**TITLE:**

Isolation and direct neuronal reprogramming of mouse astrocytes

**AUTHORS AND AFFILIATIONS:**

Bob A. Hersbach1,2,3, Tatiana Simon2, Giacomo Masserdotti1,2\*

1Institute of Stem Cell Research, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany

2Department of Physiological genomics, Biomedical Center Munich, Ludwig-Maximilians University, Planegg-Martinsried, Germany

3Graduate School of Systemic Neurosciences, BioCenter, Ludwig-Maximilians University, Planegg-Martinsried, Germany

\*Correspondence to Giacomo Masserdotti: giacomo.masserdotti@helmholtz-muenchen.de

**SUMMARY:**

Here we describe a detailed protocol to generate highly enriched cultures of astrocytes derived from different regions of the central nervous system of postnatal mice and their direct conversion into functional neurons by the forced expression of transcription factors.

**ABSTRACT:**

Direct neuronal reprogramming is a powerful approach to generate functional neurons from different starter cell populations without passing through multipotent intermediates. This technique not only holds great promises in the field of disease modeling, as it allows to convert, e.g., fibroblasts from patients suffering neurodegenerative diseases into neurons, but also represents a promising alternative for cell-based replacement therapies. In this context, a major scientific breakthrough was the demonstration that differentiated non-neural cells within the central nervous system, such as astrocytes, could be converted into functional neurons *in vitro*. Since then, *in vitro* reprogramming of astrocytes into neurons have provided substantial insights into the molecular mechanisms underlying forced identity conversion and the hurdles that prevent efficient reprogramming. However, the results from *in vitro* experiments performed in different labs are difficult to compare due to differences in the methods used to isolate, culture and reprogram astrocytes. Here, we describe a detailed protocol to reliably isolate and culture astrocytes with high purity from different regions of the central nervous system of mice at postnatal ages via magnetic cell sorting. Furthermore, we provide protocols to reprogram cultured astrocytes into neurons via viral transduction or DNA transfection. This streamlined and standardized protocol can be used to investigate the molecular mechanisms underlying cell identity maintenance, the establishment of a new neuronal identity as well as the generation of specific neuronal subtypes and their functional properties.

**INTRODUCTION:**

The mammalian central nervous system (CNS) is highly complex, consisting of hundreds of different cell types, including a vast number of different neuronal subtypes1-6. Unlike other organisms or tissues7-9, however, the mammalian CNS has a very limited regenerative capacity: neuronal loss following traumatic brain injury or neurodegeneration is irreversible and often results in motor and cognitive deficits 10. Aiming to rescue brain functions, different strategies to replace lost neurons are under intense investigation11. Among them, direct reprogramming of somatic cells into functional neurons is emerging as a promising therapeutic approach12. Direct reprogramming, or transdifferentiation, is the process of converting one differentiated cell type into a new identity without passing through an intermediate proliferative or pluripotent state13-16. Pioneered by the identification of MyoD1 as a factor sufficient to convert fibroblasts to muscle cells17,18, this method has been successfully applied to reprogram several cell types into functional neurons19-21.

Astrocytes, the most abundant macroglia in the CNS 22,23, are a particularly promising cell type for direct neuronal reprogramming for several reasons. First, they are widely and evenly distributed across the CNS, providing an abundant *in loco* source for new neurons. Second, astrocytes and neurons are developmentally closely related, as they share a common ancestor during embryonic development, the radial glial cells24. The common embryonic origin of the two cell types seems to facilitate neuronal conversion as compared to reprogramming of cells from different germ layers19,21. Furthermore, patterning information inherited by astrocytes through their radial glia origin is also maintained in astrocytes 25-27 and seems to contribute to the generation of regionally appropriate neuronal subtypes28-30. Hence, investigating and understanding the conversion of astrocytes into neurons is an important part of achieving the full potential of this technique for cell-based replacement strategies.

The conversion of *in vitro* cultured astrocytes into neurons has led to several breakthroughs in the field of direct neuronal reprogramming, including: i) the identification of transcription factors sufficient to generate neurons from astrocytes15,19,31; ii) the unravelling of molecular mechanisms triggered by different reprogramming factors in the same cellular context32 and iii) the impact of developmental origin of the astrocytes in inducing different neuronal subtypes28,29,33. Furthermore, *in vitro* direct conversion of astrocytes led to identify several major hurdles that limit direct neuronal reprogramming efficiency34,35, such as the increased reactive oxygen species (ROS) production34 and differences between mitochondrial proteome of astrocytes and neurons35. Hence, these observations strongly support the use of primary cultures of astrocytes as a model for direct neuronal reprogramming to investigate several fundamental questions in biology12, related to cell identity maintenance, roadblocks preventing cell fate changes as well as the role of metabolism in reprogramming.

Here we present a detailed protocol to isolate astrocytes from mice at postnatal age with very high purity, as demonstrated by isolating astrocytes from the murine spinal cord 29. We also provide protocols to reprogram astrocytes into neurons via viral transduction or DNA plasmid transfection. Reprogrammed cells can be analyzed at 7 days post transduction (7 DPT) to assess various aspects, such as reprogramming efficiency and neuronal morphology, or can be maintained in culture for several weeks, to assess their maturation over time. Importantly, this protocol is not specific to spinal cord astrocytes and can be readily applied to isolate astrocytes from various other brain regions, including the cortical gray matter, midbrain and cerebellum.

**PROTOCOL:**

This protocol describes the isolation of astrocytes from the spinal cord of postnatal mice and their direct reprogramming into functional neurons (**Figure 1A)**. In this protocol, spinal cord tissue is collected from mice at day 2-3 after birth. Usually, 6-8 spinal cords are collected for astrocyte isolation. Importantly, the protocol can be readily applied to isolate astrocytes from other regions of the nervous system, such as the cortex, midbrain and cerebellum. For these regions, the protocol works best when cells are obtained 5-7 days after birth. Typically, 2 cortices, 6 midbrains and 6 cerebelli are needed per experiment. When aiming to isolate astrocytes from regions that are not mentioned in this protocol, the age and input material should be experimentally determined.

1. **Preparation of dissection, dissociation and culture reagents**

Note: Prepare all culture reagents within a biological safety cabinet and work sterile using only autoclaved or sterile equipment. Dissection and dissociation reagents can be prepared outside of a biological safety cabinet.

* 1. Prepare culture flasks by coating a T25 culture flask with poly-D-lysine (stock 1 mg/mL; working solution 20 µg/mL) in H2O for a minimum of 2 hours. Afterwards, rinse 3x with H2O and let air dry.
	2. Prepare dissection buffer by adding 5 mL of 1M HEPES buffer solution to 500 mL of Hank’s balanced salt solution (HBSS).
	3. Prepare 1 C-tube (see Table of Materials) per 6 spinal cords by adding 1950 µL buffer X (Neural Tissue Dissociation Kit; see Table of Materials). Store on ice during dissection.
	4. Prepare enzymatic digestion master mix by adding 20 µL buffer Y (Neural Tissue Dissociation Kit; see Table of Materials) and 10 µL buffer A (Neural Tissue Dissociation Kit; see Table of Materials) per C-tube. Mix well and store at 4°C until needed.
	5. Prepare 1x Phosphate Buffered Saline (PBS) with Calcium and Magnesium and place on ice.
	6. Prepare basic culture medium by adding 5 mL of Penicillin-Streptomycin, 5 mL of 45% D-Glucose and 5 mL of glutamine supplement (final concentration 2mM; see Table of Materials) to 485 mL of DMEM/F12. Basic medium is stable at 4 °C for 4 weeks.
	7. Prepare astrocyte culture medium by adding 1 mL of B27-supplement and 5 mL of Fetal Bovine Serum (FBS) to 44 mL of basic culture medium. Supplement medium with EGF and basic-FGF (bFGF) (10ng/mL each).
	*Note*: EGF and bFGF should be added just before use to the appropriate amount of medium.
1. **Materials for dissection**

For the dissection of spinal cord tissue, it is recommended to use:

* 1. A large forceps with bent tip
	2. A small forceps with bent tip
	3. One pair of small forceps
	4. A small scissor
	5. A small spatula
1. **Spinal cord tissue dissection**

The following procedure follows the animal care guidelines of our institution. Please make sure to comply with the animal care guidelines of the institution where the dissection is performed.

*Note*: Dissection of tissue can be performed outside of a biological safety cabinet.

**3.1** Sacrifice mice at P2-P3 by decapitation without anesthetizing the animal. Place the torso in a 35 mm petri-dish and keep on ice.

**3.2** Open the skin with scissors, remove vertebra, extract the spinal cord and place in dissection buffer on ice.

**3.3** Under a stereotactic dissection microscope, remove the meninges from the isolated spinal cords and transfer dissected and cleaned spinal cord tissue to a C-tube.

1. **Magnetic activated cell sorting (MAC-sorting)**
	1. Add 50 µL of Enzyme P (Neural Tissue Dissociation Kit; see Table of Materials) and 30 µL of previously prepared enzyme mix to each C-tube.
	2. Invert the C-tubes and place them on a heated dissociator (see Table of Materials), making sure that all tissue is collected in the lid of the tube.
	3. Run the 37\_NTDK\_1 dissociation program (run time is approximately 22 min).
	4. Shortly before the end of the program, place a 70 µm strainer (see Table of Materials) on a number of 15 mL tubes equal to the number of C-tubes used and pre-wet strainer with 2 mL of ice-cold PBS.

*Note*: 1X PBS should be stored on ice during the entire MACS procedure.

* 1. After completion of the program, remove C-tubes and briefly centrifuge them to collect the tissue at the bottom of the tubes.
	2. Transfer the dissociated tissue from the C-tubes to the 15 mL tubes prepared in Step 4.4. by passing it through the strainer. Leave strainer on 15 mL tubes.
	3. Rinse C-tube with 10 mL of PBS to collect leftover tissue and collect it in the same 15 mL tubes used in step 4.5 by passing through the strainer once more.
	4. Centrifuge 15 mL tubes containing dissociated tissue at 300 x g for 10 min at room temperature.
	*Note*: Cool the centrifuge down to 4 °C after this step.
	5. Remove the supernatant without disturbing the cell pellet before resuspending the cells in 80 µL of PBS and adding 10 µL of blocking reagent (Mouse anti-ACSA-2 MicroBead Kit; see Table of Materials). Gently mix by pipetting.
	6. Incubate at 4 °C for 10 min in the dark (fridge).
	7. Add 10 µL of anti-astrocyte cell surface antigen-2-coupled beads (Mouse anti-ACSA-2 MicroBead Kit; see Table of Materials) and gently mix by pipetting.
	8. Incubate for 15 min at 4 °C in the dark.
	9. Wash cells by adding 3 mL of 1X PBS and centrifuge at 300 x g for 10 min at 4 °C.
	10. While centrifuging, assemble a magnetic separator (see Table of Materials) by placing the appropriate number of magnetic sorting columns (see Table of Materials) onto the separator with a 15 mL collection tube underneath. Rinse the columns with 500 µL of 1X PBS.
	11. Remove supernatant and resuspend the cells in 500 µL of 1X PBS before transferring cell suspensions to the magnetic columns. Let drain by gravity.
	12. Wash the 15 mL tubes that contained the cells with 500 µL of 1X PBS and apply to column.
	13. Wash columns with 500 µL 1X PBS for an additional 2 washes.
	14. Elute cells by removing the column from the separator, adding 800 µL of astrocyte culture medium and pushing cells out of the column with the included plunger.
	15. Plate cells in the previously prepared culture flasks by adding 4.2 mL of astrocyte culture medium supplemented with EGF and bFGF and culture at 37 °C and 5% CO2.
	*Note*: Coating culture flasks is not necessary for astrocytes isolated from other brain regions but provides a better substrate for astrocytes isolated from the spinal cord.
	16. Culture cells for approximately 7 days until confluent.

*Note*: When cells are not confluent after 7 days, it is possible to wait up until 10 days before plating them. After 10 days, cells do not proliferate anymore, and the reprogramming efficiency declines.

1. **Seeding of astrocytes for reprogramming**

The following steps have to be performed under a biological safety cabinet with a safety level 1 (SL1).

* 1. Prepare 24-well plates with PDL coated glass coverslips in the same way culture flasks were prepared previously.
	*Note*: To determine the number of plates needed it is important to consider that astrocytes are plated at a density of 50-55.000 cells per well in a 24-well plate. Usually, isolating 6 spinal cords from P2 mice yields around 1x106 cells.
	2. Aspirate media from the T12.5 culture flasks containing the cultured astrocytes and wash once with 1X PBS.
	3. Detach the astrocytes from the culture flask by adding 0.5 mL of 0.05% Trypsin/EDTA and incubate at 37 °C for 5 min. Gently tap the side of the flask to release cells from the culture flask surface and check detachment under a brightfield microscope.
	4. Stop trypsinization with 2.5 mL of astrocyte culture medium and collect cell suspension in 15 mL tubes. Centrifuge at 300 x g for 5 min.
	5. Aspirate supernatant and resuspend cells in 1 mL of astrocyte culture medium. Calculate cell concentration using a haemocytometer or an automated cell counting system.
	6. Based on the number of cells, dilute the cell suspension with fresh astrocyte medium in order to obtain a solution of 100-110.000 cells per mL. Supplement the medium with EGF and bFGF (both 10 ng/ mL).
	7. Add 500 µL of cell suspension, equivalent to 50-55.000 cells, to each well of the previously prepared 24-well plates and culture cells at 37 °C and 5% CO2.
1. **Forced expression of transcription factors**
	1. The day after plating, inspect the 24-well plates to make sure cells have adhered to the coverslips.

*Note*: before proceeding with the protocol, it’s essential to properly design the experiment. In particular, it’s important to always include a negative control for the reprogramming, namely a condition where no reprogramming factor is expressed. For instance, when using vectors carrying the cDNA for the reprogramming factor and a reporter (e.g., green fluorescent protein (GFP), DsRed), the negative control is represented by the same vector carrying only the reporter. When expressing multiple factors carrying different reporters, the negative control should be accordingly adjusted.

* 1. Based on the experimental aim and available resources, the forced expression of reprogramming factors can be achieved by viral transduction (see step 6.3) or DNA transfection (see step 6.4).
	2. Astrocytes can be reprogrammed by transducing the cells with the retro- or lentivirus carrying the genetic information to express the reprogramming factor(s) of interest:
		1. Transduce cells with high titer virus (1x1010-1x1012 particles / mL) by adding 1 µL directly to the astrocyte medium. This ensures a high infection rate.
		2. Culture the cells at 37 °C for 24-48 h astrocyte medium containing viral particles before proceeding with Section 7 or 8, depending on the purpose of the experiment.

*Notes*: Different promoters can be used to drive the expression of the transgenes (see also “Representative Results”). Constitutive promoters (e.g., CMV, CAG) induce the expression of the transgene earlier than inducible promoters; however, both types of promoter types have been successfully used to reprogram cells into neurons.

The use of retro- or lentivirus requires the approval of government authorities and must be performed under a biological safety cabinet within a laboratory with safety level 2 (SL2).

* 1. DNA plasmids can also be introduced into astrocytes via DNA transfection:

*Note*: This can be done under a biological safety cabinet approved for SL1.

* + 1. Before transfection, obtain transfection reagent, a plasmid DNA Maxiprep of the desired construct, fresh astrocyte medium and serum-reduced medium (see Table of Materials).
		2. Calculate the required amount of serum-reduced medium (see Table of Materials) by considering that each well of a 24-well plate requires 300 µL of serum-reduced medium. Add the appropriate amount of serum-reduced medium to a 50 mL tube and warm it to 37 °C.
		3. When the serum-reduced medium is warm, aspirate astrocyte medium from all wells and collect it in a 50 mL tube. Filter the collected astrocyte medium with a 0.45 µM syringe filter to remove detached cells.
		4. Add an equal volume of fresh astrocyte medium to the filtered medium to obtain a solution sufficient to add 1 mL of astrocyte medium per well. Maintain the astrocyte conditioned medium in the incubator at 37 °C until use (step 6.4.14).

*Note*: The culture medium is re-used as it contains several secreted factors that support the viability of the culture.

* + 1. Add 300 µL of pre-warmed serum-reduced medium to each well and place the 24-well plate back to the incubator.
		2. Prepare solution A, consisting of DNA and serum-reduced medium. Per well a total of 0.6 µg total DNA can be used and should be added to 50 µL of serum-reduced medium. Solution A is stored at room temperature until use.
		*Note*: typically, a technical triplicate per condition is considered. Therefore, a mix for 3.5 reaction is prepared (e.g., 2.1µg of total DNA diluted in 175 µL od serum-reduced medium) to ensure enough material to transfect 3 well of a 24-well plate.
		3. Prepare solution B, composed of the transfection reagent and serum-reduced medium. Per well 0.75 µL of transfection reagent is added to 50 µL of serum-reduced medium. As this solution is common to all transfection conditions it can be prepared in bulk, thus reducing the variability between transfections.
		4. Incubate solution B at room temperature for 5 min.
		5. Add solution B to solution A drop by drop at a 1:1 ratio and gently mix. Do not vortex.
		6. Incubate solution A+B for 20-30 min at room temperature under the hood.
		7. Repeat Step 6.4.6 – 6.4.9. for all transfection conditions.
		8. After 20-30 min, add solution A+B to each well drop by drop for a final volume of 100 µL.
		9. Gently shake the plate and place the cells back in the incubator at 37 °C for 4 h.
		10. After 4 h, remove transfection medium and add 1mL of the pre-warmed astrocyte conditioned medium prepared in step 6.4.4.
		11. Maintain cells for 24-36 h before proceeding with Section 7 or 8, depending on the purpose of the experiment.
1. **Reprogramming of astrocytes (7 days analysis)**
	1. Prepare neuronal differentiation medium by adding 1 mL of B27-supplement to 49 mL of basic culture medium (see step 1.6).
	2. After 24-48 h, depending on whether cells have been transduced or transfected (see steps 6.3 and 6.4 respectively), replace astrocyte medium with 1 mL of neuronal differentiation medium per well and culture cells at 37 °C and 9% CO2.

*Note*: Cells can also be kept at 5% CO2 if no 9% CO2 incubator is available. However, neuronal reprogramming is more efficient under these conditions.

* 1. (Optional) To increase the reprogramming efficiency, neuronal differentiation medium can be supplemented with Forskolin (final [30 µM]) and Dorsomorphin (final [1 µM]) when replacing the astrocyte medium to differentiation medium.
	2. (Optional) When opting to treat cells with Forskolin and Dorsomorphin, a second dose of Dorsomorphin 2 days after the initial treatment is recommended. This second treatment is added directly to the culture medium.
	3. Direct reprogramming of murine astrocytes into neuronal cells normally occurs within 7 days after changing the medium. Hence, 7 days after the initiation of neuronal reprogramming cells can be fixed or collected for downstream analysis.
1. **Reprogramming of astrocytes into mature neurons (long term cultures)**
	1. Prepare neuronal differentiation medium by adding 1 mL of B27-supplement to 49 mL of basic culture medium (see step 1.6).
	2. After 24-48 h, depending on whether cells were transduced or transfected (see steps 6.3 and 6.4 respectively), replace astrocyte medium with 1 mL of neuronal differentiation medium supplemented with Forskolin (final [30 µM]) and Dorsomorphin (final [1 µM]) per well and culture cells at 37 °C and 9% CO2.

*Note*: Cells can also be kept at 5% CO2 if no 9% CO2 incubator is available. However, neuronal reprogramming is more efficient under these conditions.

* 1. Repeat Dorsomorphin treatment 2 days after the initial treatment by adding it directly to the neuronal differentiation medium.
	2. 7 days after the start of neuronal reprogramming, prepare maturation medium by adding 6 µL of N2, 1.2 µL of NT3 (stock 20 µg/mL), 1.2 µL of BDNF (stock 20 µg/mL), 1.2 µL of GDNF (stock 20 µg/mL) and 1.2 µL of cAMP (stock 100 mM) to 189.2 µL of neuronal differentiation medium.
	3. Supplement neuronal differentiation medium present in each well with 200 µL of maturation medium.

*Note*: Maturation medium is supplemented only for the first treatment. All subsequent treatments are done by partially replacing the neuronal differentiation medium as described below.

* 1. Repeat the treatment with small molecules by removing 200 µL of neuronal differentiation medium and adding 200 µL of fresh maturation medium twice a week for up to 6 weeks.
	2. During the maturation, cells can be used for electrophysiological experiments (typically, at day 21 or later) or fixed for downstream analysis (e.g., immunofluorescence).

**REPRESENTATIVE RESULTS:**

Primary cultures of astrocytes typically reach 80-90% confluency between 7 to 10 days after MAC-sorting and plating (**Figure 1B**). Generally, a single T25 culture flask yields around 1 - 1.5 x 106 cells, which is sufficient for 20 – 30 coverslips when seeding cells at a density of 50-55.000 cells per well. The day after plating, cells typically cover 50-60% of the coverslip surface (**Figure 1C**). At this stage, cultures consist almost exclusively of astrocytes, while other cell types, such as neuroblasts, are virtually absent (**Figure 1D**)29.

Reprogramming factors can be delivered to astrocytes either via retro- or lentiviral transduction or through transfection of DNA plasmids. Usually, viral transduction infects more cells as compared to transfection. As direct neuronal conversion causes a substantial amount of cell death 34,35, retro- or lentiviral transduction is preferred to maximize the number of cells for analysis. Different promoters can be used to control the expression of the reprogramming factors: constitutive (e.g., CMV, CAG) 15,32, inducible (e.g., Tet-responsive elements, Tet-ON) 21 or cell type specific (e.g., GFAP promoter) 36,37. When using constitutive or cell type specific promoters, astrocytes start to express detectable levels of the transgenes, based on fluorescent reporter expression (e.g., GFP, DsRed), within 24 hours after the gene delivery, with each cell independent to the others; conversely, inducible promoters allow to synchronize the activation of the expression of the transgenes among the cells transduced, as they are activated following the addition to the culture medium of a small molecule (e.g., doxycycline). Detectable levels of the transcription factors are usually reached 18-20 hours after the activation of the promoter. In most cases, the peak of the reprogramming factor expression is reached at around 48 hours, with lentivirus-mediated expression taking slightly longer.

While transcriptional changes following reprogramming factors expression can be detected as early as 4 hours32, robust changes occur after 24 hours and later 29,32. Morphological changes follow transcriptional changes and first signs of conversion can be observed around 3 days post transduction/transfection (3 DPT). At 7 DPT, induced neuronal cells are clearly distinguishable from astrocytes: their soma is smaller than control or un-reprogrammed astrocytes, they have long processes and they are positive for neuronal marker βIII-tub and are negative for astrocyte marker GFAP (**Figure 1E**). However, it is worth noting that some cells can be either positive for both GFAP and βIII-tub, suggesting that the neuronal program has been induced but the astrocyte identity has not been inhibited, or negative for both markers, indicating the repression of the astrocyte identity but the absence of induction of the neuronal cascade. In either case, the cells usually maintain an astrocyte morphology.

For more functional analyses, such as electrophysiology or the evaluation of the generated neuronal subtypes, cultures are generally maintained for a minimum of 21 DPT and treated with maturation medium. At 21 DPT, many induced neurons are capable of firing action potentials and are positive for the mature neuronal marker NeuN as well as for the pan-synaptic protein Synaptophysin (**Figure 1F**).

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Overview of astrocyte culture and reprogramming.** **A.** Timeline of astrocyte-to-neuron direct conversion. Each black line represents an important step in the protocol. **B.** Representative brightfield images of cultured spinal cord-derived astrocytes after 7 days in culture. Pictures were taken using a brightfield microscope and 10x objective. Scale bar represents 100 µm. **C.** Representative brightfield images of spinal cord astrocytes one day after re-plating at a density of 55.000 cells per well in a 24-well plate. Images were taken using a brightfield microscope and a 10x objective. Scale bar represents 100 µm. **D.** Immunofluorescence image of a βIII-tub, Sox9, GFAP triple staining on astrocytes fixed one day after plating to demonstrate culture purity. Cells were fixed in 4% paraformaldehyde for 10 min and washed twice with 1x PBS. Cells were blocked using a 3% BSA, 0.5 % Triton-X 100 in 1x PBS solution. Primary antibodies were diluted at the proper concentration (e.g., anti-GFAP 1:250; anti-βIII-tub 1:250; anti-Syp1 1:500) in blocking solution and incubated for 2 hours at room temperature. Cells were washed three times with 1x PBS and incubated with fluorophore-conjugated secondary antibodies for 1 hour at room temperature. Coverslips were washed three times with 1x PBS before mounting with Aqua Poly/Mount. Images were acquired using an AxioM2 epifluorescence microscope and a 40x objective. Scale bar represents 20 µm. **E.** Immunofluorescence image of a βIII-tub, DsRed double staining to demonstrate astrocyte to neuron conversion with Ascl1 after 7 DPT. Protocol of immunofluorescence and image acquisition are equal to what is described above. Scale bar represents 20 µm. **F.** Immunofluorescence images of a βIII-tub, DsRed, Synaptophysin 1 (Syp1) triple staining to demonstrate neuronal maturity after 21 DPT of reprogramming with Ascl1. Protocol of immunofluorescence and image acquisition are equal to what is described above. Scale bar represents 20 µm.

**DISCUSSION:**

Primary cultures of murine astrocytes are a remarkable *in vitro* model system to study direct neuronal reprogramming. In fact, despite being isolated at a postnatal stage, cells express typical astrocyte markers29, retain the expression of patterning genes28,29, and maintain the capacity to proliferate, similar to *in vivo* astrocytes of a comparable age38. After MACS-mediated isolation, cells first adhere to the flask and then start to proliferate, giving rise to highly enriched astrocyte cultures29. Importantly, cultured astrocytes do not dedifferentiate into a multipotent cell state nor get immortalized. Furthermore, they do not spontaneously generate neurons following the expression of a reporter protein (e.g., DsRed or GFP), but maintain an astrocyte identity. Also, they do not proliferate indefinitely, but rather slowdown their proliferation and transition into a more mature stage, which reduces the efficiency of direct neuronal reprogramming32,39.

There are several steps that are critical in this protocol: first, it is essential to carefully isolate the region of interest and remove any contaminating tissues. For example, to prepare spinal cord astrocytes, the spinal cord is extracted from the vertebrae and the dorsal root ganglia (DRG) are carefully removed. Second, converting cells undergo significant cell death34, which has a negative impact on the transduced cells as well as the overall culture due to the stimulation of phagocytosis by surrounding astrocytes as well as altered media osmolarity. Therefore, it is important to replace the astrocyte medium with an adequate volume of differentiation medium – usually 1 mL/well of a 24 well plates. Additionally, the transfection of plasmid DNA is an easy and more accessible approach compared to viral transduction, which requires an approved safety level 2 cell culture room. However, transfection rate and reprograming efficiency are lower compared to viral-mediated delivery of the reprogramming factors. Therefore, transfection can be used as a fast method to test the reprogramming potential of new candidate reprogramming factors or to screen pools of factors. Regarding neuronal maturation, reprogrammed cells usually become electrophysiologically active at around 3 weeks. Though not required, treating the cells with small molecules increases both survival as well as maturation of the reprogrammed cells, leading to a higher density of induced neurons and a more mature morphology.

Although the described method to isolate and culture astrocytes is robust and reliable, a few aspects need to be considered. First, while conventional methods based on mechanical dissociation of tissue yield an overall higher number of cells in culture per tissue dissected40, a MAC-sorted approach requires the dissection of tissue from 6-8 pups to isolate an adequate number of cells for subsequent experiments. Furthermore, the isolation of astrocytes is based on the expression of the ATPase Na+/K+ transporting subunit Beta2 protein (Atp1b2), recognized by the antibody ACSA-241. In principle, astrocytes not expressing Atp1b2 would be lost in the preparation, therefore causing a bias in the preparation. Although we cannot exclude that this is the case, our analysis of MACS flow-through revealed that few cells in the negative fraction were immunoreactive for the astroglia markers Sox9, suggesting the high efficiency of MAC-sorting protocol. A second caveat related to Atp1b2 is related to its expression. Atp1b2 is specifically expressed by astrocytes at postnatal stage, while in the mouse adult brain other cell types express it, in particular myelinating oligodendrocytes and ependymal cells27. Therefore, a careful dissection of the area of interest and a myelin removal step is required to isolate astrocytes from adult brains.

Compared to other methods for isolating astrocytes, MACS-based approach ensures high purity of the cultures (>90% of Sox9+ cells) and provides a standardized procedure to isolate astrocytes from different regions of the CNS. This is particularly important when comparing cultures from different CNS regions, as the culture purity obtained by classical mechanical dissociation can vary remarkably (>80% GFAP+/DAPI from cortical gray matter, ~50% GFAP+/DAPI from spinal cord)15,29. A standardized protocol reduces such variability and provides a common starting point for *in vitro* reprogramming experiments. This allows, for instance, to systematically compare the molecular identity of astrocytes from different regions28,29, investigate the impact of the developmental origin on the reprogramming efficiency and the subtype identity of the induced neurons.

In summary, *in vitro* direct neuronal reprogramming of optimized cultures of astrocytes is a very powerful approach to unravel universal as well as region-specific molecular mechanisms of astrocyte-to-neuron conversion, providing essential information to design better and more effective strategies for *in vivo* direct conversion of resident CNS astrocytes.

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**DISCLOSURES:**

No conflicts of interest declared.

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