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Corresponding Author:	kathrin meyer Nationwide Children's Hospital Columbus, OH UNITED STATES
Corresponding Author's Institution:	Nationwide Children's Hospital
Corresponding Author E-Mail:	kathrin.meyer@nationwidechildrens.org
Order of Authors:	Kathrin Meyer
	Cassandra Dennys
	Julieth Sierra-Delgado
	Shrestha Ray
	Annalisa Hartlaub
	Florence Roussel
	Yacidzohara Rodriguez
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TITLE:

In vitro Modeling for Neurological Diseases using Direct Conversion from Fibroblasts to Neuronal Progenitor Cells and Differentiation into Astrocytes

AUTHORS AND AFFILIATIONS:

Cassandra N. Dennys[1], Julieth A. Sierra-Delgado[1], Shrestha Sinha Ray[1], Annalisa M. Hartlaub[1], Florence S. Roussel[1], Yacidzohara Rodriguez[1], and Kathrin Meyer[1,2]

[1]Center for Gene Therapy, The Research Institute at Nationwide Children's Hospital, Columbus, Ohio, USA

[2]College of Medicine, The Ohio State University, Columbus, Ohio, USA

Cassandra N. Dennys: Cassandra.Dennys-rivers@nationwidechildrens.org
Julieth A. Sierra-Delgado: Julieth.SierraDelgado@nationwidechildrens.org
Shrestha Sinha Ray: Shrestha.SinhaRay@nationwidechildrens.org
Annalisa M. Hartlaub: Annalisa.Hartlaub@nationwidechildrens.org
Florence S. Roussel: Florence.Roussel@nationwidechildrens.org
Yacidzohara Rodriguez: Yacidzohara.Rodriguez@nationwidechildrens.org
Kathrin Meyer: Kathrin.Meyer@nationwidechildrens.org

Correspondence to:
Kathrin Meyer at Kathrin.Meyer@nationwidechildrens.org

KEYWORDS:

In vitro modeling, direct reprogramming, conversion, fibroblasts, neuronal progenitor cells, induced astrocytes

SUMMARY:

We describe a protocol to reprogram human skin-derived fibroblasts into induced Neuronal Progenitor Cells (iNPCs), and their subsequent differentiation into induced Astrocytes (iAs). This method leads to fast and reproducible generation of iNPCs and iAs in large quantities.

ABSTRACT:

Research on neurological disorders focuses primarily on the impact of neurons on disease mechanisms. Limited availability of animal models severely impacts the study of cell type specific contributions to disease. Moreover, animal models usually do not reflect variability in mutations and disease courses seen in human patients. Reprogramming methods for generation of induced pluripotent stem cells (iPSCs) have revolutionized patient specific research and created valuable tools for studying disease mechanisms. However, iPSC technology has disadvantages such as time, labor commitment, clonal selectivity and loss of epigenetic markers. Recent modifications of these methods allow more direct generation of cell lineages or specific cell types, bypassing clonal isolation or a pluripotent stem cell state. We have developed a rapid direct conversion method to generate induced Neuronal Progenitor Cells (iNPCs) from skin fibroblasts utilizing retroviral vectors in combination with neuralizing media. The iNPCs can be differentiated into

neurons (iNs) oligodendrocytes (iOs) and astrocytes (iAs). iAs production facilitates rapid drug and disease mechanism testing as differentiation from iNPCs only takes 5 days. Moreover, iAs are easy to work with and are generated in pure populations at large numbers. We developed a highly reproducible co-culture assay using mouse GFP+ neurons and patient derived iAs to evaluate potential therapeutic strategies for numerous neurological and neurodegenerative disorders. Importantly, the iA assays are scalable to 384-well format facilitating the evaluation of multiple small molecules in one plate. This approach allows simultaneous therapeutic evaluation of multiple patient cell lines with diverse genetic background. Easy production and storage of iAs and capacity to screen multiple compounds in one assay renders this methodology adaptable for personalized medicine.

INTRODUCTION:

Understanding underlying disease mechanisms is critical for neurological diseases as it aids in the development of potential therapeutic strategies. While animal models have historically been the gold standard for researching diseases of the nervous system, the direct translation of potential therapies into a clinical setting often shows limited success[1-3]. Reasons for the lack of translation are lack of variability of genetic background and mutations in mice, incomplete display of disease phenotypes and variation in drug sensitivity or dosing in human patients that are not pictured well in inbred mouse strains. Furthermore, for many rare neurological disorders, no or few animal models are available. Studying disease mechanisms directly in relevant human cell types could facilitate research and improve translation to the clinic. For CNS disorders, primary human cells are difficult to retrieve and represent a very limited source as biopsies are invasive or can only be retrieved post-mortem at the end-stage of the disease. In the past 15 years, the development of cellular reprogramming technology has rapidly expanded the ability to model human neurological and neurodegenerative diseases in vitro.

Traditional methods of cell reprogramming involve reprogramming fibroblasts or other cell types into induced pluripotent stem cells (iPSCs) capable of differentiating into cell types from all three germ layers[4]. Takahashi and Yamanaka were the first to show that re-expression of four stem cell transcription factors is sufficient to redirect somatic cells to become iPSCs[5]. iPSCs can then be further differentiated into specific cell types. However, the drawback of this process is that it is labor intensive and time consuming to generate and isolate stable stem cell clones. Somatic mutations or specific aberrancies of the mother cell are also maintained in the stem cell clone and can impact results of the study[6]. In addition, increasing evidence suggests that the reprogramming process to stem cells erases valuable epigenetic changes that could influence cellular disease mechanisms[4,7,8]. In recent years, researchers focused on modified reprogramming methods allowing a more direct generation of different cell types while bypassing the pluripotent stem cell state[9-12] (reviewed in [13,14]). Initial breakthroughs revealed in vitro combinatorial reprogramming of fibroblasts to cardiomyocytes[15], neurons[16] and hepatocytes[17] by ectopic expression of multiple lineage-specific transcription factors or microRNAs[18]. This was followed by studies directly reprograming cells to model neurological disorders[19,20]. As mentioned above, such direct reprogramming or conversion protocols have potential advantages over classical iPSC protocols including speed and the ablation of the clonal isolation step. Moreover, evidence suggests that these protocols allow to maintain a larger

amount of epigenetic information related to the age of the patient and likely the same holds true for disease relevant markers[7,8].

To date, most in vitro research on neurological disorders has been focused primarily on neurons. However, it is well known that other cell types such as astrocytes, microglia and oligodendrocytes play a critical role in disease pathology and progression of neurodegenerative disorders such as Alzheimer's disease (AD), Amyotrophic Lateral Sclerosis (ALS), Parkinson's disease (PD), Huntington's Disease (HD), Multiple Sclerosis (MS), and other neurological pathologies such as Rett syndrome (RTT), sleep disorders, addiction, epilepsy, lysosomal storage disorders, depression, migraine and pathological pain[21-26]. Astrocytes are the most abundant cell type in the central nervous system (CNS) and until recently, they have been broadly neglected from a pathological point of view[7]. They are now known to have a critical role in supporting almost all homeostatic functions of the healthy CNS. Additionally, it is apparent that they have an important pathological impact and are very valuable in understanding disease mechanism and evaluating therapeutic strategies[19].

In the current description, we detail a protocol for direct conversion of human patient fibroblasts into induced Neuronal Progenitor Cells (iNPCs) using retroviral vectors expressing the Yamanaka reprogramming factors (Klf4, Oct3/4, Sox2 and c-Myc) and subsequent exposure to neuralizing media. Previous studies have used different combinations of transcriptional factors for direct reprogramming to neural cells (reviewed in [14]), but the factors used in the described protocol are broadly available either as plasmids for in house production of viral vectors, or as commercially available ready to go viral reprogramming kits. The resulting iNPCs can be further differentiated into induced neurons (iNs)[27], oligodendrocytes (iOs)[24] and astrocytes (iAS)[19] to study their role in neurological diseases. Our protocol does not involve time-consuming generation of iPSCs which most available protocols utilize for derivation of human astrocytes[28]. Approximately 98% to 100% of directly reprogrammed iNPCs can differentiate into GFAP-positive astrocytes[29] as compared to only 2% using direct conversion method from fibroblasts to astrocytes[30]. Recently, a comparative transcriptomic study using our described reprogramming method has shown that iNPCs reprogrammed from donor fibroblasts can retain ageing features at transcriptional and functional level similar to age matched postmortem astrocytes and primary astrocytes[29]. Thus, this conversion protocol is a powerful tool to investigate disease mechanisms and evaluate novel therapeutic approaches for age-related neurodegenerative and neurological disorders.

Here we describe the protocol utilized to generate iNPCs and further differentiation into iAs, which are the most suited for larger scale small molecular screening assays. Disease mechanisms and drug testing with iAs can be done using different methodologies such as co-cultures with neurons or metabolic and biochemical analysis. The advantage of this system is the speed and ease of maintenance of these cell lines compared to iPSCs. Moreover, iNPCs can be frozen in small portions for iAs differentiation which facilitates drug testing under constant conditions over extended periods of time. Overall, comparison of larger sample numbers becomes possible this way which opens the door to evaluate therapeutic potential of compounds in a larger and more representative patient population.

PROTOCOL:

[Place Table 1 here].

1. Direct conversion of adult human fibroblasts to neuronal progenitor cells

NOTE: A schematic timeline of this protocol can be reviewed in Meyer (2014)[19].

1.1. Use primary human skin fibroblasts that can be obtained from cell banks or skin biopsies[31]. Culture cells at 37 °C in a 5% CO_2 tissue culture incubator. Passage cells for at least 1-2 passages post thawing prior to use in the experiment. Once the cells are ready, coat a well of a 6-well plate with human fibronectin (1:200 diluted in PBS) at room temperature for 15-20 min.

1.2. When the cells are ready to be plated, remove the fibronectin from the dish and immediately add cell solution or 2 mL of fibroblast media (**Table 1**) – do not let the dish dry out prior to adding media. Depending on how fast the cells grow, plate 150,000 (fast growing) - 200,000 (slow growing) fibroblasts in two wells of a human fibronectin coated 6-well plate each.

NOTE: The cells should be around 70% confluent for viral transduction to be able to keep them in the same plate for several more days after transduction. It can be beneficial to seed at two different densities in order to have a choice the next day for conversion.

1.3. The day after seeding, check the fibroblasts under the microscope and verify cell density (between 70-85%) and even seeding throughout the well for optimal results.

1.4. Transduce one well with all four retroviral vectors (Oct3/4, Klf4, Sox2 and c-Myc) – use a multiplicity of infection (MOI) of 10 for fast growing or 15 for slow growing fibroblasts (transduction efficiency should exceed 70% or more positive cells for each vector). Add regular fibroblast medium to viral mix to a total volume of 1 mL per well and incubate overnight at 37 °C in a 5% CO_2 tissue culture incubator. Leave the second well untreated and return cells to tissue culture incubator.

NOTE: Retroviral vectors can be purchased by a vendor or made in-house, protocols are available online[32].

1.5. The day after transduction, wash cells 1x with PBS and add 1 mL of fresh fibroblast medium.

1.6. The next day, change medium to conversion medium. Wash cells 1x with PBS to get rid of the residual fibroblast medium, and then add 1 mL of conversion medium. Change the media of the untreated well to conversion media as well.

NOTE: Some morphological changes can be observed even by changing the media on the fibroblasts that did not receive retroviral vector treatment, thus having an untreated well

(optional) will be helpful when determining effects of the viral vectors. Cells should start changing morphology 2-4 days after switching to conversion media.

1.7. Observe cells under the microscope and watch for changes in morphology (**Figure 1**). Cell media should be replaced daily throughout the conversion process (1-2 mL of conversion media, **Table 1**) and for subsequent iNPC culture. Change medium by carefully removing 70% of medium from the well while making sure that the cells remain covered in the remaining 30% of media.

NOTE: Media with growth factors in it needs to be consumed within 1 week of preparation.

1.7.1. Continue to observe cells and change media daily (1-2 mL of conversion media).

1.7.2. Some cell lines start forming loose elevated round formations growing in ball like structures or loose neuronal spheres (**Supplemental Figure 1** and [19,33]). Take care to not detach these cells. If the balls detach, they can be collected and re-plated in a new fibronectin coated well of a 6-well plate.

NOTE: If they are expanded on their own, the cells will have reduced diversity compared to the initial plate and may represent a distinctly different cell line. We recommend combining these cells with the rest of the converted cells at the next round of splitting.

1.8. After about 5-6 days in conversion media (up to 12 days for difficult cell lines) or when cells become very dense, passage them 1:2 or maximum 1:3.

NOTE: It is very important to keep the cells at a high density throughout the conversion process.

2. Conversion and iNPC splitting procedure

2.1. Warm up cell detachment solution (e.g., Accutase) and coat an appropriate number of wells of a 6-well with fibronectin (1:200 in PBS, 1 mL of coating volume) for 15–20 min at room temperature. Fibronectin coating can be longer without negative impact, but care should be taken to not shorten the coating time.

2.2. Wash cells carefully with PBS without detaching any cells (use the wall of the well to apply PBS gently to the cells). Carefully remove PBS with pipettes or by aspiration.

2.3. Add 0.5 mL of cell detachment solution and incubate 2-3 min at 37 °C in a tissue culture incubator. Check under the microscope to verify that most cells have detached. If all cells have come off, add 2 mL of fresh conversion media and gently pipette up and down 2-3 times to dissociate clumps (if required).

2.4. Collect the cells in a 15 mL tube and add additional 3 mL of media to further dilute the cell detachment solution. Wash the well with the extra media first to ensure all cells have been

collected.

2.5. Centrifuge for 4 min at 200 x g at room temperature.

NOTE: Converting cells or iNPCs are extremely sensitive to the presence of cell detachment solution in the media. It is absolutely necessary to remove the residual enzyme by centrifugation and withdrawal of supernatant.

2.6. Remove supernatant from cell pellet and resuspend the cells in 1 mL of fresh medium. Gently pipette up and down 2-3 times to resolve cell clumps. Remove fibronectin from coated 6-wells and add 1 mL of fresh media to each well immediately (do not let fibronectin dry). For a split ratio of 1:2, add 0.5 mL of the cell suspension in one well and the other 0.5 mL in a second well.

2.6.1. Culture cells at 37 °C in a tissue culture incubator.

2.7. Distribute the cells by gently shaking the 6-well in north-south-east-west direction (not circular) and put in tissue culture incubator.

2.8. Observe the cells under the microscope the next day and change media (1-2 mL). Change media every day until the cells are ready to be split again.

NOTE: At this point, the cell proliferation should start to accelerate, and cells should be ready for a second split within 2-3 days (4 days for very slow growing lines).

2.8.1. At this new split, seed one well of cells in conversion media and the other well in NPC media containing only FGF at increased concentration without EGF/heparin (**Table 1**).

NOTE: Some cell lines reach a stagnant state in conversion media after about 10 days and switching to NPC media might help with cell growth. In addition, NPCs have a more specific, smaller shape, when grown in NPC media[19]. At this point, the iNPCs are ready for differentiation and further use.

2.9. Keep changing media (1-2 mL) of the iNPCs daily.

2.9.1. Expand NPCs into 10 cm dishes by combining two or three confluent wells of a 6-well. The media volume for 10 cm dishes is typically 12-15 mL.

2.9.2. At the next split, further expand these cells to three or four 10 cm dishes (12-15 mL of media) for generations of large numbers of cell stocks. 10 cm dishes are usually split at 1:3 or 1:4 every 3-4 days depending on proliferation rate.

3. Generating induced astrocytes from NPCs

3.1. To make astrocytes from fresh iNPCs, seed iNPCs (in 10 cm fibronectin coated plates) directly in 10 mL astrocyte medium (**Table 1**) so that they are around 10% confluency the following day. Culture cells at 37 °C in a 5% CO_2 tissue culture incubator.

NOTE: The recommended seeding density is usually 50 µL of the cell resuspension of a 10 cm dish of NPCs, provided they are diluted to a final volume based on their split ratio (e.g., 3 mL for a 1:3 split, 4 mL for a 1:4 split, etc.).

3.2. Change medium (10 mL astrocyte media) of the iAs three days after plating. Keep the cells differentiating for 5-7 days.

NOTE: iAs do not tolerate multiple passages well, therefore it is better to make fresh astrocytes every time you split the NPCs.

3.3. To seed for experimentation, split the astrocytes using trypsin or cell detachment solution as described in steps 2.1-2.7. Recommended seeding densities are included in **Table 2**.

[Place Table 2 here].

4. **Preparing and defrosting portions for astrocyte differentiation from frozen NPC stocks (alternative to step 3)**

NOTE: As an alternative to maintaining iNPCs in culture, astrocytes can also be produced directly from a frozen stock. To do so, the iNPCs are frozen in smaller portions. **Table 3** shows suggested freezing and thawing volumes for portions to be defrosted into a 10 cm dish containing 10 mL of Astrocyte media.

[Place Table 3 here].

4.1. To make astrocytes from frozen iNPC stocks, remove portion stock vial from liquid nitrogen storage tank and quickly thaw at 37 °C. As soon as cells are defrosted, pipette cell solution in a 15 mL tube containing 5 mL astrocyte media.

4.2. Centrifuge for 4 min at 200 x g and room temperature. Remove the supernatant and resuspend in 1 mL of fresh astrocyte medium.

4.3. Add 9 mL of fresh astrocyte medium to a fibronectin coated 10 cm dish and add 1 mL of cell solution. Gently distributing cells in north-south-east-west motion. Culture cells at 37 °C in a 5% CO_2 tissue culture incubator.

REPRESENTATIVE RESULTS:

This protocol allows the rapid and easy generation of iNPCs directly from human skin fibroblasts using retroviruses containing the Yamanaka factors. This method allows bypassing the stem cell state and the need for clonal selection, thereby avoiding clonal variation. Important steps to keep

in mind while cells are undergoing the conversion process are the fibroblast seeding density, media pH and keeping the cells at an optimal confluency. Examples of optimal splitting confluency and morphology changes during the conversion process can be found in **Figure 1**.

[Place Figure 1 here].

After the conversion process is complete, the NPCs will show strongly reduced expression or no expression of fibroblast markers and morphology, and express NPC specific cell markers (**Figure 2**). Moreover, they can also be used for generating different cell lines, like iNs, IOs, and IAs.

[Place Figure 2 here].

In our experience, iAs in particular are very valuable for drug and disease mechanism testing as they can be generated in pure populations (98% or more GFAP positive cells[29]) at reproducible large numbers. iAs can be differentiated from iNPCs by taking a small aliquot of cells during splitting or a previously frozen iNPC portion and directly plating in astrocyte media. Important considerations during this process are maintaining the iNPCs initial seeding density low (**Figure 3** and **Figure 4**), as a high density has been shown to hinder the differentiation process (**Figure 4**) and paying additional attention to the media pH, as acidification can activate even healthy astrocytes.

[Place Figure 3 here].

[Place Figure 4 here].

FIGURE AND TABLE LEGENDS:

Figure 1. Representative images of the conversion process after adding the retroviral mix of Yamanaka factors. Fibroblasts were seeded and transduced twenty-four hours later with Yamanaka factors. Two days post virus (DPV), media was changed to conversion media. Cells were cultured in conversion media until they were ready to passage (13 DPV). Cells were seeded post passage to reach 80% confluence the following day (14 DPV). Subsequent passages maintained this density until neuronal progenitor cells begin rapidly dividing. Scale bar is 200 µm.

Figure 2. Cells that undergo the conversion process express cell-specific markers. Patient fibroblasts (A), induced Neuronal Progenitor Cells (iNPCs (B) and induced Astrocytes (iAs (C) cells were seeded on glass coverslips in a 24 well tissue culture treated plate. Cells were immunostained for: fibroblast specific cell markers (A), Vimentin (green) and TE7 (red), iNPC specific cell marker (B), Nestin (red), and iAs cell marker (C) GFAP (purple) and iNPC marker Nestin (green). Scale bar is 50 µm.

Figure 3. Representative images of the iAs generation process from iNPCs. iNPCs are seeded in Astrocyte media (Table 1) at a low seeding density. A good seeding density is about 10% on the first day post seeding (DPS) (left); however, this density can be adjusted according to the growth rate of cells. Typical iAs morphology can be observed after 5 DPS (middle). In some cases,

aberrant astrocyte morphology can be observed, with long, spiky extensions. This change is indicative of astrocyte activation and can be secondary to disease state or incorrect culturing techniques (right). Scale bar is 200 µm.

Figure 4. iNPC seeding density affects the iAs differentiation efficiency. Control iNPC lines were seeded at low (A) and high (B) density in Astrocyte media to demonstrate effects of seeding density on differentiation. 5 DPS, the low-density line expressed astrocyte-specific markers, while the high-density line shows a mixture of iNPC and iAs markers with a marked iNPC-like morphology. Scale bar is 50 µm.

Supplemental Figure 1. Additional figures of the conversion process after adding the retroviral mix of Yamanaka factors. Images of the conversion process at 12 DPV (1 day before passage) and 19 DPV (6 days after passage). Note the difference on morphology between cells before and after passage, at 12 DPV cells have a ball-like structure morphology. After a passage and several days on conversion media (table 1) cells start displaying a NPC-like morphology. Scale bar is 200 µm.

Table 1. Media recipes for all cell types included in the protocol. Media should be filtered after mixing all components with either a Sterile Vacuum Filtration System for big volumes or syringe and 0.2 um syringe filters. Conversion media (with factors) must be prepared fresh every week.

Table 2. Recommended seeding density of iAs per plate area. Typical number of iAs seeded to generate a monolayer on the most common tissue culture plates.

Table 3. Instructions on how to portion iNPC to generate iAs. Proportion of iNPC suspension and freezing media to generate portions. Note that the final DMSO concentration is 10% when cell suspension is mixed with freezing media.

DISCUSSION:

In summary, the direct conversion is a fast and easy method to generate induced neuronal progenitor cells from patient skin fibroblasts. This method is advantageous because of its speed as well as the large number of cells generated that are easy to maintain. Moreover, increasing evidence suggests that direct reprogramming methods remove less epigenetic marks compared to classic iPSC reprogramming technology, therefore leaving the disease environment more intact[4,7,8]. The differentiation of iNPCs to astrocytes is very straight forward and highly reproducible even between multiple independent laboratories[19,29,33]. iNPCs can be frozen in small portions and thawed directly for differentiation purposes, which helps reduce variation between experiments since passage numbers can be kept similar. Astrocytes play a crucial role in disease progression in many neurological diseases and are therefore an interesting cell type to work with. The astrocytes can be used for mechanistic studies, metabolic studies or drug testing assays and are usually grown as monolayers. Moreover, astrocytes can be combined in co-culture systems with mouse or human neurons to assess the impact of astrocytes on neuronal survival and morphology, both of which are good readouts for drug testing[34].

In order to successfully use this protocol, a few points and potential limitations need to be kept in mind. The human skin fibroblasts for example vary greatly in their cell division rate as well as response to viral vectors. Since these are not standardized cell lines, they are as variable as mankind itself, each line is different. As for many reprogramming methods, it is easier to reprogram fibroblasts that have a moderate to fast cell division rate versus cells that are barely replicating. The replication rate can be impacted by the skin biopsy quality and processing itself, by the passage number of the fibroblasts as well as handling. When working with primary skin fibroblasts, it is important to not let the cells get too dense to avoid induction of senescence. If the fibroblasts do not grow well, it is best to try a new stock or a lower passage, although in some cases, the disease itself could also play a role. Cell division can sometimes be improved by using 15% or 20% FBS in the fibroblast media compared to the standard 10%.

The quality of the reprogramming viral vectors is of high importance for success. In our hands, retroviral vectors were more efficient compared to lentiviral vectors or other non-integrating viruses; however, this could be dependent on the viral vector quality and purity. Commercial retroviral vector kits usually specify the viral vector concentration and often also a multiplicity of infection for a certain cell line. It is important to keep in mind that primary fibroblasts differ from the cell line used by the companies to determine viral vector uptake. Furthermore, even when ordering the same commercial kit, variation between batches is common. Moreover, the uptake of viral vectors also varies between fibroblast cell lines. Some lines could be more sensitive and therefore transduced cells could die, while other cell lines might be more resistant to viral uptake. Thus, determining transduction efficiency for a given cell line can be important especially if the cell line is growing slowly or previous conversion attempts failed. In our hands, the best way to evaluate how much of a specific retroviral vector is needed for conversion is to perform an immunofluorescent staining with several dilutions of the viral vector. The number of cells expressing the transgene for each vector can then be counted, with the aim of a transduction efficiency of 70% or higher. In our experience, similar MOIs can be used for most cell lines however the MOI can be adjusted if necessary.

During the conversion process, it is critical to not split the cells too early. The time until the cells are ready to be split depends on multiple factors including the primary cell line and its characteristics, the quality of the viral vector used and the media quality. Importantly, the success rate of the conversion is much higher when the cells are kept at high density. Even if the cells start to change morphology, it is better to leave the cells in the first well longer than splitting them prematurely. If the split occurs too early the conversion can halt in its progress and lead the cells to differentiate back to fibroblast-like shape and behavior. Furthermore, diluting them too early can result in more elongated cell bodies compared to the small and compact cell bodies of the NPCs observed previously. Thus, the first splits should always be conservative; it is better to keep the cells too dense with a 1:1 or 1:2 split compared to diluting them too much. Some cell death is expected following the initial split. Of note, the cells always look worse (flatter) the first day after splitting, which is normal. Best judgments on cell shape should be made 2-3 days later. Importantly, the quality of the growth factors and media components is crucial as well. It is important to prepare small amounts of media containing growth factors so that the fully prepared media will only be used for 5-7 days maximum. Do not keep the media at room

temperature or 37°C for extended periods of time. Use quickly and refrigerate as soon as the media change is performed. In addition, keeping the volume of media low at 1 mL instead of 2 mL can help especially for cell lines that are more resistant to converting. If the cells consume the media rapidly, which can be observed by a change in media color form red to yellow (acidification rate), adjust the volume of the media to up to 2 mL. Rapid media consumption is a positive sign and often observed at later stages of conversion alongside increased proliferation.

NPCs are also very sensitive to the presence of accutase in the media and it is very important to keep the accutase incubation time short. We recommend an incubation period of 2-4 minutes, check cells frequently under the microscope to see when they have detached. It is essential to centrifuge the cells after splitting to remove the enzyme mix from the media. It is also important to keep the passage number in mind as cell lines can change upon extended culturing leading to alteration of expression profiles. Thus, it is important to freeze many cell stocks at early passages. Cells should be frozen in 10% DMSO and 90% NPC media and stored long term in liquid nitrogen. Due to the lack of serum in this media it is critical to ensure a slow freeze using freezing chambers and once frozen overnight, immediately transfer to liquid nitrogen. While no clonal selection is performed in this protocol, it is possible that extended culturing and passaging leads to natural selection of an initial cell population with favorable growth and survival rate. Therefore, it is important to keep passage number and handling in mind when performing experiments. We prefer to use lower passages for experiments, if possible, we do not exceed passaging the cell lines for more than 20 times. Since NPCs grow rapidly, large numbers of stocks can be frozen at lower passages for experiments. Cell lines with particularly high growth rates are at higher risk of media acidification. Thus, as cell lines grow with different speed, the amount of media might need to be adjusted based on proliferation. If the cells are continuously left in highly acidified media, it can influence the health of both control and disease cell lines. This causes even healthy astrocytes to become reactive, affecting their use in further differentiation and experiments.

For astrocyte differentiation, it is important to keep the cells at low density as high density will prevent the cells from differentiating and they will keep proliferating at higher rates. Thus, seeding density needs to be adjusted for each cell line dependent on their proliferation rate. We recommend trying different seeding densities and perform stainings/RT-PCR with astrocyte markers to ensure proper differentiation. IAs quality can be assessed alongside healthy controls using co-culture assays with neurons. Provided disease pathology does not lead to a reactive phenotype, neurons should remain viable in contact with iAs for several days. Astrocyte morphology can vary greatly between individual cell lines and can be impacted by the disease phenotype as well. Moreover, in diseases where astrocytes are heavily impacted, they might show a reactive phenotype with large, elongated extensions.

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DISCLOSURES:

The authors have nothing to disclose.

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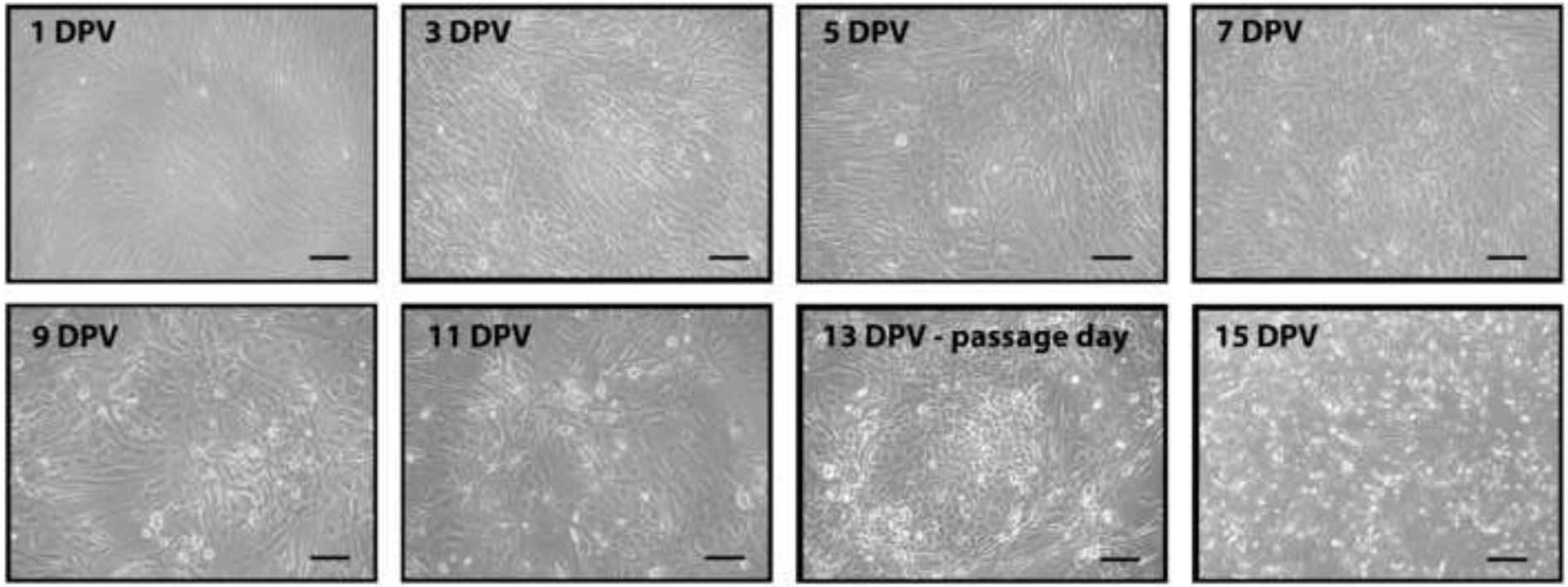
575

Figure 1



Background

Figure 2

Click here to access/download;Figure;Figure 2. IF w cell type markers.tif

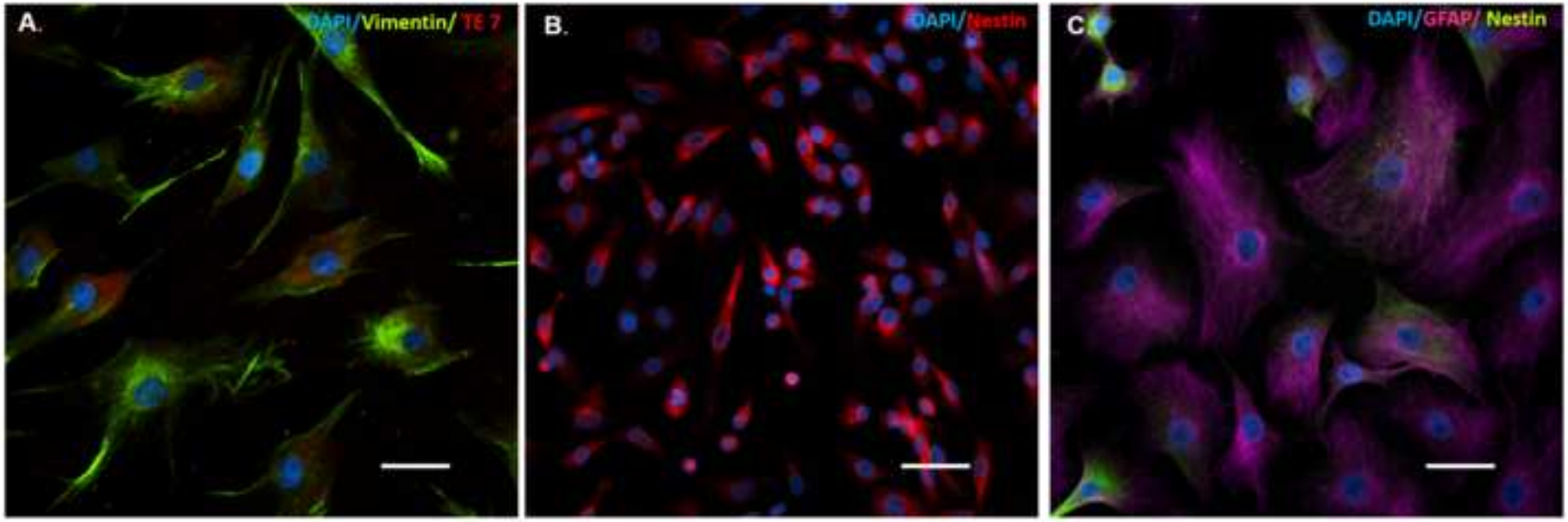
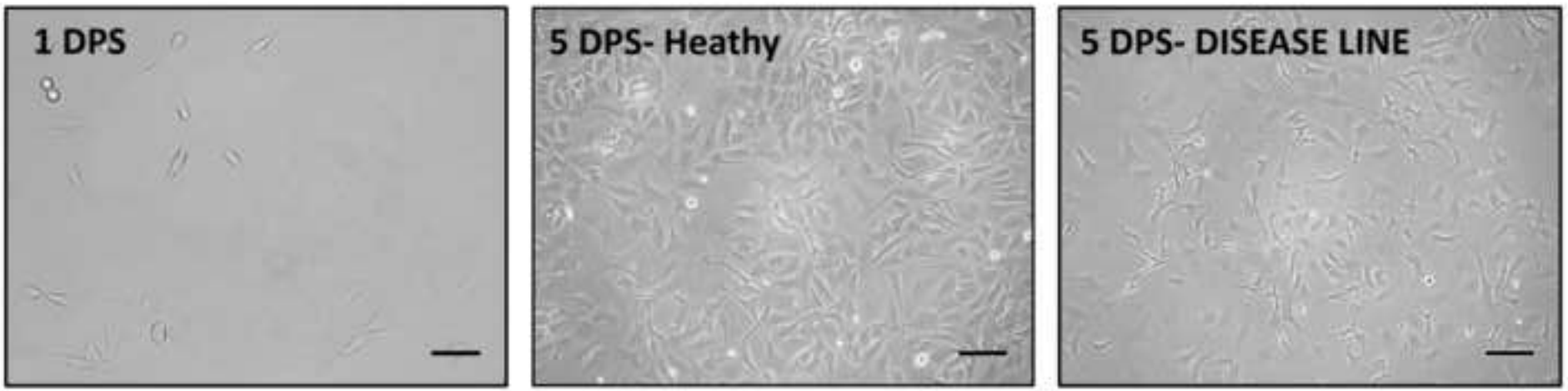


FIGURE 2. No numbers.tif

Figure 3



NEW FIGURE 3-Representative images of the Astrocyte generation process from i

Figure 4

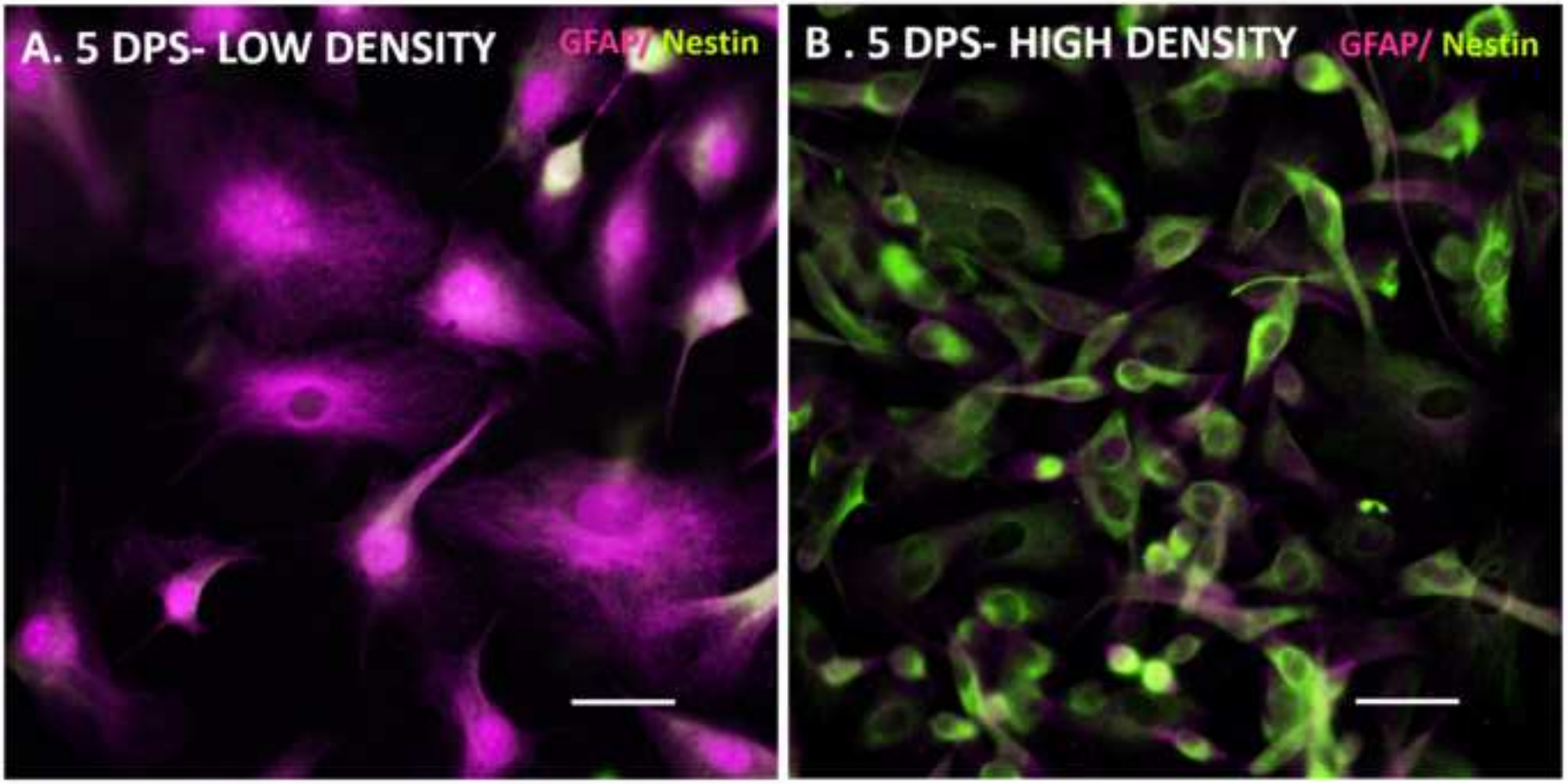


FIGURE 4 las SEEDING IF.tif

Table 1. Media Prepa

Media	Reagent
Fibroblast media	DMEM, high glucose, GlutaMA
	Fetal Bovine Serum
	Antibiotic-Antimycotic
Base media	DMEM/F12 + Glutamax
	N-2
	B-27
	Antibiotic-Antimycotic
Conversion media	Base media
	FGF
	EGF
	Heparin
Neural Progenitor Cell (NPC) Media	DMEM/F12 + Glutamax
	N-2
	B-27
	Antibiotic-Antimycotic
	FGF
Astrocyte Media	DMEM, high glucose, GlutaMA
	Fetal Bovine Serum
	Antibiotic-Antimycotic
	N-2

Media must be filtered after mixing all components. Conversion med

ıration

Amount to mix	Final concentration (%)
500 mL	89
50 mL	10
5 mL	1
500 mL	97
5 mL	1
5 mL	1
5 mL	1
50 mL	99.9
1 µL	0.02 (20 ng/mL)
1 µL	0.02 (20 ng/mL)
50 µL	0.1 (5 µg/mL)
500 mL	96.9
5 mL	1
5 mL	1
5 mL	1
10 uL	0.002
500 mL	89
50 mL	10
5 mL	1
1 mL	0.2

dia (with factors) must be prepared fresh every week.

Table 2

Click here to access/download;Table;Table 2. Recommended seeding density of Astrocytes.xlsx

Plate Type	Astrocytes per Well
384-well	2500
96-well	10000
24-well	40000
6-well	150000

Table 3

Click here to access/download;Table;Table 3. Instructions on iNPC portions for freezing.xlsx

Portion Size	Cell Suspension (µL)	Freezing media (µL)	Total volume (µL)
4x	400	400	800
2x	200	200	400

Defrosts Into
Two 10-cm dishes
One 10-cm dish

Table of Materials

Click here to access/download;Table of Materials;Materials Table Final.xlsx

Name	Company
100mm x 2mm Style dish, Cell culture treated, Nonpyrogenic	Corning
15 ml conical screw cap centrifuge tubes, copolymer polypropylene	USA Scientific
50mL Conical Centrifuge Tubes	USA Scientific
Antibiotic-Antimycotic (100X)	Gibco
B-27 Supplement (50X), serum free	Invitrogen
Cryogenic vials 1.2ML	Corning
DMEM, high glucose, GlutaMAX Supplement, pyruvate	Gibco
DMEM/F-12, GlutaMAX supplement	Gibco
DMSO	Sigma
Dulbecco's Phosphate Buffered Saline (PBS)	Gibco
EZ Retrovirus iPSC Reprogramming Kit	ALSTEM
Fetal Bovine Serum, certified	Gibco
Fisherbrand Sterile Syringes for Single Use	Fisher
Heparin sodium salt from porcine intestinal mucosa	Sigma
Human Plasma Fibronectin Purified Protein	Millipore Sigma
Isopropanol (technical grade)	Fisher Scientific
Mr. Frosty	Thermo Fisher
N-2 Supplement (100X)	Gibco
Recombinant Human EGF	Preprotech
Recombinant Human FGF-basic	Preprotech
StemPro Accutase Cell Dissociation Reagent	Gibco
Stericup Quick Release-GP Sterile Vacuum Filtration System	Millipore Sigma
Tissue culture plate, 6 well, Flat bottom with Low evaporation lid	Fisher
Trypsin-EDTA (0.05%), phenol red	Invitrogen
Whatman Puradisc 25 syringe filters, 0.2 µm, PES sterile	Millipore Sigma

Catalog Number	Comments/ Description
430167	Tissue culture
1475-1611	Used for lifting and centrifuge cells
1500-1811	Media preparation
15240062	Antibiotic with antifungal activity for media preparation
17504044	For NPC and base media
CLS430658-500EA	For freezing cell stocks
10569010	For fibroblast and Astrocyte media
10565042	For NPC and base media
D2438-50ML	For freezing cell stocks
14190136	Referred in protocol as PBS. For fibronectin dilution and cell wash
RF101	Retrovirus containing the Yamanaka factors. Virus can also be made in-house.
16000-036	Referred in protocol as FBS . For Fibroblast and Astrocyte media
14-955-461	Filter media (50mL)
H3149-10KU	Referred in protocol as Heparin. Used in conversion media. Powder diluted in ultrapure water. Final stock concentration of 5000X
FC010-10MG	Referred in protocol as Fibronectin, used in 1:200 dilution for coating.
S25372A	For freezing cell stocks
5100-0001	For freezing cell stocks
17502048	For Astrocyte, NPC and base media
AF-100-15	Referred in protocol as EGF, used in conversion media. Powder diluted in PBS, final concentration of 1mg/mL, stored in small frozen aliquots
100-18B	Referred in protocol as FGF, used in NPC and conversion media. Powder diluted in PBS, final concentration of 1mg/mL, stored in small frozen aliquots
A1110501	Referred in protocol as Accutase. Used for lifting during the conversion process and NPCs.
S2GPU05RE	Media filtration
08-772-1B	Tissue culture
25300062	Lifting of Fibroblasts and Astrocytes
80-2502	Filter media (50mL)

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January 11, 2021

Answers to Editorial and Reviewers comments

We would like to thank all reviewers for their effort and important constructive comments. We are excited to submit a further improved version of the protocol manuscript. Please see our answers to each point and comment below.

Editorial and production comments:
Changes to be made by the Author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.
3. 1.1: How much cell solution or fibroblast media is added? Please specify all volumes used throughout.
4. What are the culture conditions throughout?
5. Please discuss limitations of the protocol in the discussion.

We thank the editors for the suggestions, we have reviewed and proof-read the manuscript as well as added requested media amounts. Limitations of the protocol such as growth rate of the fibroblasts, quality of the viral vector used and impact of specific disease phenotypes on conversion rate are carefully elaborated in the discussion section.

Changes to be made by the Author(s) regarding the video:
1. Please increase the homogeneity between the video and the written manuscript. Ideally, all figures in the video would appear in the written manuscript and vice versa. The video and the written manuscript should be reflections of each other.
2. Furthermore, please revise the narration to be more homogenous with the written manuscript. Ideally, the narration is a word for word reading of the written protocol.
3. Text
00:03 - 00:03 It's hard to read the title with everything capitalized. Also, the numbers following the names should be superscripted. Please remove the caps and superscript the numbers.
00:05 - 00:05 The title needs to fade out with the names. Here you can see the title fades out after the person fades up, covering their face. Please fade out the title at the same time as the names, and allow a second of black before the person fades in.
01:01 - 01:01 It looks like all the text is capitalized throughout. Please only capitalize the first letter of every word.
13:45 - 13:45 Please add the person's name and title as you did with the beginning.
4. Pacing
00:34 - 00:34 The person looks off camera to the script and there's an awkward pause here. I recommend removing this part and to fix the cut in the video, add footage of the protocol for the remainder of this interview.

00:59 - 00:59 The video should fade out before the text fades in. Please add a half second of black after the fade out before fading up on the text.
02:33 - 02:33 The pacing goes from quick to slow right here, then speeds back up after four sentences. Maybe find a middle ground.
04:42 - 04:42 The pacing starts to slow down here. Please maintain the same pace throughout.
07:58 - 07:58 Please fade out the table first, then face up on the title.
09:22 - 09:22 Please fade out video, then fade up title.
10:36 - 10:36 Fade out video frist, THEN fade up title.
10:41 - 10:41 Please fade up on the video, don't cut to the video.
11:12 - 11:12 Instead of cross dissolving from the microscope to the counting, please just do a straight cut to the counting.
11:14 - 11:14 Extend the counting shot to give it enough time before the table comes in.
11:43 - 11:43 Fade out table first, then fade up title.
12:24 - 12:24 Instead of dissolving between these figures, please fade out the previous figure THEN fade in on the next figure.
13:39 - 13:39 Fade out first, then fade in title.
14:16 - 14:16 Fade out before she looks up from the camera, then fade in the title after.
5. Pacing is all over from fast to slow and back to fast. It's also almost 15 minutes long. I feel that when listing the parts that needs to be combined, instead of listing every single part, have a text list come up with what's needed and have the narration refer to this list.
6. Audio
01:11 - 01:11 I can hear the narrator hitting the spacebar to stop recording. Please remove any unscripted audio such as breaths and odd sounds like that.
02:09 - 02:09 There's a cut in the audio that's very audible, as if the person starts to breath and it gets cut off. Remove this by fading out before the breath happens.
03:26 - 03:26 The last word gets cut off, then a weird breath happens here. Please adjust the edit so the word ""Media"" doesn't get cut off, and remove the breath following this.
06:52 - 06:52 I hear paper rustling. Please remove any odd sound that's not scripted.
08:52 - 08:52 Please remove the thump sound
11:33 - 11:33 The narration sounds like it changes in tone or quality. It's as if this narration was recorded at separate times. Please maintain the quality throughout when recording the narration."
7. Composition
11:49 - 11:49 The background should be white to match with the figures background.

Please upload a revised high-resolution video here:
https://www.dropbox.com/request/vaszLxivjhU4wQwgX6Gd?oref=e

We thank the reviewer for this thorough review and we believe we have addressed all comments listed in the new version of the protocol.

Reviewers' comments:
Reviewer #1:
Major Concerns:
1. The video should include the media composition table when dictating - otherwise it's hard to follow, specially as they form quite bit of the video. Authors mentioned monitoring morphological changes in the text as well in the video. Examples of some of these intermediate morphology should be included in the video/supplementary - these can be helpful supplement to the main figures.

We thank the reviewer for these thoughtful suggestions and have implemented the changes in the video and as additional material. Examples of intermediate morphology are also shown in figure 1.

2. Scale bar for fig 2b is off - it can't be the same as a and c. Please check.

We verified and found the scalebar to be correct, the iNPCs are much smaller/compacter cells compared to astrocytes and fibroblasts.

3. Authors need to refer previous works in the introduction and/or discussion on direct conversion of mouse and human fibroblasts into induced astrocytes using viral over expression (eg: Caiazzo et al., 2014). Discussion should include how their protocol is better than these previous work briefly.

We thank the reviewer for this important comment and have added additional sections and references to previous work done as suggested in line 86-96 in the introduction as follows:

In recent years, researchers focused on modified reprogramming methods allowing a more direct generation of different cell types while bypassing the pluripotent stem cell state[9-12] (reviewed in [13,14]). Initial breakthroughs revealed in vitro combinatorial reprogramming of fibroblasts to cardiomyocytes[15], neurons[16] and hepatocytes[17] by ectopic expression of multiple lineage-specific transcription factors or microRNAs[18]. This was followed by studies directly reprograming cells to model neurological disorders[19,20].

as well as have added additional information on the advantage of this protocol in lines 115 and following. Briefly, a major advantage is the use of widely available reprogramming factors that can be purchased easily as a plasmid for in house vector production or even as commercial reprogramming kits as ready to go viral vectors. Moreover, this protocol allows the production of highly pure iAstrocytes with a rapid differentiation of only 5 days.

4. Is the expected %induced astrocytes obtained from fibroblasts mentioned anywhere? If not, that is a vital information helpful to include. It is understandable that their will be variability but providing a ballpark range is helpful.

We thank the reviewer for pointing out this shortcoming, we have added the following information to the introduction – we have also added new citations of a recent publication using this method:

Approximately 98% to 100% of directly reprogrammed iNPCs can differentiate into GFAP-positive astrocytes[26] as compared to only 2% using direct conversion method from fibroblasts to astrocytes[27].

Minor Concerns:
These are suggestions for ease of following the protocol:
1. The steps are fine but there are too many sub steps which seems more like 'notes'.
For example: Steps 1.1.2, 1.4.1, 1.6.1-3, 2.8.1-2, 2.9.1-3 might be better off as 'notes' rather than steps.

Step 4 should be note and Step 4.1 should be Step 4. Steps 3.1 should also be 'note'.

2. Also, these sub steps/notes could be made more compact and shorter.

3. Step 1.6 needs to refer to image/s showing the morphological changes mentioned.

We are grateful to the reviewer for these suggestions and have reduced the number of sub steps in the protocol, we agree that this helps with the flow of the protocol. All suggestions have been followed in the new version of the protocol.

Reviewer #2:

Manuscript Summary:

These authors describe a method for generating astrocytes from human fibroblasts. They utilize a technique involving retroviral transduction of reprogramming factors (Oct3/4, Sox2, Klf4, and c-Myc) commonly found in the generation of induced pluripotent stem cells. Presumably the various media components (EGF/FGF/Heparin) allow the conversion to neural progenitor cells from a pseudo-pluripotent state. Overall I think having this technique hosted on JOVE will be a benefit to those interested in utilizing this technique to study a variety neurological diseases. The protocol itself is clearly organized and easy to follow in the manuscript and the accompanying video was also clear and easy to follow.

Major Concerns:

NA

We thank the reviewer for recognizing the value of the protocol.

Minor Concerns:

1. I would like to see the introduction expanded at least briefly to better describe the similarities/differences in marker expression profiles for all astrocyte tissue regardless of the source (primary, iPSC derived, direct conversion, etc.). How do the authors suggest we best gauge the quality of these derived astrocytes? Just through being able to express the markers discussed in this paper, RT-PCR, or other cellular functions? Besides speed, does this method offer other benefits?

The quality of the astrocytes is best established using a combination of different evaluations including qRT-PCR as well as immunofluorescent stainings for example for GFAP. We have added a brief description in the introduction in line 122-127 in regards to characterization of the iastrocytes. That paragraph also now contains a reference to a recently published study in which iAs made through this protocol are compared to astrocytes made by classical reprogramming technique and in which we demonstrate that the iAs maintain epigenetic marks to a greater extent compared to iPS derived astrocytes. Multiple publications have used cells generated with this method and a many different functional assays were run, we believe listing them in the introduction would go beyond the scope of this protocol.

2. I'm curious if other methods (such as electroporation) have been attempted.

We have attempted lentiviral vectors, which also work, but show a reduced success compared to the retroviral constructs as well as sendai virus (non-integrative). While the sendai virus attempt worked in one of our collaborators laboratories with the same protocol described here, it did not work in our hands. We might retry this again at some point. However, we did not try electroporation. We believe short term expression of the transcription factors might make the conversion more difficult, but it should be possible (based on the results observed with sendai virus by our collaborator).

3. Do the authors have supportive evidence regarding their statement starting on line 336? They claim "the method allows...avoiding clonal variation." Would the retroviral induction not create cells with varying amounts of viral load? The fact that they are not isolating clones does not mean that all of the cells in this system are identical or that one treatment to the next will result in identical cell types or recreate phenotypes. In fact, Meyer et al. referenced in this manuscript appears to include a clonal selection step. Are there new data that suggests this isn't necessary?

We thank the reviewer to bring up this point. We have added this to the limitation section in the discussion as follows:

While no clonal selection is performed in this protocol, it is possible that extended culturing and passaging leads to natural selection of an initial cell population with favorable growth and survival rate. Therefore, it is important to keep passage number and handling in mind when performing experiments. We prefer to use lower passages for experiments, if possible, we do not exceed passaging the cell lines for more than 20 times. Since NPCs grow rapidly, large numbers of stocks can be frozen at lower passages for experiments.

The initial publication Meyer et al 2014 did not perform clonal selection but mentions that some cell lines create ball like structure that can be collected, dissociated and expanded – balls that detach from the main dish can be dissociated and grown individually OR they can be dissociated and re-added to the main pool of cells. To clarify this important point, we have added the following notes into the protocol under step 1.6.1:

Note: Some cell lines start forming loose elevated round formations growing in ball like structures or loose neuronal spheres (Supplemental image 1 and [19,33]). Care should be taken to not detach these cells. If the balls detach, they can be collected and re-plated in a new fibronectin coated well of a 6-well plate.

Note: If they are expanded on their own, these cells will have reduced diversity compared to the initial plate and may represent a distinctly different cell line. We recommend combining these cells with the rest of the converted cells at the next round of splitting.

We do not perform clonal selection in our protocols and none of the published studies used a clonal selection step. Another aspect that we have not published is that we have converted the same cell line in two independent laboratories and have gotten very similar results in the functional assays.

4. Line 414: "increasing evidence suggests that direct reprogramming methods remove less epigenetic marks compared to classic iPSC reprogramming technology..."Please add references.

We have added references to support this statement.

5. Figure 2. The authors stain three different stages of astrocyte development with three different stain sets. This limits the reader's ability to visualize how these markers may be turning on or off during this conversion process. Can DAPI/Vimentin/TE 7 be shown for all three stages? Much of this data is in Meyer et al. already, but it would be nice to show a more clear picture of this process here.

We don't have this particular staining for all three cell types and were unable to perform the additional experiment over the holidays. However, several previous publications that are cited in the protocol show marker expression as well as micro array comparison between the individual cell types including fibroblasts, iNPCs and astrocytes. We think these publications do cover this aspect extensively.

6. In Table 1 can the concentrations be added to the Conversion media list for EGF, FGF, and heparin? It wouldn't take more room up than "see Material list for conc" and it would allow a user to more easily utilize the table.

We have added the concentration to the table

Reviewer #3:
Manuscript Summary:
The Authors described a a protocol for direct reprogramming of human patient fibroblasts into induced neuronal progenitor cells (iNPCs) by expressing the Yamanaka factors. The resulting iNPCs can be further differentiated astrocytes. The authors also claimed that the iNPCs can be frozen in small portions for astrocyte differentiation over extended periods of time.

Major Concerns:
The reprogramming of fibroblast into NPC is not a novel technology in the field. The authors should make brief discussion on the development of such technology in the field either in the introduction or the discussion section.

We thank the reviewer for this reminder and we have added a brief discussion of previous work in the introduction as follows:

Initial breakthroughs revealed in vitro combinatorial reprogramming of fibroblasts to cardiomyocytes[11], neurons[12] and hepatocytes[13] by ectopic expression of multiple lineage-

specific transcription factors or microRNAs[14]. This was followed by studies directly reprograming cells to model neurological disorders[15,16].

The authors did not and should have specified the design of the expression vector they used. This is crucial as the promoter of choice could affect how successful the conversion would be. On the other hand the timeline of conversion is not very clear from the text and the authors did not include a schematics of the conversion timeline in their figures. It is strongly recommended that the authors include a schematics showing the design of the expression vectors and the timeline of the entire conversion protocol.

We thank the reviewer for this suggestion, however, this protocol can be used with different reprogramming vectors for expression is driven by different promoters including commercial kit that is cited – we tried to find the promoter information but it was not available on the homepage. We have included the catalogue number of the reprogramming kit in the materials table.

We have added the following sentence in the beginning of the protocol for the schematic:

Note: A schematic timeline of this protocol can be reviewed in Meyer (2014)[15].

Since the authors did not describe the design of the expression vector, it is not clear if the Yamanaka factors are silenced upon conversion. Authors should address this issue by staining markers or qPCR for the Yamanaka factors and discuss the potential alternation of the cell fate if those factors continue to express in the NPCs generated.

We did not have enough time to perform these additional experiments during the timeframe we were given for review over the holiday period and we believe these would go beyond the scope of this protocol publication. The cells created with this protocol have been tested in many functional assays and have proven to be highly useful for disease modelling. Several articles are cited underlining this point.

The authors claimed that the iNPC generated could be frozen into aliquots and thawed for future experiment. The authors should have shown data to demonstrate the efficacy to thaw the frozen cells and to generate astrocytes based on that.

We thank the reviewer for these thoughtful suggestions.

Several of the cited articles are using cells stored in this fasion, including the recently published article for which we added a new citation: Gatto, N. et al. Directly converted astrocytes retain the ageing features of the donor fibroblasts and elucidate the astrocytic contribution to human CNS health and disease. Aging Cell. 10.1111/acel.13281 e13281, (2020).

The data supporting this aspect are spread out throughout multiple large projects and we did not run a isolated study to proof this point. In the short time we had to revise the protocol manuscript during the holiday period, we were unable to perform this additional experiment. However, based on the published work, we are highly confident that these cells can be stored in this manner.

The authors have described a series of morphology changes along the conversion process like " loose elevated round formations growing in ball like structures" which is not shown in the figure. The author should shown these morphology changes as they described so to give the readers a full picture of what to expect during the conversion.

The images for Figure 1 is either not having enough contrast or the resolution is low. The morphology change along the conversion is not very clearly demonstrated in Figure 1.

We thank the reviewer for his valuable comments, we have further improved the images in figure 1 to ensure better visibility. In addition, we have added more images in supplementary figure 1 as well as referenced 2 papers that show ball like structures as follows:

Note: Some cell lines start forming loose elevated round formations growing in ball like structures or loose neuronal spheres (Supplemental image 1 and [19,33]).

Minor Concerns:
It is recommended that the authors to include the staining of one more marker for both the NSC and astrocytes in order to be more affirmative of the identity of the cell type generated. qPCR of multiple markers for each cell type is also recommended.

Thanks very much for this suggestion, multiple studies cited in this protocol article have done extensive testing of various markers in fibroblasts, iNPCs and iAs. We think these previous experiments cover this aspect and additional experiments would go beyond the scope of this protocol publication.

Again we would like to thank all reviewers and editors for the comments and suggestions, we believe these have improved the manuscript significantly.

Sincerely,

Kathrin Meyer, Ph.D
Assistant Professor - Department of Pediatrics
The Ohio State University College of Medicine
Principal Investigator - Center for Gene Therapy

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kathrin.meyer@nationwidechildrens.org

Supplemental Figure 1 (Figures, Permissions, etc.)

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