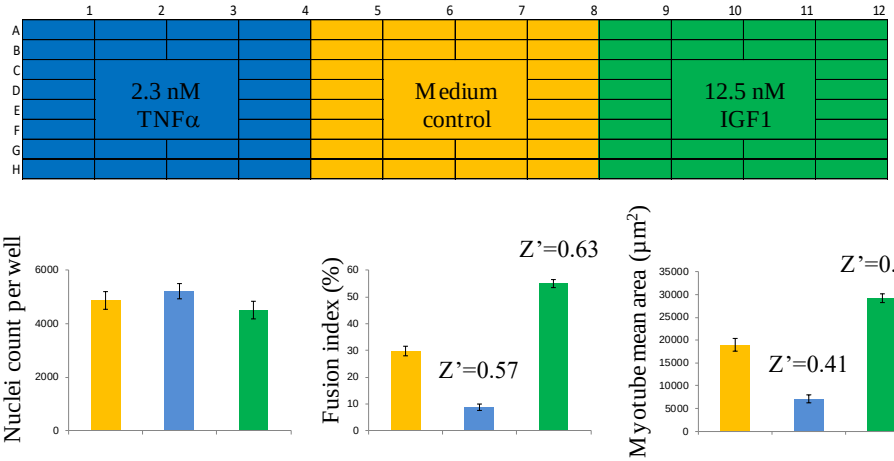
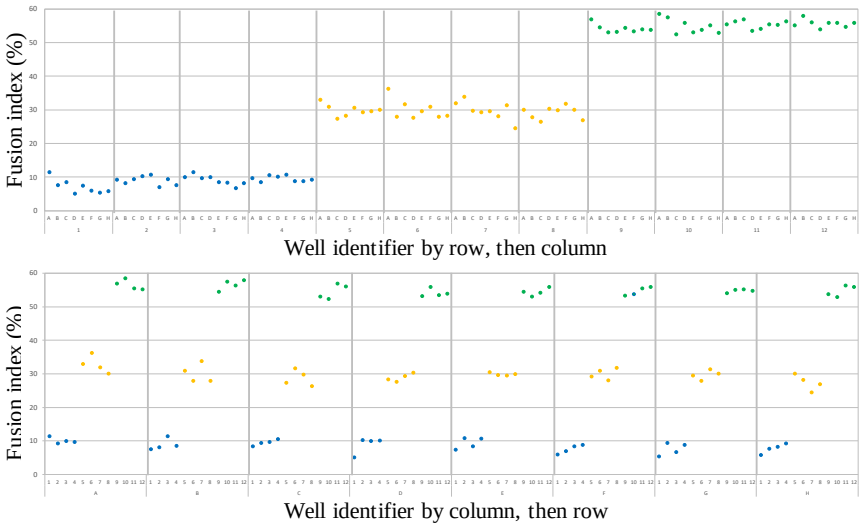
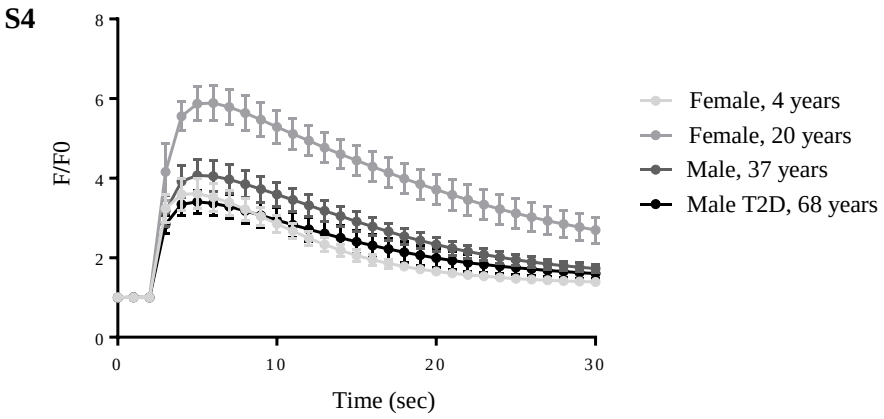



**S3E**

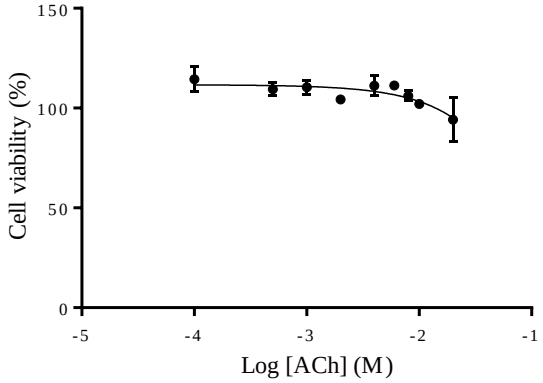


**Supplementary Figure S3A, S3B, S3C, S3D and S3E. Dose-response curves of part of the reference compound panel used to validate the pharmacological relevance of the MyoScreen model (male, 21 years) and a typical CYTOOplate showing assay validation results.** Shown are hypertrophy-inducing trichostatin A (**A**) and atrophy-inducing interleukin-1β (IL1β) (**B**) and myostatin (**C**) with their effect on myotube differentiation (green), size (red) and viability (blue). Values are normalized against control vehicle-treated myotubes. $EC_{50}$ and $IC_{50}$ from the fit curves are shown for fusion index and mean area endpoints. n = 3 wells. (--): values that could not be calculated due to a too small range of applied concentrations. (**D**) Assay validation plate layout showing high (12.5 nM IGF1) and low (2.3 nM TNFα) signal wells and medium control wells (n=32 wells each condition). The non-normalized data was used to measure assay quality which was validated here by the presence of CV <15% and Z'-factor values of >0.3 for the parameters fusion index and mean myotube area for both atrophy and hypertrophy inducing compounds. Results for signal means and S.D concerning the nuclei count, FI and myotube mean area are shown in the graphs below. (**E**) Scatter plot for the results in D with each data point showing fusion index (%) for each individual well of the 96-well plate. Wells are presented in a row-wise (upper plot) and column-wise (lower plot) fashion to better assess noise level of the assay as well as visually check for edge effects and drift patterns. Slight edge effects are present in outer wells which is not surprising given the long duration of the assay. Whenever possible, edge wells were avoided for analyses but are shown here for transparency.

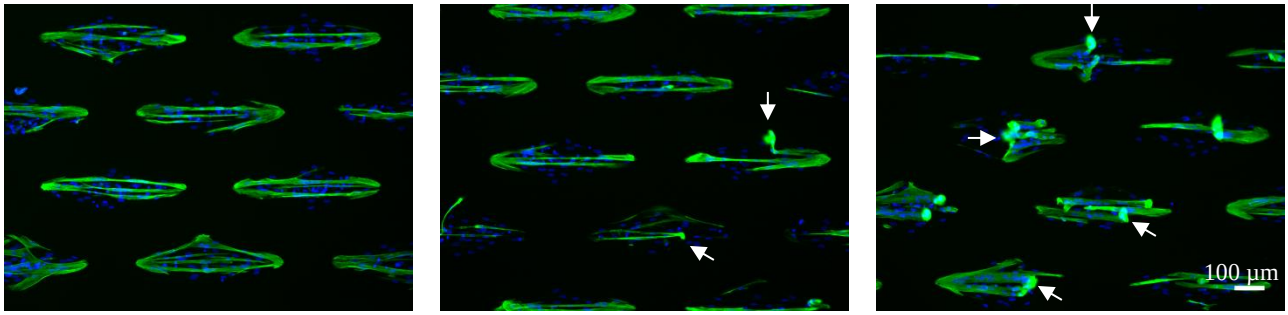



**Supplementary Figure S4. Characterisation of $Ca^{2+}$ transients in myotubes cultured from 4 different donor samples.** Time course of intracellular $Ca^{2+}$ flux after stimulation of day 9 differentiated myotubes with 20 µM ACh, evaluated by monitoring Fluo-4 fluorescence. n = 5 wells. Myotubes from a female 20-year old donor display the highest peak levels of intracellular calcium and there is no apparent correlation of increase in intracellular calcium levels with donor age.

**S5A**




**S5B**



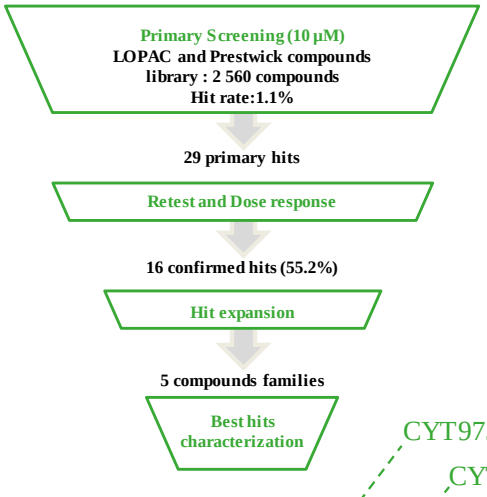

Control cells
No stimulation

Control cells with
100 µM ACh stimulation

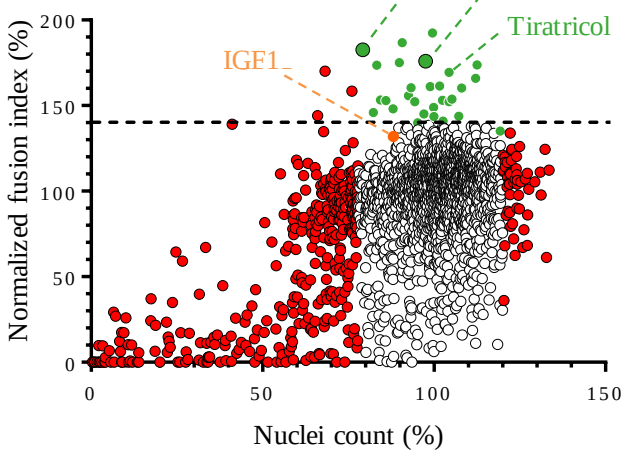
IGF1 treated cells with
100 µM ACh stimulation

**Supplementary Figure S5A and S5B. Setting up the contractile activity assay.** (**A**) Effect of ACh on myotube viability. Myotubes (male, 21-years) were differentiated for 5 days and treated with ACh from 100 µM to 20,000 µM for 5 mins. Measurement of cell viability using an ATP-based assay showed no evidence for myotube toxicity at any ACh concentration. (**B**) Representative images of myotubes (female, 20-years) differentiated for 5 days in the absence (Control) or presence of IGF1, and subsequently stimulated or not with 100 µM ACh. Note the presence of contracted myotubes (labelled with anti-troponin T antibody in green) as retracted structures on the micropatterns (white arrows) that is increased in the case of IGF1 treated myotubes and the lack of contracted myotubes in the absence of ACh stimulation. Nuclei are stained with Hoechst (blue).

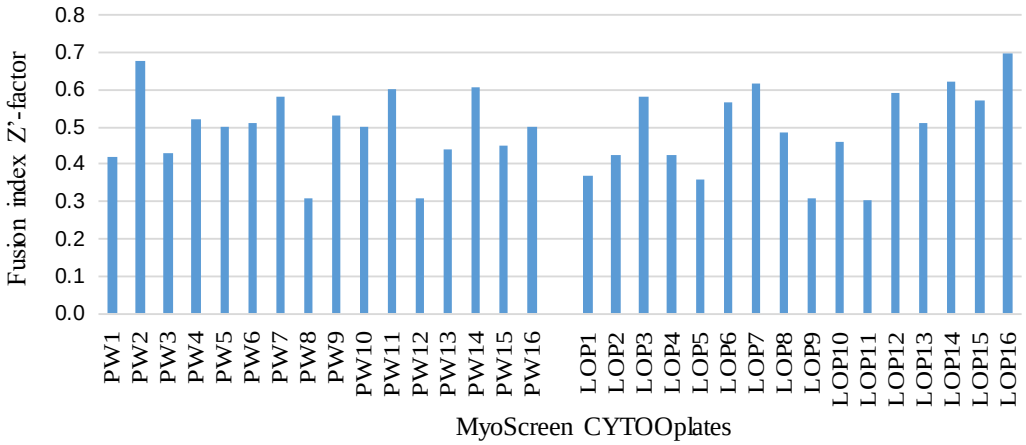
S6A



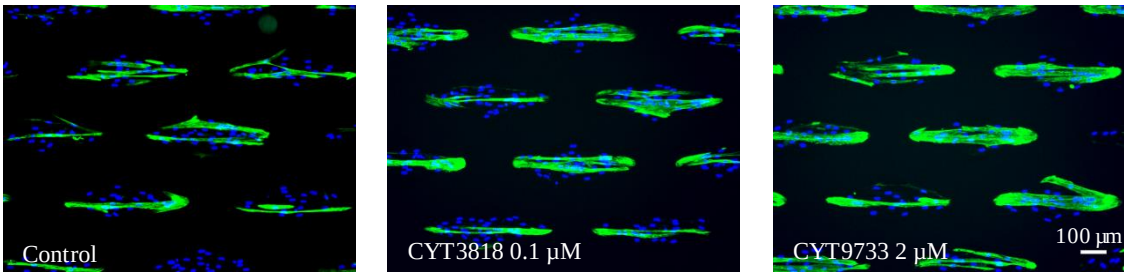

S6B




S6C




S6D



**Supplementary Figure S6A, S6B, S6C and S6D. Primary screen and characterization of hits inducing hypertrophy.** (**A**) Workflow of the screening strategy from the primary screen using the phenotypic hypertrophy/atrophy assay to compound selection resulting in secondary assay characterization of 2 compounds. (**B**) Correlation plot of FI versus nuclei count for the full 2560 compounds of the screen with red dots indicating cytotoxic (<78% viability) and pro-proliferative compounds (>120% viability) that were excluded from further analysis. The dotted line indicates the >140% fusion index cut-off used to define positive hits shown in green. Coded compounds as well as tiratricol are indicated with labels as well as IGF1. The screen was performed using a male, 21-year old donor. (**C**) Z'-factor data graph showing values for each assay CYTOOplate of the primary screen (male, 21-year old). All 32 plates have Z' ≥ 0.3 passing the acceptance criterion for imaging screens. Values were calculated from 3 vehicle control wells versus 3 positive control wells (18.75 nM IGF1). PW: Prestwick plates LOP; LOPAC$^{1280}$ plates (**D**) Typical images of differentiated myotubes (female, 20-years) after treatment for 4 days with 0.1 µM CYT3818 or 2 µM CYT9733. Troponin T identifies myotubes in green and Hoechst marks the nuclei in blue.

**Supplementary Movie S1**. **Cholinergic stimulation of intracellular calcium release and myotube contraction.** Movie from time-lapse video microscopy simultaneously showing calcium flux changes in fluorescence images (top) and myotube contraction in phase contrast images (bottom) in response to addition of 20 µM ACh at t=3 seconds on myotubes (male, 19-years) differentiated for 8 days. Calcium is detected by loading myotubes with the calcium indicator Fluo-4 AM.

**Supplementary Movie S2. KCl stimulation of intracellular calcium release and myotube contraction.** Movie from time-lapse video microscopy simultaneously showing calcium flux changes in fluorescence images (top) and myotube contraction in phase contrast images (bottom). KCl at 20 mM was added at t=3 secs on myotubes (male, 19-years) differentiated for 8 days. Calcium is detected by loading myotubes with the calcium indicator Fluo-4 AM.

**Supplementary Movie S3**. **Intracellular calcium release and myotube contraction in response to 4-CmC stimulation of myotubes.** Movie from time-lapse video microscopy simultaneously showing calcium flux changes in fluorescence images (top) and myotube contraction in phase contrast images (bottom). 4-CmC at 400 µM was added at t=3 secs on myotubes (male, 19-years) differentiated for 8 days. Calcium is detected by loading myotubes with the calcium indicator Fluo-4 AM.