Stem Cell Research (2011) 7, 256–263

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METHODS AND REAGENTS

Computer-Aided 2D and 3D quantification of human stem cell fate from *in vitro* samples using Volocity high performance image analysis software

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Received 8 April 2011; received in revised form 24 May 2011; accepted 26 May 2011
Available online 7 June 2011

Abstract Accurate automated cell fate analysis of immunostained human stem cells from 2- and 3-dimensional (2D-3D) images would improve efficiency in the field of stem cell research. Development of an accurate and precise tool that reduces variability and the time needed for human stem cell fate analysis will improve productivity and interpretability of the data across research groups. In this study, we have created protocols for high performance image analysis software Volocity® to classify and quantify cytoplasmic and nuclear cell fate markers from 2D-3D images of human neural stem cells after in vitro differentiation. To enhance 3D image capture efficiency, we optimized the image acquisition settings of an Olympus FV10i® confocal laser scanning microscope to match our quantification protocols and improve cell fate classification. The methods developed in this study will allow for a more time efficient and accurate software based, operator validated, stem cell fate classification and quantification from 2D and 3D images, and yield the highest ≥94.4% correspondence with human recognized objects.

Introduction

Human stem cells have potential to be used for treatment of multiple diseases and trauma (Nelson et al., 2010; Anderson et al., 2008). To better understand the properties of stem cells and the ability of these to repair human conditions, many laboratories are routinely culturing and differentiating stem cells in vitro. Accordingly, the numbers of samples processed for fate analysis are increasing exponentially, and accurate automated cell fate analysis would be an enormous improvement in this field.

In vitro cultured stem cells are commonly classified and quantified from 2D or 3D images, and different image acquisition platform types used to collect the data have their advantages. Confocal microscopy is a common tool for image acquisition of z-stacks of optical slices of cells or tissues after immunohistochemistry, and it has the advantage of allowing for numerous adjustments, e.g. aperture size, image dimension, and scan speed that affect image

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doi:10.1016/j.scr.2011.05.005

quality, the rate of image capture, and file size. These parameters define not just the quality of 3D images, but also the time needed for image capture and the size of data files, which matters especially when dealing with the large number of samples common when testing effects of variable culture conditions such as small molecules and drugs on stem cell differentiation.

Depending on the number of optical slices, acquisition time is often shorter for 2D images than z-stacks, especially when using conventional laser scanning confocal microscopes. On the other hand, stem cells typically show heterogenous cell morphology, high number of overlapping cell processes, and small distances between individual cells, which make object recognition in 2D challenging. Identification becomes even more difficult when more than one cell fate marker is used at once; in particular when the proteins of interest are located in the cytoplasm and co-expressed in a proportion of the cells undergoing fate selection (Walton et al., 2006; Rieske et al., 2007). There are several image analysis software tools available that can analyze 2D images, however the major disadvantage is their inability to distinguish partly overlapping objects due to lack of z-axis data (Hamilton, 2009). 2D images are also regularly used for human based manual image analysis to classify and quantify stem cell progeny. However, manual cell counting methods are often an inconsistent and error prone process in which the objects are subjectively scored either positive or negative, and can result in variability of data within experiments between researchers as high as 20%. Conversely, software based image analysis can produce repeatable measurements of not just intensity, but also volume and textures that would not be detectable by a human observer (Hamilton, 2009; Huang and Murphy, 2004).

3D data analysis in z-stacks of optical slices could overcome the majority of classification errors associated with x and y, but requires advanced software and high volume data management. Volocity® (PerkinElmer Inc.) is one of several commercially available advanced high performance 3D-4D image analysis software packages that can measure all volumetric pixels (i.e. voxels in total volume of z-stacks of optical sections) and therefore gain better signal overlap detection in all x, y and z axes improving compiling of cell structures as well as fate classification (Rueden and Eliceiri, 2007). In this report, we have A) created 2D and 3D cell classification protocols for the high performance 3D-4D image analysis software (Volocity®, Perkin Elmer Inc.) to quantify cytoplasmic and nuclear markers in human neural stem cells (hNSCs) after *in vitro* differentiation. Both protocols can be modified to suit a specific user's needs. They allow for semi-automated, more time efficient and accurate stem cell fate classification and quantification, although data verification by operator is still highly recommended. We also show how to B) optimize laser scanning confocal microscope (Fluoview® FV10i; Olympus America Inc.) image acquisition settings to collect 3D information for Volocity® quantification analysis in a more time efficient and accurate manner. C) Finally, we assessed Volocity® based operator validated cell fate analysis in experimental conditions, and compared the time efficiency and validity between software based and human based manual image analysis.

Results

Protocols for stem cell fate analysis using Volocity® high performance image analysis software

We created two Volocity® protocols to study hNSC *in vitro* differentiation. hNSC are a commonly studied cell type in basic science and translational research, and the cells can be characterized by the ability to proliferate, to self-renew, and to differentiate into three specialized neural-cell types: neurons, astrocytes and oligodendrocytes. These fate choices can be distinguished from each other by using several commercially available antibodies. One Volocity® protocol is for 3D images to classify and quantify Hoechst-counterstained hNSC progeny expressing cytoplasmic markers (Supplementary Fig. 1), such as neuron-specific ß-Tubulin III (ß-TubIII) and glial fibrillary acidic protein (GFAP) expressed by mature astrocytes and undifferentiated hNSCs. The other protocol was designed for 2D images to classify and quantify the cells expressing nuclear markers (Supplementary Fig. 2), such as oligodendrocyte lineage marker Olig2. Our protocols can be downloaded from the Supplementary Material (.assf) and tested/modified to suit a user's specific needs or dataset using a free Volocity® demo version (http://cellularimaging.perkinelmer.com/downloads/).

Optimization of confocal laser scanning microscope image acquisition settings improves time efficiency and accuracy of software based object recognition

The time of image capture varies depending on acquisition platform and number of z-stacks of optical slices. To enhance time efficiency of cell fate analysis, we optimized the image acquisition settings of confocal laser scanning microscope Olympus Fluoview® FV10i to match our Volocity® cytoplasmic cell marker classification protocol (Supplementry Fig. 1). This method can be adapted to optimize other confocal microscope settings to match Volocity® or other software based image analysis protocols. In addition to excluding out of focus light, confocal microscopy has the advantage of allowing for numerous adjustments to optimization, e.g. aperture size, image dimension, scan speed, and the rate of image capture. The FV10i confocal aperture size ranges from 1 to 2.5; the aperture determines the angle of light that comes into focus in the image plain. Smaller values (with narrow aperture size) result in a sharp focus at the image plain with less diffraction, whereas larger values (with wider aperture size) result in an image that is sharp at the plane where the lens is focusing but blurred otherwise (McNally et al., 1999). To determine the optimal aperture size for Volocity® post-processing, we compared images of 12 z-stacks of optical sections captured with 10x objective with 2.5 optical zoom at each of the four aperture sizes (Supplementary Fig. 3A). Aperture sizes ranging from 1.0 to 2.5 each provided a consistent image, however the image captured at aperture size 1.0 had the sharpest resolution and allowed for the greatest amount of detail discernment. Wider aperture sizes let more light through so that the images appear brighter, but the resolution was actually lower. Therefore, we selected to use an aperture size of 1 for FV10i confocal image acquisition with 10x objective and 2.5

optical zoom. For other confocal systems, the user should test different apertures empirically.

FV10i confocal image acquisition can also be tuned using three different image resolutions (256×256, 512×512 and 1024×1024) and five variable scanning speeds (1x, 2x, 4x, 8x and 16x). On an FV10i, the scanning speeds coorespond to the level of Kalman averaging. The acquisition time of only one hNSC sample (e.g. cell line A in an comparison of A, B and C cell lines, or cell fate in culture condition 1 versus 2 in a multi-condition experiment) consisting of 10 images with 12 z-stacks of 2 μm optical sections captured with 10x objective with 2.5 optical zoom, can vary from 7 minutes to 5 hours depending on FV10i image resolution and scan speed settings (Supplementary Fig. 3B). Therefore, optimization of the acquisition parameters is critical for time efficiency of the data collection phase of any given experiment. Before optimization of the image acquisition settings, we first assessed how big an effect FV10i image resolution and scan speed has on Volocity® object recognition in our samples. Volocity® 3D image analysis is based on recognition of overlapping or touching objects with different color intensities. The unprocessed Volocity® classification data of hNSC fate includes information on ß-TubIII+Hoechst+neurons (green touching blue objects), GFAP+Hoechst+astrocytes (red touching blue objects), and ß-TubIII+GFAP+Hoechst+ immature neural progenitors (green touching red touching blue objects). Additionally, non-labeled Hoechst+only cells (blue only objects) as well as cytoplasmic areas positive only for ß-TubIII+(green only objects), GFAP+(red only objects), and ß-TubIII+GFAP+(green and red only objects) are classified (Fig. 1). Differences in object recognition can be seen between low versus high resolution images (Fig. 1) demonstrating that both resolution and scan speed affect the software based object recognition.

Software based operator validated object recognition of hNSC fate using cytoplasmic markers is most accurate from high 1024×1024 resolution 3D images captured with FV10i 4x "balanced" scan speed setting

We started optimization of FV10i image acquisition settings for Volocity® software based object recognition using the cytoplasmic cell fate marker classification protocol by comparing numbers of Hoechst+ objects (total number of the cells) detected in 3D images captured with different image resolutions and scan speeds (Fig. 2A). Our data revealed that the number of Volocity® recognized Hoechst+ objects agreed best with experienced human based manual image analysis when the FV10i images were captured with high 1024×1024 resolution (Fig. 2A). We also analyzed Volocity® object recognition of different cell types, specifically neurons, astrocytes and immature progenitors, from images captured with all three resolutions either on

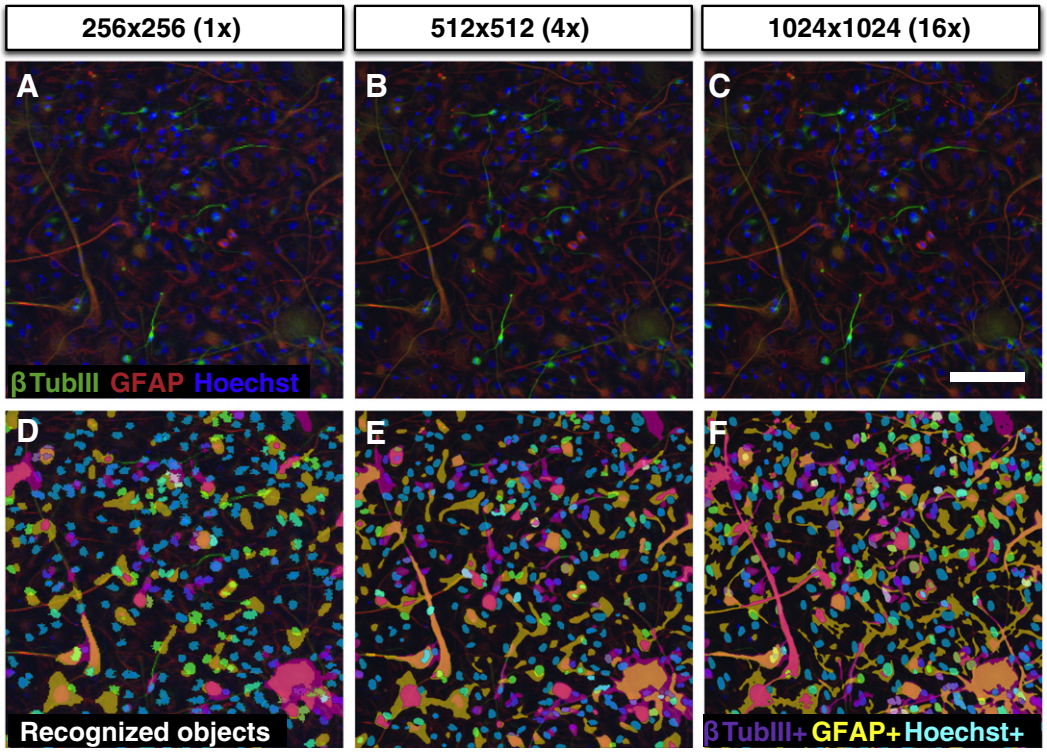

Figure 1 Volocity® software protocol for classification of the cytoplasmic cell fate markers is sensitive to image quality. An image of a single area of interest was acquired at multiple confocal laser scanning microscope FV10i image acquisition settings: A) 256×256 resolution and 1x scan speed, B) 512×512 resolution and 4x scan speed, and C) 1024×1024 resolution and 16x scan speed. D-E) When the images (A-C) were analyzed using the Volocity® software cytoplasmic cell fate classification and quantification protocol, increases in object detection/recognition are clearly visible from left to right in concordance with the image quality. Volocity® data images include in ß-TubIII+, GFAP+ß-TubIII+GFAP+, ß-TubIII+Hoechst+, GFAP+Hoechst+, ß-TubIII+GFAP+Hoechst+and Hoechst+objects. Scale 100 μm.

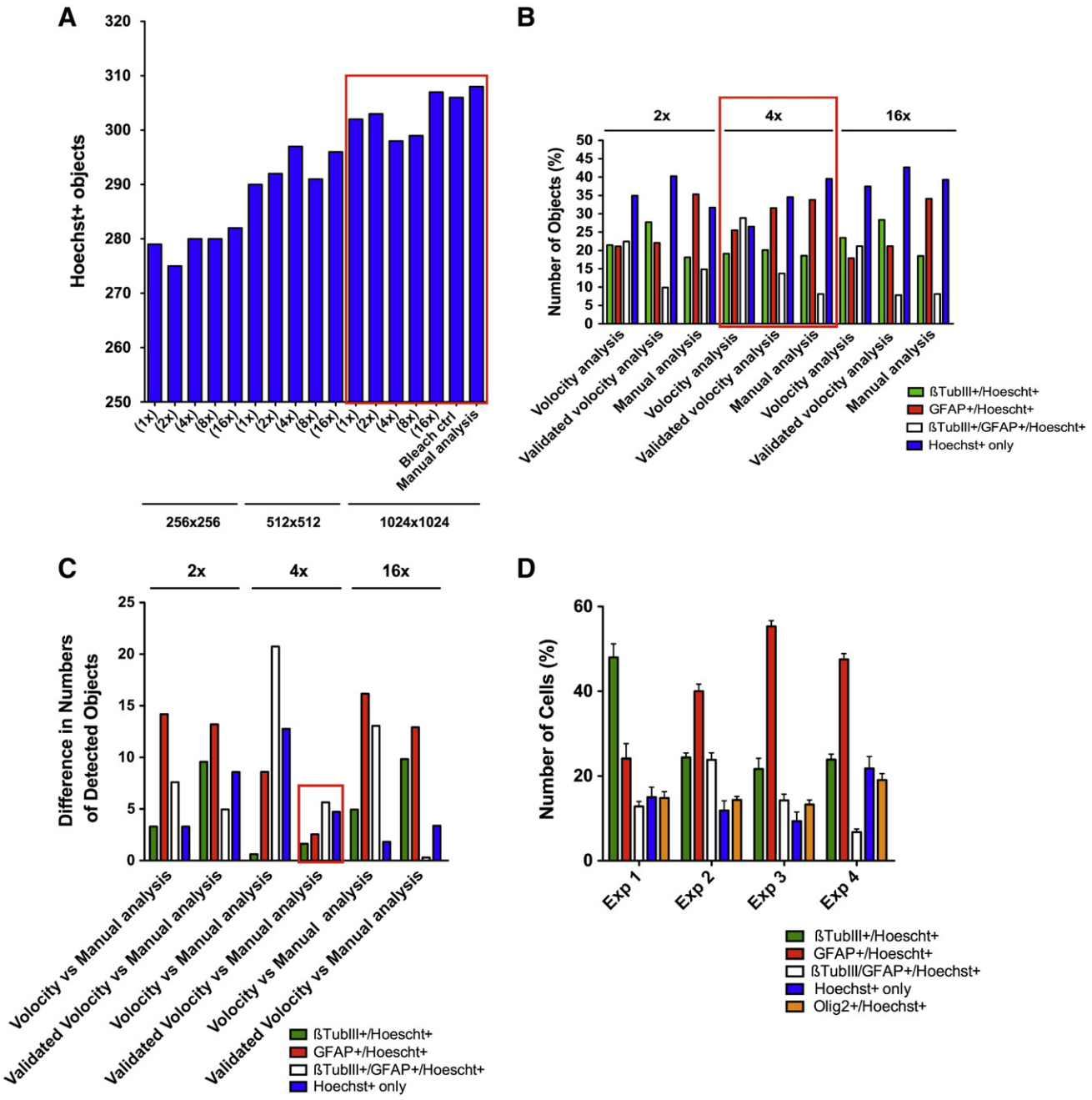

Figure 2 Volocity® based cell fate analysis is most accurate when images are captured with high 1024×1024 resolution and FV10i "balanced" scan speed (4x), and when the software based object recognition is validated by correcting possible mislabeled objects. A) Volocity® data analysis of Hoechst+objects across different FV10i image capture resolutions (256×256, 512×512 and 1024×1024) and scan speeds (1x, 2x, 4x, 8x and 16x). Number of the Hoechst+objects analyzed from images captured at 1024×1024 resolution (red outline) show the highest concordance with human based manual cell quantification (manual analysis). The effect of prolonged laser exposure on the sample was minimal (bleach control). B) For optimization of FV10 scan speed settings the number of ß-TubIII+Hoechst+, GFAP+Hoechst+, ß-TubIII+GFAP+Hoechst+and Hoechst+recognized objects between unassisted Volocity® analysis, Volocity® based operator validated, and human based manual image analysis were compared. Numbers of the Volocity® based operator validated were in the highest concordance with the manually counted objects when analyzed from the images acquired with a 4x "balanced"scan speed setting (red outline). C) The lowest overall difference between numbers of the Volocity® based operator validated, and the human based manual analysis recognized objects was detected in image captured with the 4x "balanced" scan speed setting (red outline). D) An example of Volocity® based operator validated analysis of four individual hNSC *in vitro* differentiation experiments using the cytoplasmic and nuclear cell fate classification protocols. Within each experiment (x-axis), 8 or 10 images were used for internal comparison of neural-astroglial or oligodendroglial differentiation. Error bars represent SEM± between images within a single experiment.

speed (2x) or high quality (16x) scan speeds. Similar to the number of software recognized Hoechst + objects, comparison of these three cell types suggested that the highest image capture resolution provided/yielded the best accuracy for the software based object recognition (Supplementary Fig. 4A).

Next, we evaluated the effect of FV10i scan speed on Volocity® capacity to recognize the cells of interest. Adjustments in scan speed influence the signal-to-noise ratio of confocal captured images via Kalman averaging, with slower scans decreasing overall background noise. However, slow scan speeds also increase the amount of time required to capture an image and may increase the risk of phototoxicity. Accordingly, adjustments in scan speed should be made to achieve the fastest scan rate while maintaining adequate image resolution for Volocity® cell fate quantification. For this analysis, we captured images using high 1024×1024 resolution with three scan speed settings: high quality (16x), balanced (4x), and speed (2x) scans. When numbers of Volocity® recognized ß-TubIII + Hoechst + neurons, GFAP + Hoechst + astrocytes, ß-TubIII + GFAP + Hoechst + immature neural progenitors, and only Hoechst + cells were compared to those of experienced human based manual image analysis, we found 0.5% to 20.8% difference in numbers of recognized cell types between these two methods depending on scan speed (Figs. 2B and C).

Visual observation of the Volocity® data images revealed that in cases of high hNSC density, the software made some quantification errors by labeling a proportion of cells that had a small cytoplasm-nucleus ratio and were located closely to neighboring cell types as triple positive. Consequently, we decided to address the cell density mislabeling issue by confirming the Volocity® object recognition and correcting mislabeled objects manually via a human operator "data validation" step. This decreased the difference between number of Volocity® and human recognized objects into the range of 0.3-13.2%, depending on the scan speed (Figs. 2B and C). Software based object recognition after data validation showed the highest correspondence with human recognized objects when analyzed from high resolution images captured with balanced scan (4x). Under these conditions, the greatest magnitude of difference between the numbers of human and Volocity® recognized objects ranged from 1.6 to 5.6%, depending on the cell type (Fig. 2C). In conclusion, Volocity® based, operator validated cell classification and quantification of hNSC progeny using the cytoplasmic cell fate marker protocol showed the highest ≥94.4% accuracy when analyzed from FV10i image captured with high 1024×1024 resolution and balance (4x) scan speed settings (Figs. 2C and 3).

Software based operator validated image analysis of hNSC progeny is 2 fold faster and shows greater validity than human based manual image analysis

After finding the optimal FV10i image acquisition settings, we tested Volocity® based object recognition using our cytoplasmic and nuclear cell fate classification protocols (Supplementary Figs. 1 and 2) to analyze cell proportions of three *in vitro* hNSC derived lineages: neurons, astrocytes, oligodendrocytes and immature neural progenitors in experimental conditions. Proportions of the cells positive for cytoplasmic ß-TubIII and GFAP were analyzed from 3D images captured with previously described FV10i settings, where as numbers of the nuclear cell fate marker Olig2 positive cells were analyzed from 2D images captured using a standard inverted microscope combined with digital camera. Comparison of the time efficiency between human and Volocity® based operator validated image analysis revealed that the software based analysis was at least 2-fold faster. In high cell density samples, careful human based manual classification/quantification of hNSC progeny using the cytoplasmic markers took approximately 40 minutes per 2D image. When the same image was analyzed using a 3D data set with Volocity® software, including the human based validation step, the analysis time was decreased by half.

Volocity® based operator validated analysis of four individual *in vitro* differentiation experiments with identical experimental conditions revealed that the number of ß-TubIII + cells varied from 21.6% ± 3.2 to 48% ± 2.4, GFAP + cells from 24.1% ± 3.5 to 55.3% ± 1.3, and nuclear Olig2+ cells from 13.3% ± 1.0 to 19% ± 1.5 between the four experiments (Fig. 2D). Normal human brain parenchyma derived GFAP + cells have been shown to transiently co-express early neural and neuronal cell markers, such as ß-TubIII and GFAP, during early *in vitro* differentiation into either neurons or astrocytes (Walton et al., 2006; Rieske et al., 2007). A two week *in vitro* differentiation period is not long enough for full cell fate commitment in hNSCs, and the cultures included a 6.7% ± 1.4 to 23.8% ± 1.7 proportion of cells that not fully differentiated and co-expressed low levels of both ß-TubIII and GFAP (Figs. 2D and 3C, G). A majority of the *in vitro* differentiated cells were still positive for nestin, a marker for undifferentiated hNSCs and progenitors, and only the most mature cells showed typical round bipolar neuronal or astrocytic morphology (data not shown). The greatest variation between the four individual experiments was found in the proportions of neurons 26.3% ± 6.2 and astrocytes 31.2% ± 6.6, and the lowest in the proportion of Olig2 positive oligodendrocyte lineage cells 5.8% ± 1.3 (Fig. 2D). This kind of variation after hNSC *in vitro* differentiation is very common due to changes in intrinsic/extrinsic factors between the experiments (Jensen and Parmar, 2006).

Volocity® based, operator validated image analysis was not just more time efficient but also showed better validity than human based manual image analysis. Data collected with the software based classification protocols showed very high internal validity within each 4 experiment indicating greater consistency. When using Volocity® software, the greatest standard error of mean (SEM) between the cell proportions within a single experiment (8 images/experiment) for cytoplasmic fate markers was ± 3.5% (Fig. 2D). Data collected with nuclear cell fate classification protocol showed even higher internal validity, and the greatest SEM between the proportions of Olig2+ cells within a single experiment (10 images/experiment) was only ± 1.5% (Fig. 2D).

We also evaluated the reproducibility of human based manual image analysis by individual users for a single image. When a single 2D image, rotated and flipped three different ways, and containing a number of cells positive for ß-TubIII and GFAP was analyzed by three individuals with variable levels of cell classification experience, the average difference between the analyzers varied from 4% ± 1.0 to 12.3% ± 4.0, depending on

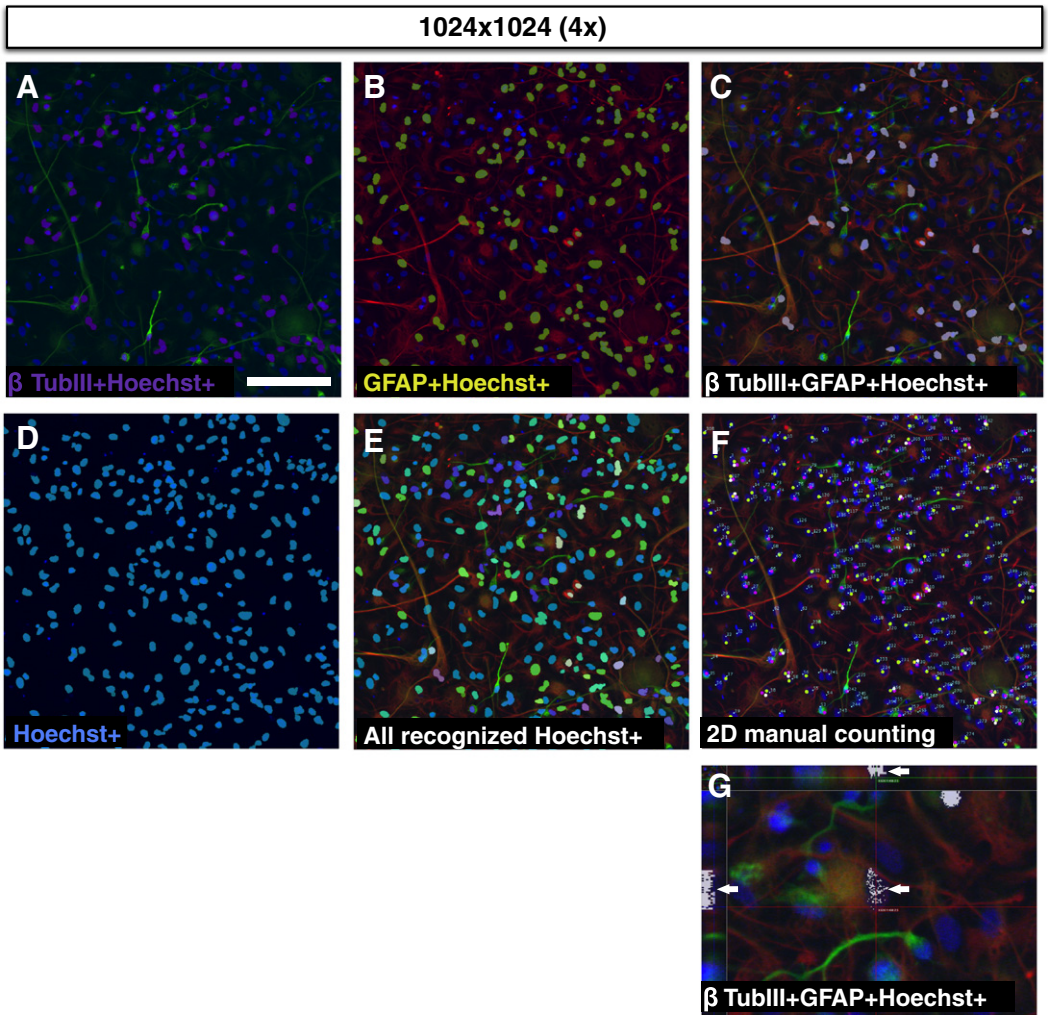

Figure 3 Extended focus data images of Volocity® software recognized operator validated cell types after hNSC *in vitro* differentiation. The cells were immunostained with antibodies against ß-TubIII (green) and GFAP (red) using Hoechst (blue) as a nuclear counterstain, and the images acquired using a 1024x1024 resolution and 4x "balanced"scan speed settings. Nuclei of Volocity® recognized A) ß-TubIII + cells (purple), B) GFAP + cells (yellow), C) ß-TubIII + GFAP + immature neural progenitors (white), D) cells with Hoechst + (blue), and E) all the classified cells (overlapping purple, yellow, white and blue). Volocity® image analysis classifies objects using 3D information as belonging to different "groups" and then determining if those classified objects "overlap or touch each other". Quantification of different cell proportions requires exclusion of cytoplasmic areas that do not touch Hoechst + nuclei (ß-TubIII+, GFAP+, and ß-TubIII + GFAP + objects only). F) An example of 2D image after human based manual image analysis of neural and astroglial lineages: ß-TubIII + cells (purple dots), GFAP + astrocytes (yellow dots), ß-TubIII + GFAP + immature neural progenitors (white dots), and Hoechst + nuclei (numbers). G) XYZ image projection of a Volocity® recognized ß-TubIII + GFAP + immature neural progenitor (white nuclei) showing that the cell is co-expressing both ß-TubIII and GFAP (yellow) in 3D. Scale 100 μm.

the cell type (Supplementary Fig. 4B). The maximal difference between human analyzers was as high as 23.3%. Human based manual image analysis also showed relatively low internal validity; the maximal difference between three repeated analyses within each individual varied between 2.3% and 18.2%, depending on the cell type and the analyzer's level of experience.

Discussion

Based on this study commercially available Volocity® high performance image analysis software (PerkinElmer Inc) based, operator validated image analysis of hNSC fate was not just more time efficient but also showed greater validity than human based manual image analysis. We showed that optimization of 3D acquisition platform settings seems to be not only important for the time efficiency of but also for the accuracy of the software based object recognition and sensitivity, and therefore recommended especially when using conventional laser confocal microscope systems.

Volocity® image analysis software is not restricted only to the image acquisition systems used in this study. The software supports approximately 90% of the current image acquisition platform file formats currently on the market. Beside the Olympus microscope system detailed above, we

have also used these hNSC fate classification protocols successfully to quantify 3D images captured using a Zeiss ApoTome® acquisition platform. The protocols presented in this paper are Volocity® specific but fairly robust, so these can be modified to suit a specific user's needs for fate analysis of other cell types and could be adapted to work in other image analysis software packages. However, cell type, immunocytochemistry protocol and image capture related factors such as cell size, antibody concentrations, laser/gain settings, objective magnification and z-stack interval are variables that may require minor modification of numerical parameters, such as object size and intensity as long as the image quality is similar to the outlined quality. Users should always optimize their protocols to match experienced human based manual image analysis for each cell type of interest.

As previously reported, human based hNSC fate analysis was found to be a very subjective and inconsistent method even when human observers are experienced. The greatest variation in human based manual cell quantification both between individuals and between repeated analyses by a single individual was found in the number of cells positive only for Hoechst, GFAP, and ß-TubIII and GFAP double positive immature progenitors, suggesting that humans have a limited capacity to apply an accurate reference baseline to object volumes and different color intensities, e.g. for the size of nuclei and different levels of red and green mixed together. Low internal and external validity of human based image analysis can affect interpretability of human stem cell *in vitro* differentiation data.

Conversely, Volocity® software made some quantification errors, especially when two cytoplasmic cell fate markers together with nuclear counter staining were analyzed at once. The software based quantification errors were consistent and the number of the errors was cell density dependent. A human operator "data validation" step to confirm Volocity® object recognition and correct mislabeled objects manually gave higher $\geq$94.4% accuracy, but also increased the time of the analysis. Nonetheless, Volocity® software based, operator validated, hNSC fate classification and quantification was still 2 fold faster than the human based quantification alone. Plating the cells in lower density may also overcome the problem with the mislabeled objects. However, when working with certain types of cells, such as hNSCs, this can be technically challenging because of the relatively long *in vitro* differentiation period required or the practical limitation that the cells prefer growing in high density. Protocol modifications, such as adding "erode objects" decreased the number of software based quantification errors, but at the cost of losing some correctly labeled cells. The software based object separation was found to be less demanding if using only one cell fate marker at once or if the objects of interest were strictly overlapping each other, e.g. nuclear Olig2 and Hoechst, which in some cases allows software based image analysis in 2D. The validation of software based object recognition is highly recommended for better accuracy, especially when quantifying cells in high densities.

Finally, Volocity® based cell image analysis requires careful standardization of immunocytochemistry protocols and image acquisition parameters in order to avoid unnecessary data variations. Despite of these limited disadvantages, when high numbers of samples need to be quantified, Volocity® based cell quantification can improve both productivity and interpretability of *in vitro* differentiation data across different researchers and research groups.

Materials and Methods

hNSC culture and immunocytochemistry

Fetal derived hNSCs were differentiated on Polyornithine and Laminin coated 8- well-chamber slides in Ex-Vivo based serum free medium (Bio Sciences) supplemented with 10 ng/ml glial cell line derived neurothropic factor (GDNF) (PeproTech), 10 ng/ml brain derived neurothropic factor (BDNF) (PeproTech), and 1 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen) for 14 days *in vitro*. For immunocytochemistry, the cells were fixed with 4% PFA, permeabilized and blocked in PBS solution supplemented with 0.1% Triton-X (Sigma), 5% goat serum (Jackson ImmunoResearch) and 1% Bovine serum albumin (Sigma). The cells were stained using primary antibodies monoclonal mouse anti- ß III tubulin (1;500, MMS-435P, Covance), polyclonal rabbit anti-GFAP (1:1000, Z0334, DakoCytomation) and goat anti-hOlig2 (1:100, AF2418, R&D Systems), and secondary antibodies Alexa Fluor 488 goat anti-mouse IgG (1:1000, Invitrogen), Alexa Fluor 555 goat anti-rabbit IgG (1:2000, Invitrogen), Alexa Fluor 488 donkey anti-goat IgG (1:1000, Invitrogen) and Hoechst 33342 (H1399, Invitrogen). The antibodies used did not show non-specific binding to each other (Supplementary Fig. 4).

Confocal image capture settings

For 3D data analysis of cytoplasmic cell fate markers, a total of ten z-stacks of optical slices in 2 μm intervals were captured using a Fluoview® FV10i (Olympus America Inc) confocal laser scanning microscope with 10x objective and 2.5 optical zoom for a final magnification of 25x. Laser/gain settings were as follows: 5%/53% (Hoechst), 4%/38% (Alexa Fluor 488), and 3%/42% (Alexa Fluor 555), and aperture sizes of 1.0, 1.5, 2.0, or 2.5 were tested. For optimization of FV10i acquisition settings, we compared Volocity® object recognition at scan speeds of 1x, 2x, 4x, 8x, or 16x at image resolutions of 256×256, 512×512, or 1024×1024. "Bleach Control" is a second scan at the longest exposure, performed subsequent to all other scans used for quantification and it was used to determine if prolonged exposure to confocal lasers affects cell fate quantification. For classification and quantification analysis of nuclear cell fate markers, 2D images were captured using Olympus Inverted System Microscope IX71 combined with Olympus U-CMAD3 digital camera and a 20x objective.

Classification and quantification of cytoplasmic or nuclear cell fate markers in hNSC progeny using Volocity® protocols and human based manual image analysis

Volocity® based cell quantification was performed using created protocols (Supplementary Fig. 1 and 2). Images were imported into Volocity® as tiff- or multiple tiff- file format. The software supports several other image acquisition

platform file formats (Supplementary Fig. 6). Briefly, the objects of interest were found using standard deviation (SD) intensity and then either excluded or retained based upon color and size. A uniform filter was used to remove noise from the system and measurements were performed to identify cells as either ß-TubIII + Hoechst+, GFAP + Hoechst+, ß-TubIII + GFAP + Hoechst + or only Hoechst + cells. Similar measurements were also made to identify cells as Olig2 positive. These protocols can be downloaded from Supplementary Material (.assf). For validation of Volocity® data, the software based object recognition was confirmed by visual observation of Volocity® data images by an experienced user, and the mislabeled objects were corrected manually. For human based manual image analysis, 3D images were flattened and ß-TubIII + Hoechst+, GFAP + Hoechst + and ß-TubIII + GFAP + Hoechst + objects were manually counted in Adobe® Photoshop® software in collaboration with two individuals blinded to the outcome in order to minimize number of possible classification errors. To assess accuracy of human based manual image analysis, three individuals with variable level of experience analyzed one 2D image three times at different time points. To decrease the possibility of memorization, the image was rotated and flipped in three different ways prior to analysis. External and internal validity of human based image analysis was assessed respectively by comparing numbers of recognized objects between the individuals and each analysis time points.

Conclusions

Accurate and efficient quantification of human stem cell lineage and fate is critical for the many assays required for the translation of basic stem cell research to clinical therapeutics for disease or trauma. Conventional human based manual cell fate analysis is not just time consuming but a subjective and inconsistent method. The Volocity® software based human neural stem cell classification and quantification protocols presented in this paper allow for semi-automated, 2 fold faster, and more accurate image analysis of cytoplasmic and nuclear cell fate markers after *in vitro* differentiation. These factors improve both productivity and interpretability of *in vitro* differentiation data across different researchers and research groups. Altogether, these data suggest that Volocity® image analysis software can be used as a precise tool in conjunction with both inverted and confocal laser scanning microscope image acquisition platforms. The Volocity® software protocols presented in this paper are not limited only to human neural stem cell fate analysis or the image acquisition platforms used in this study; with small modifications, such as adjustments in object size, color intensities, and z-step interval, these protocols can be used for quantification of other humans stem cell lineages and in conjunction with other microscope image capture systems.

Acknowledgments

We thank Paul Miller, Imaging Specialist of PerkinElmer, and Brendan Brinkman, Confocal and Multiphoton Specialist of Olympus America Inc. for their technological support and advice. This study was supported by California Institute for Regenerative Medicine (CIRM) ETA grant (TR2-01767), a CIRM Postdoctoral Training grant (TG2-01152) and a CIRM Bridges grant (TB1-01182).

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.scr.2011.05.005.

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