We recently presented a feasible protocol that enables researchers to perform single cell tracking by time-lapse video-microscopy, and which can be followed by data post-processing[22]. This protocol can be easily adapted to multiple neural populations by following some specific premises described in the manuscript, thereby addressing fundamental questions regarding the cell biology or lineage progression of these cells. There are some important requirements and considerations that must be taken into consideration in order to successfully design and perform a live imaging experiment. First, time-lapse video-microscopy needs adequate hardware, including microscopy systems, most frequently brightfield and phase contrast microscopes, and usually in combination with motorized components supplying a level of automation that is vital for long-term experiments (i.e.: stages, shutters, and filters). In addition, incubation systems and permeable plates are necessary to maintain the correct CO_2 saturation and pH during the experiment, which is indispensable to ensure cell viability throughout. Moreover, the detection of molecular markers commonly requires epifluorescence devices. Epifluorescence is needed to detect specific cell labelling produced by the use of transgenic animals or exogenous cell labelling achieved by transfection or transduction. Furthermore, identifying the nature of the progeny often involves post-imaging immunocytochemistry, which may also rely on epifluorescence. Besides the microscopes and their accessories, time-lapse video microscopy also requires robust data storage systems and reliable software to process the enormous -amount of data produced during long term live imaging experiments. An average experiment over 120 hours in which brightfield images are acquired every 5 min will require around 120-150 gigabytes of storage on a computer linked to the microscope. In our manuscript, we use the NIS-Elements software from Nikon to control the number of imaging fields, the frequency of acquisition and the duration of the experiment, although these features can be readily be controlled by any imaging software that contains a time-lapse module. It is also important that when setting the experimental conditions, over-exposure to transmitted, or particularly fluorescent light, should be avoided as this may compromise cell viability due to its inherent phototoxicity. Hence, it is wise to define an adequate interval to establish a balance between the temporal resolution of the analysis and the potential cell death. We also describe the use of dedicated software for single cell tracking that was developed by the group of Dr Timm Schroeder, namely The Tracking Tool (tTt)[23]. This software is freely available, and it can be downloaded, together with the instructions for its installation and use, at: https://www.bsse.ethz.ch/csd/software/tTt-and-qtfy.html.

The correct hardware and software settings constitute only part of a successful live imaging experiment. There are also crucial issues regarding the cell populations under study that must be adequately adjusted in order to obtain data of sufficient quality. Probably the most critical issue is the density of the cells in culture. If the cell density is too high or the efficiency of the dissociation is poor, the clumps present in the culture may hinder single cell tracking. Conversely, a low density may compromise the viability of the cells and thus, researchers must standardize their cultures to ensure an optimal cell density prior to commencing the live-imaging experiments. In this line, the researchers need to bear in mind that variations within independent cultures might be high, making indispensable the analysis of enough number of movies and proper sample sizes to guaranty solid readouts. Conclusions based in single or few selected image sequences may lead to misinterpretations. In our manuscript, we define suitable culture conditions and cell densities to successfully image adult NSCs[8], postnatal cortical astrocytes[24], mouse neuroblastoma cells and cerebellar astrocytes[25]. In addition, frequent adjustment of the focal distance is essential to obtain high-quality images, a necessary condition for flawless single cell tracking. Likewise, maintaining stable conditions of humidity, temperature and CO_2 ensures cell viability throughout the experiment.

Although live imaging experiments provide the researcher with a remarkable amount of data regarding the cell biology of selected cell populations, this approach also has important limitations that must be borne in mind. First, the low-density conditions necessary to ensure reliable single cell tracking makes the use of biochemical assays unfeasible. Moreover, such low-density cultures often compromise the viability of isolated cells over longer periods, limiting the duration of the experiments. Conversely, while rapidly dividing populations often do not suffer the problems of viability, they may rapidly become confluent, presenting a similar restriction to the duration of the live imaging experiment. Second, it is important to consider that cell isolation creates an environment that is not necessarily representative of their physiological niche. While this may have some negative effects, given the loss of signals that may be important for physiological cell behavior, it also represents an opportunity to study the specific role of each extrinsic factor that defines the neural niche, as well as environmental factors that may reproduce pathological conditions, representing one of the true benefits of live imaging experiments[9, 10, 17, 26-29].

In summary, the protocol described in our manuscript constitutes a useful resource for researchers in the field of neuroscience. However, given the limitations described here, it is important to dedicate some effort to the future development of methods aimed at following the behavior of single cells *in vivo* with minimal interference to the physiological environment[11].

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