**Supplementary Information**

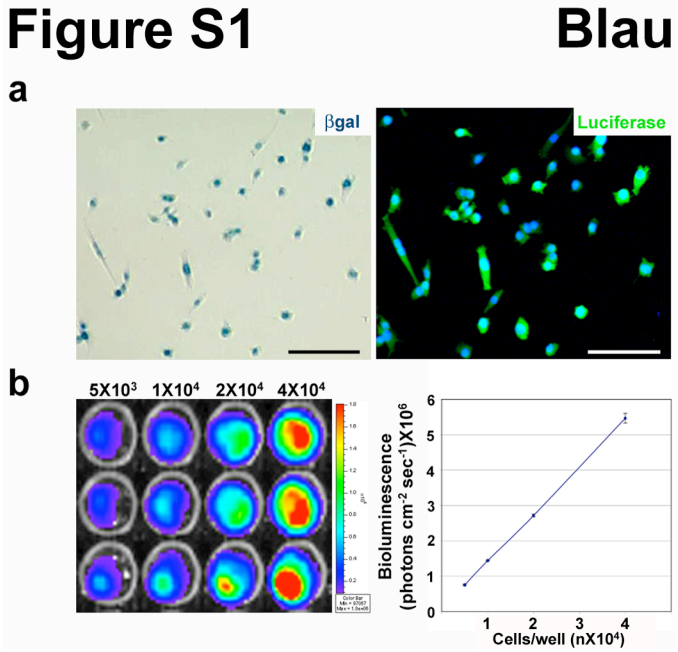


**Figure S1 | Linearity of bioluminescence imaging *in vitro*. a**, Primary myoblasts were isolated from *Myf5-nLacZ/FLuc* double transgenic mice (β-gal, left panel, and Luciferase, right panel). Scale bars, 100μm. **b**, Increasing numbers of *Myf5-nLacZ/FLuc* myoblasts were plated in a 96-well plate and imaging was performed immediately after plating. Bioluminescence image of the 96-well plate (number of cells plated is indicated on the top, color scale on the right: minimum, $1.0X10^5$ photons $cm^{-2}$ $sec^{-1}$, maximum, $18.0X10^5$ photons $cm^{-2}$ $sec^{-1}$; left). Graph of bioluminescence data represented as average ± s.e.m. (n=5; P≤0.0001; right).

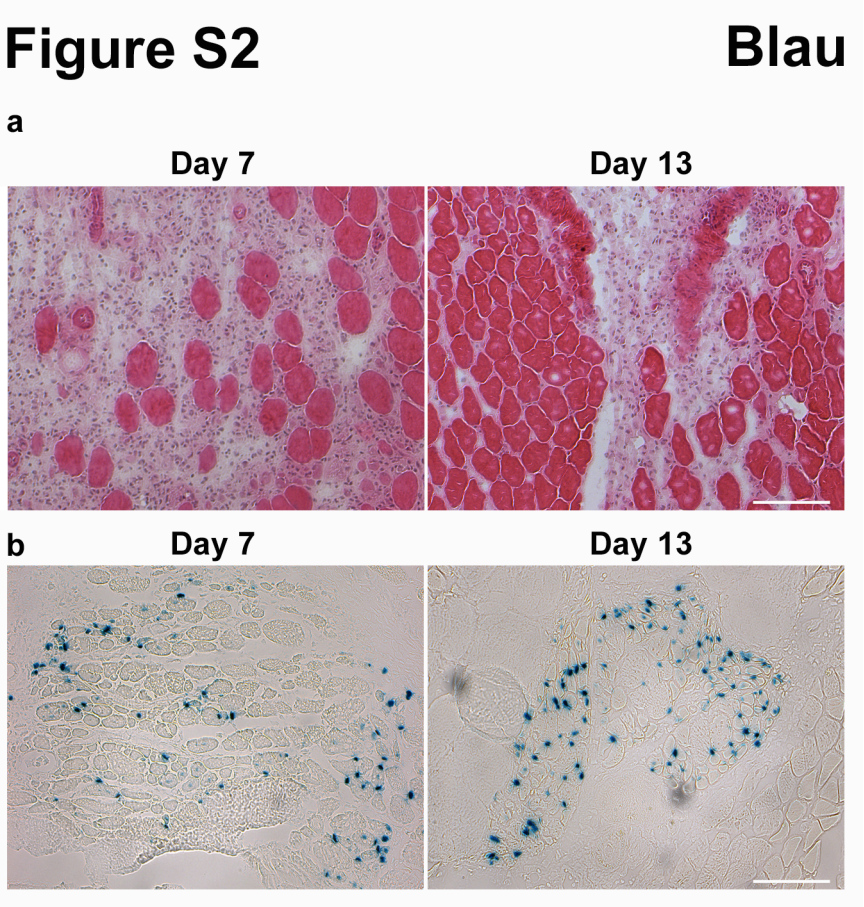


**Figure S2 | Muscle regeneration timing in irradiated legs of NOD/SCID mice after notexin damage. a**, Legs of NOD/SCID mice were irradiated with 18Gy. Two months later, Tibialis Anterior muscles were damaged with Notexin and tissue harvested at the days indicated above. Regeneration is still ongoing 13 days post damage in irradiated tissues. H&E staining indicates myofibers in red, and infiltrating immune cells in purple. Scale Bars, 100 μm. **b**, Legs of NOD/SCID mice were irradiated with 18Gy and 500 muscle stem cells from double transgenic mice were transplanted (as in Fig. 3). After engraftment plateau was reached (7 weeks), muscles were damaged with Notexin and harvested at the

days indicated on top. β-galactosidase histochemistry revealed several Myf5$^{+}$ cells at day 13, indicative of ongoing regeneration. Scale Bars, 120 μm.

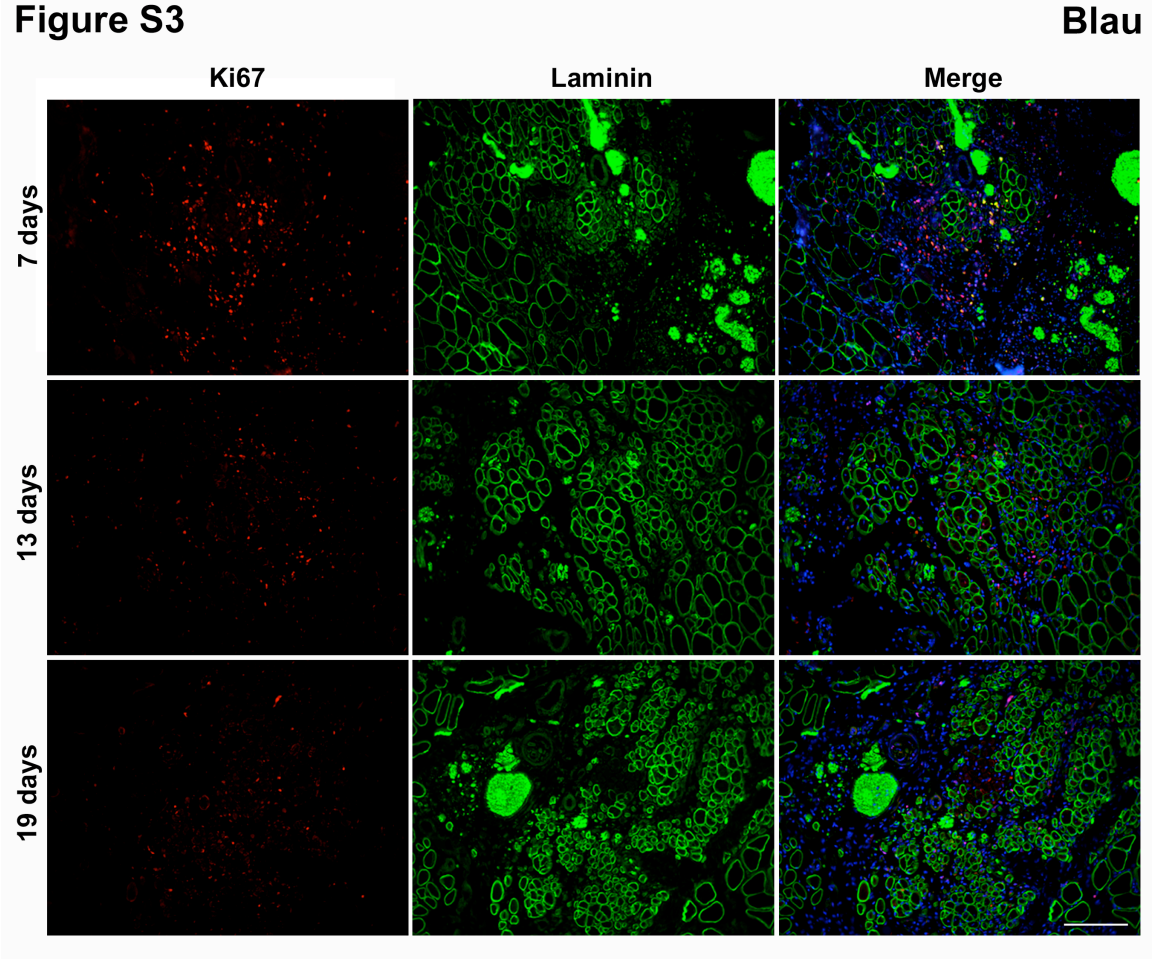


**Figure S3 | Cell proliferation persists during NTX-induced muscle regeneration.** Legs of NOD/SCID mice were irradiated with 18Gy and transplanted with muscle stem cells. 4 weeks later, Tibialis Anterior muscles were damaged with Notexin and tissue harvested at the days indicated on the left and immunostained for the proliferation marker Ki67 and for the basal lamina marker Laminin. Regeneration is still ongoing 19 days post damage in these experimental conditions, as shown by the presence of proliferating cells ($Ki67^{+}$) and small newly forming myofibers. Nuclei (blue), Ki67 (red), Laminin (green). Scale Bars=130μm.

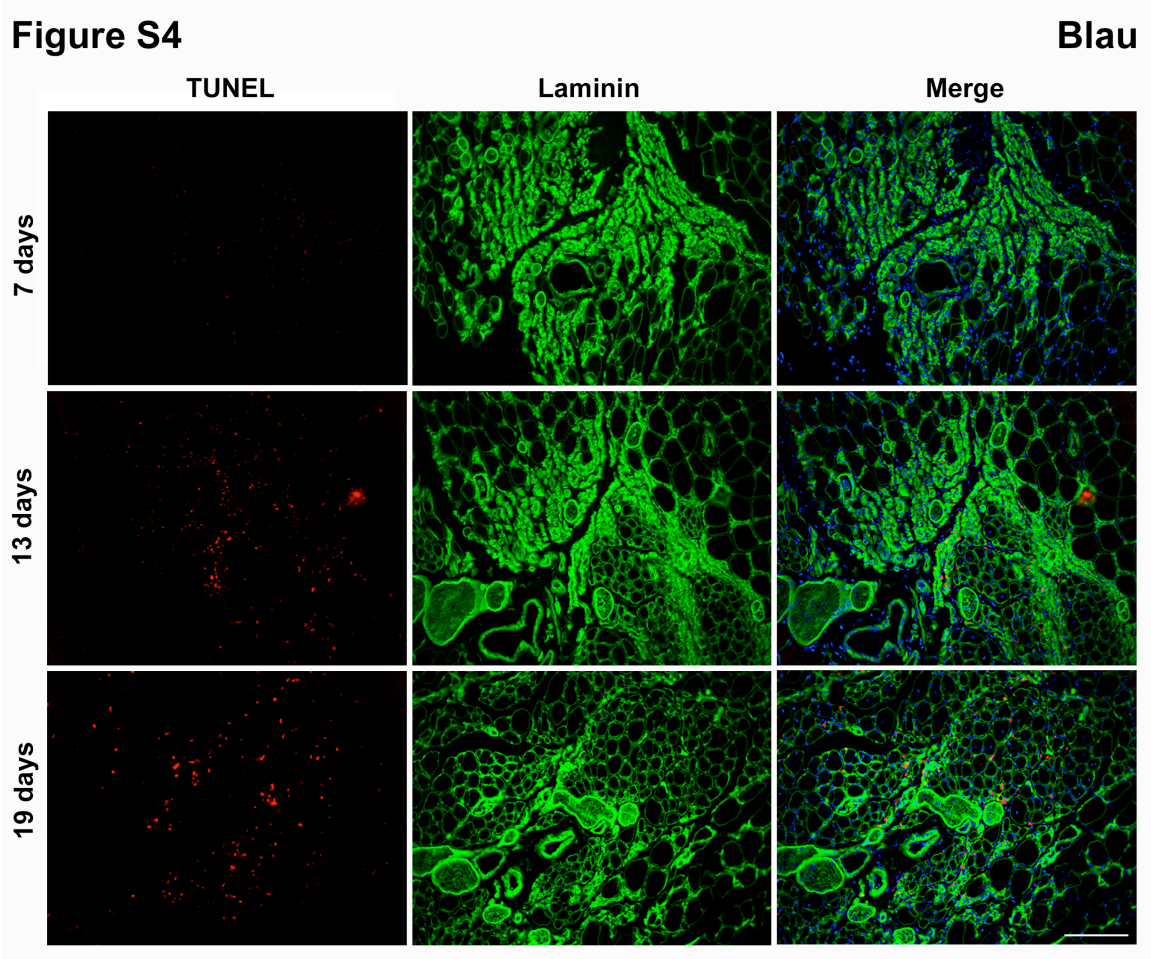


**Figure S4 | Apoptosis increases over time during NTX-induced muscle regeneration.** Legs of NOD/SCID mice were irradiated with 18Gy and transplanted with muscle stem cells. 4 weeks later, Tibialis Anterior muscles were damaged with Notexin and tissue harvested at the days indicated on the left and immunostained for apoptotic cells (TUNEL) and for the basal lamina (Laminin). Apoptotic cells are visible, and they increase in number over time from 7 to 19 days post injury, indicating a role of cell death in tissue homeostasis during regeneration. Nuclei (blue), TUNEL (red), Laminin (green). Scale Bars=130μm.

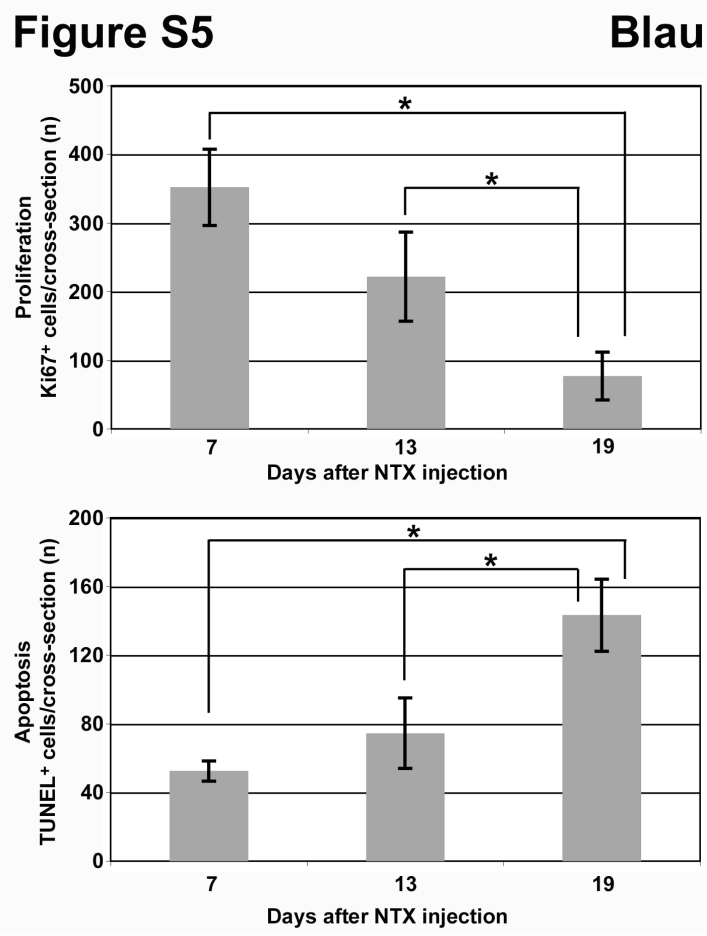


**Figure S5 | Time course of muscle regeneration in irradiated/transplanted NOD/SCID mice.** Quantification of mouse muscles shown in Figures S3 and S4. High numbers of proliferating ($Ki67^+$) cells are present at day 7 post-notexin injury (top panel), and they keep proliferating at day 13 and day 19, consistent with the peak of luciferase activity (Fig. 3a). Conversely, cell death ($TUNEL^+$ cells) progressively increases over time and is highest at day 19 (bottom panel), when luciferase activity starts decreasing (Fig. 3a). The graphs show average±s.e.m. (n=4, asterisk=$P<0.05$). These results show that during muscle regeneration a balance between cell proliferation and death is involved in

restoring tissue homeostasis and support the time-course of luciferase activity shown in Figure 3.

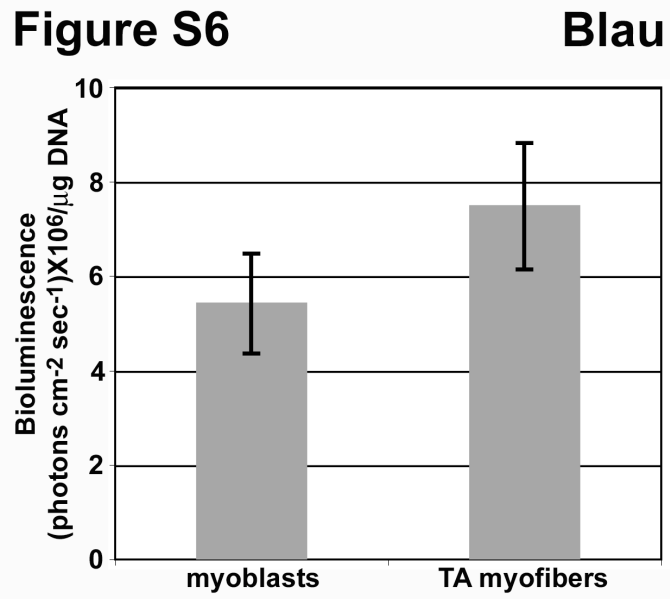


**Figure S6 | Luciferase activity is not affected by muscle differentiation.** Proliferating myoblasts or TA myofibers derived from transgenic mice expressing constitutive luciferase were lysated and assayed in a 24-well plate for Luciferase activity. Bioluminescence per microgram of DNA is not significantly different between myoblasts and myofibers, indicating that this assay is a useful readout for quantifying numbers of donor-derived nuclei in transplanted muscles. Graph of bioluminescence values/μg DNA represented as average ± s.e.m. (n=4; P>0.05).

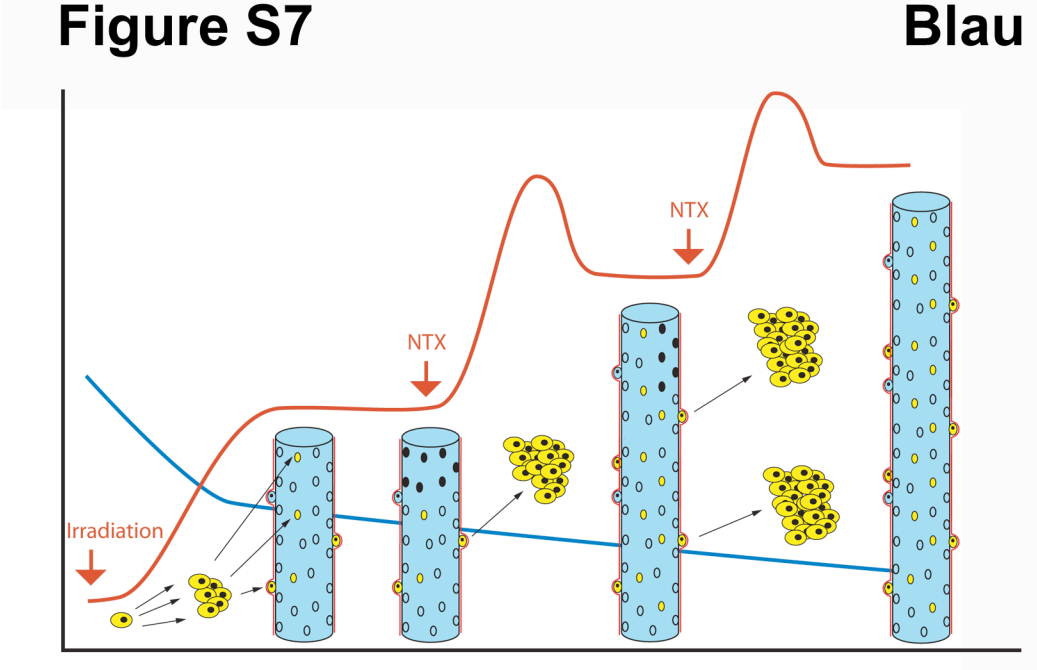


**Figure S7 | Schematic representation of the dynamics of muscle stem cell behavior *in vivo: three waves of proliferation*.** Using bioluminescence imaging of transplanted muscle stem cells, the magnitude and kinetics of their proliferative response (red line) relative to more committed myoblasts (blue line) was analyzed in a manner previously not possible. (a) Following transplant into muscles depleted of endogenous stem cells by irradiation, a first wave of approximately 100-fold expansion of cells occurs within two weeks, after which a plateau is reached, indicating that homeostatic conditions have been obtained. (b) Following injury by NTX injection, muscle stem cells undergo a second wave of rapid 80-100-fold proliferation within 2 weeks. (c) Following another NTX injury a third wave of expansion of similar magnitude and time course is observed. Only stem cells exhibit this behavior. Myoblasts, the more specialized

mononucleated progeny of stem cells, are incapable of yielding such waves of proliferation. Muscle fiber drawing was adapted from Collins et al. [3].

**Methods**

Animals.

All protocols were approved by the Stanford University Administrative Panel on Laboratory Animal Care. *Myf5-nLacZ* transgenic mice and *L2G85* (*Fluc*) strain ubiquitously expressing luciferase from the ACTB promoter were used to generate the double transgenic animals. The GFP transgenic mice were a kind gift from Irving Weissman (Stanford University, California). The NOD/SCID immunodeficient mice were purchased from the Jackson Laboratories.

Cell Transplantation, Notexin Damage and Imaging.

NOD/SCID mice were anesthetized with isofluorane and shielded in a lead-jig so that only the legs were exposed to the radiation source. A single dose of 18Gy was administered to the legs and cell transplantation was performed on the same day. Freshly isolated muscle stem cells or primary myoblasts from the same double transgenic mice were resuspended in 2.5% goat serum in PBS and a 10μl of cell suspension (with different cell concentrations, as indicated in the text) was injected intramuscularly into the Tibialis Anterior (TA) muscles of recipient mice. For local tissue injury, mice were anesthetized with isofluorane and a single 10μl injection of notexin (10μg/ml, Latoxan, France) was injected into the TAs of recipient mice.

Cell Culture.

Cells were isolated from muscle tissue by enzymatic dissociation as described above. Cells were plated on dishes coated with Laminin (Roche) in F10/DMEM

(50/50)+15%FBS+2.5ng/ml bFGF (GM) for proliferation and in DMEM+2% horse serum (DM) for differentiation.

Image acquisition of immunofluorescence and histology.

Images of muscle transverse sections were acquired using an epifluorescent microscope (Axioplan2; Carl Zeiss MicroImaging, Inc.), Fluar 20X/0.75 objective lens, and a digital camera (ORCA-ER C4742-95; Hamamatsu Photonics). The software used for acquisition was OpenLab 4.0.2 (Improvision). Images of cell cultures were acquired using a laser-scanning confocal microscope (LSM510; Carl Zeiss MicroImaging, Inc.) using a Plan NeoFluar 20X/0.50 objective lens and maximum optical sections with the LSM software. All images were composed and edited in Photoshop 7.0 (Adobe). Background was reduced using brightness and contrast adjustments, and color balance was performed to enhance colors. All the modifications were applied to the whole image using Photoshop 7.0 (Adobe).

Luciferase activity assay in protein extracts.

Myoblasts or TA myofibers were isolated from transgenic mice constitutively expressing luciferase and plated in a 24-well plate. Immediately after, cells were lysated as previously described[18]. After complete lysis, Luciferin substrate (1mM) was added to the protein extracts and bioluminescence was measured. In aliquots of the same samples, DNA was extracted, quantified with NanoDrop ND-1000 (Thermo Fisher Scientific) and luciferase activity was normalized per microgram of DNA.

Single cell RT-PCR.

*Single cell collection.* Single cells were directly sorted via FACS (Diva, BD) into PCR tubes containing 9-µl aliquots of RT-PCR lysis buffer. The buffer components included commercial RT-PCR buffer (SuperScript One-Step RT-PCR Kit Reaction Buffer, Invitrogen), RNase inhibitor (Protector RNase Inhibitor, Roche) and 0.15% IGEPAL detergent (Sigma). After a short pulse-spin, the PCR-tubes were immediately shock-frozen and stored at -80ºC for subsequent analysis.

*Two-Step multiplex nested single cell RT-PCR.* Cell lysates were first reverse-transcribed using the pairs of gene-specific primers as described by the manufacturer (SuperScript One-Step RT-PCR Kit, Invitrogen). Briefly, the RT-PCR was performed in the same PCR cell-lysis tubes by addition of a RT-PCR-reaction mix containing the gene-specific primer pairs and RNase inhibitor. Genomic products were excluded by designing and using intron-spanning primer sets for the first and second round PCR (see Table 1). Nested RT-PCR ensured greater specificity. The expected PCR-product sizes for the first and second round were approximately 450bp (external primers) and 250bp (internal primers), respectively. In the first step, the reverse transcription reactions were carried out at 55ºC for 30 min, and followed by a 2-min step at 94ºC. Subsequently, 30 cycles of PCR amplification were performed as follows: 94ºC for 20 sec; 60ºC for 25 sec; 68ºC for 30 sec. In the final PCR step, the reactions were incubated for 3 min at 68ºC. The completed reactions were stored at 4ºC.

In a second step of the nested RT-PCR protocol, the completed RT-PCR reaction from the first step was diluted 1:1 with water. One percent of these

reactions were replica transferred into new reaction tubes for the second round of PCR, which was performed for each of the genes separately using fully nested gene-specific internal-primers, for greater specificity, as indicated by the manufacturer in a total reaction volume of 20μl (Platinum Taq Super-Mix HF, Invitrogen). Thirty cycles of PCR amplification were performed as follows: 94ºC for 20 sec; 60ºC for 20 sec; 68ºC for 20 sec. In the final PCR step, the reactions were incubated for 3 min at 68ºC. The completed reactions were stored at 4ºC. Finally, the second-round PCR products were subjected to gel eletrophoresis using one fifth of the reaction volumes and 1.4% agarose gels.

**Table 1:** Primer sequences utilized for single cell PCR

| ***Multiplex genes*** | | |
|---|---|---|
| | Nested Primer Sets | |
| | ***External Primers*** [5'-3'] | ***Internal Primers*** [5'-3'] |
| Pax7 5' | gaaccacatccgtcacaaga | tttcccatggttgtgtctcc |
| Pax7 3' | gagcactcggctaatcgaac | gtcgcagtgaccgtcctt |
| Pax3 5' | aaccatatccgccacaagat | aaacccaagcaggtgacaac |
| Pax3 3' | ctagatccgcctcctcctct | ggatgcggctgatagaactc |
| Myf5 5' | agacgcctgaagaaggtcaa | ccaccaaccctaaccagaga |
| Myf5 3' | agctggacacggagctttta | ctgttctttcgggaccagac |

## References


[3] C. A. Collins, I. Olsen, P. S. Zammit et al., *Cell* **122** (2), 289 (2005).

[18] T. S. Wehrman, G. von Degenfeld, P. O. Krutzik et al., *Nature methods* **3** (4), 295 (2006).