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(**Fig. 1a** and **Supplementary Fig. 1f**,**g**). The cells assigned to subpopulation 2 expressed high levels of immediate early genes (IEGs, including *Fos*, *Jun* and other activating protein 1 complex genes), *Socs3* and heat-shock proteins (HSPs) (**Fig. 1a**, **Supplementary Fig. 2** and **Supplementary Table 1**). Interestingly, these genes have been described in several satellite cell studies[3,4,6] (**Supplementary Fig. 1h**), which suggests that we identified two functionally distinct subpopulations of SCs.

To validate the existence of the two subpopulations, we performed single-molecule RNA fluorescence *in situ* hybridization (smFISH) on cryosections of *Pax7nGFP* muscles using probes designed against the subpopulation-2-specific genes *Fos* and *Socs3* (**Supplementary Table 2**). We could not detect expression of *Fos* and *Socs3* in cryosections; however, we could detect *Fos* in SCs that had undergone dissociation or both dissociation and FACS, which demonstrated that the SC isolation procedure induces *Fos* expression in a subpopulation of the SCs (**Fig. 1b** and **Supplementary Fig. 3**). Additional experiments revealed that the duration of the dissociation protocol affects the detected bulk expression levels of the genes that are unique to subpopulation 2 (**Fig. 1c**, **Supplementary Note 1**, **Supplementary Figs. 4** and **5**, and **Supplementary Tables 3** and **4**), and this confirmed that the dissociation protocol affects the transcriptome of SCs. Our observations thus suggest that subpopulation 2 might not exist *in vivo* in uninjured muscles and that, in contrast to the current consensus[1,2], the quiescent satellite cell population might be relatively homogenous *in vivo*.

Next, we developed computational and experimental strategies to remove the dissociation-affected subpopulation of SCs. The computational solution entails the *in silico* removal of dissociation-affected cells from single-cell data sets (**Supplementary Note 2**, **Supplementary Fig. 6** and **Supplementary Table 5**). The experimental solution combines indexed FACS and robot-assisted transcriptome sequencing (SORT-Seq)[7] on SCs that are stained for mitochondrial activity (**Supplementary Note 3**) in order to effectively identify and remove dissociation-affected cells during FACS (**Fig. 1d**,**e**, **Supplementary Note 3**, and **Supplementary Figs. 7** and **8**).

Our results show that the SC isolation procedure induces transcriptome-wide changes in a subpopulation of these cells. Even though the dissociation-affected subpopulation can be relatively small, it causes a strong contaminating signal in bulk studies because of the high expression levels of the induced IEG and HSP genes. Interestingly, the genes that are induced by dissociation are also induced by muscle injury[6], which suggests that the dissociation protocol activated some of the satellite cells (**Supplementary Note 4**). Our findings thus show that what was previously considered to be a purely quiescent subpopulation of SCs is in fact contaminated with a dissociation-affected subpopulation that might reflect activated SCs. Therefore, the results of several previous bulk studies where similar dissociation protocols have been used to study 'quiescent' SCs[2–4] warrant reinterpretation (**Supplementary Note 4**).

Since similar dissociation procedures are also used to isolate cells from other tissues, our findings may be more broadly relevant. For example, a similar IEG- and HSP-expressing subpopulation that was not validated by microscopy has been described in a recent single-cell study of mouse acinar cells[8] (**Supplementary Fig. 9a** and **Supplementary Table 6**). We also identified subpopulations with high IEG and HSP expression in other single-cell data sets from our lab, including a subpopulation of osteoblast cells in a zebrafish fin data set that is highly similar to the dissociation-affected subpopulation of satellite cells (**Supplementary Fig. 9b–f** and **Supplementary Table 7**). The overlap between our satellite cell data and other data sets suggests that dissociation protocols might induce similar problems across tissues and even across species. Taken together, our results highlight the importance of single-cell resolved experiments and validation by orthogonal methods.

**Data availability statement.** Sequencing data and FACS index data are deposited under accession number GSE85755. Source data for **Figure 1** is available in the online version of the paper. A **Life Sciences Reporting Summary** is available.

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*


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#### AUTHOR CONTRIBUTIONS

S.C.v.d.B. and A.v.O. conceived and designed the project. S.C.v.d.B. and F.S. performed experiments. S.C.v.d.B., A.V. and B.S. analyzed the data. The zebrafish fin experiments were performed by J.P.-M. and analyzed by J.P.-M. and C.S.B. S.C.v.d.B. and F.S. wrote the manuscript with support from all other authors. C.R. and A.v.O. guided the project.

#### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.


**Susanne C van den Brink[1,2,5], Fanny Sage[1,5], Ábel Vértesy[1,2], Bastiaan Spanjaard[1–3], Josi Peterson-Maduro[1,2], Chloé S Baron[1,2], Catherine Robin[1,4] & Alexander van Oudenaarden[1,2]**

[1]Hubrecht Institute–KNAW and University Medical Center Utrecht, Utrecht, the Netherlands. [2]University Medical Center Utrecht, Cancer Genomics Netherlands, Utrecht, The Netherlands. [3]Berlin Institute for Medical Systems Biology, Max Delbrück Center for Molecular Medicine, Berlin-Buch, Germany. [4]University Medical Center Utrecht, Department of Cell Biology, Utrecht, The Netherlands. [5]These authors contributed equally to this work.
e-mail: a.vanoudenaarden@hubrecht.eu



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