This protocol provides detailed instructions for generating induced neuronal cells (iN cells) from fibroblast cultures by means of forced expression of the neural transcription factors Ascl1, Pou3f2 (also known as Brn2), Myt1l, and NeuroD1. The rapid and efficient production of iN cells from readily available somatic cells bypasses the need to isolate and culture pluripotent stem cells, thus providing an approachable platform for studying neural differentiation and nuclear reprogramming processes. Also included are methods for generating functional iN cells co-cultured with primary glial cells and mouse cortical neurons that are suitable for electrophysiological assays.

This manuscript provides detailed information about the following experimental procedures:

- Preparation of non-neural mouse embryonic fibroblasts
- Preparation of perinatal tail tip fibroblasts
- Preparation of primary human foreskin fibroblasts:
  - Glial cell isolation
  - Preparation of mouse cortical cultures
- Lentiviral production using HEK293T cells
- Preparing fibroblasts for lentiviral infection and culturing iN cells
- Generating mature iN cultures

Nuclear transfer experiments in *Xenopus* and mammals first demonstrated that the nuclei of differentiated somatic cells could be reprogrammed to a pluripotent state following transfer into an enucleated, unfertilized egg (Briggs and King, 1952 #1) (Wilmut, 1996 #52) (Gurdon, 2006 #3). Despite this early progress, the factors responsible for the reprogramming capability of the oocyte remained largely mysterious. The discovery that forced expression of the four transcription factors Oct4, Sox2, Klf4, c-Myc is sufficient to reprogram somatic cells to a pluripotent, embryonic stem cell-like state provided a critical breakthrough, demonstrating convincingly that a finite number of transcription factors can drive reprogramming between distantly related cell fates (Takahashi, 2006 #4). The advent of human induced pluripotent stem cell technologies allows for the in vitro generation numerous cell types that can be used for human developmental studies, disease modeling, and as a source of autologous cells for cellular transplantation therapies (Takahashi, 2007 #5)(Takahashi, 2007 #6)(Murry and Keller, 2008 #7).

These studies also suggested that other cell fates could potentially be induced by forced expression of transcription factors that are important for the development of the desired cell type in the embryo. For example, it had long been known that fibroblasts could be converted into myocytes by forced expression of the basic helix-loop-helix (bHLH) transcription factor MyoD1 (Davis, 1987 #8), however, more distantly related cells failed to undergo complete myogenic conversion (Weintraub, 1989 #9)(Schafer, 1990 #10). Similarly, forced expression of the bHLH transcription factor Ngn3 (also known as neurog3) combined with MafA and Pdxl was sufficient to convert exocrine cells of the
pancreas (acinar cells) into insulin producing β-cells in vivo, however, it could not convert fibroblasts (in vitro) or myocytes (in vivo) to β-cells (Zhou, 2008 #11). Thus, it remained unknown whether conversion between distantly related somatic cell types was possible.

Recently, we showed that a combination of the three genes Ascl1, Brn2 (also known as Pou3f2) and Myt1l could convert mouse embryonic and neonatal fibroblasts into neuron-like cells in vitro (Vierbuchen, 2010 #12). These induced neuronal cells (iN cells) express neuron-specific proteins, fire repetitive action potentials following current injection, receive excitatory and inhibitory synaptic inputs from mouse cortical neurons, and form synapses with each other when co-cultured with astrocytes (Fig. 2b,e-g). Recently, we and others have further extended these findings by demonstrating that iN cells can also be generated from human fibroblasts by defined factors (Pang, 2011 #13)(Pfisterer, 2011 #14)(Caiazzo, 2011 #15)(Yoo, 2011 #17)(Son, 2011 #18)(Qiang, 2011 #19)(Ambasudhan, 2011 #20). Thus, iN cells could prove to be a novel platform for studying the mechanisms that regulate neural fate determination and differentiation and a potential alternative to embryonic and induced pluripotent stem cell approaches for generating neurons for in vitro disease modeling or cell-replacement therapies.

**Development of the protocol:** We hypothesized that forced expression of a group of transcription factors that regulate neuronal development could stably reprogram fibroblasts into neural cells. We chose fibroblasts as a starting cell type because they are easy to obtain and culture, and had been used previously in other reprogramming studies (Davis, 1987 #8)(Takahashi, 2006 #4). To test this hypothesis, we chose 19 candidate transcription factors that are either important regulators of neural development, neuronal differentiation, or epigenetic reprogramming. We introduced all 19 of these genes into mouse embryonic fibroblasts (MEFs) with lentiviral vectors under the control of a tetracycline-inducible promoter, cultured the virally transduced fibroblasts in neural media for 4 weeks, and found that, surprisingly, neuron-like cells had been generated at a low frequency. Furthermore, by overexpressing each of the 19 genes alone, we found that expression of the basic helix-loop-helix (bHLH) transcription factor Ascl1 was sufficient to induce some neuronal properties in fibroblasts, such as neurite outgrowth and the expression of neuronal proteins, consistent with previous observations from a variety of other tissues (Perez, 1999 #21)(Farah, 2000 #22)(Berninger, 2007 #23). By coexpressing each of the remaining 18 genes with Ascl1 in 2-factor combinations we discovered a group of genes that substantially facilitated the effects of Ascl1 alone (Brn2, Brn4, Myt1l, Olig2, Zic1). When expressed together with Ascl1 as a group, this pool of 5 transcription factors further increased the efficiency of reprogramming. Using a process of elimination, we identified a minimal pool of three genes Brn2, Ascl1, and Myt1l (BAM) that are sufficient to convert fibroblasts into functional iN cells (Vierbuchen, 2010 #12).

While effective at converting mouse fibroblasts into neurons, these three factors could not efficiently generate iN cells from human embryonic fibroblasts. In order to determine a more suitable combination of factors for conversion of human fibroblasts into iN cells, we tested the effect of adding additional factors to Brn2, Ascl1 and Myt1l. We found that the addition of Neurod1 to BAM improved the efficiency of reprogramming by 2-3 fold (Pang, 2011 #13). Furthermore, the combination of Brn2 and NeuroD1 was sufficient to generate immature neuronal cells from fibroblasts, suggesting some
level of functional redundancy among these factors. Following dissociation and replating onto pre-
established mouse cortical cultures, human iN cells could receive both excitatory and inhibitory
synaptic inputs, albeit after longer periods of co-culture than required for mouse iN cells.

**Applications of the protocol:** This protocol is useful primarily as a novel model of nuclear
reprogramming and as an alternative method for the efficient derivation of neurons from non-neural
tissues. Fibroblast-to-neuron reprogramming is a valuable tool for studying nuclear reprogramming
because, similar to reprogramming to pluripotency, it involves the transition between two distantly
related cell types. Unlike iPS cell generation, which requires multiple cell divisions (Hanna, 2009
#25), iN cells can be generated from fibroblasts with a limited number of intervening cell divisions (0-2).
While iPS cells have been generated from a large variety of cell types (Hanna, 2010 #26), iN cells
have only been generated from mouse and human fibroblasts and mouse hepatocytes (Marro, 2011
#24). It is also possible to determine the functional properties of iN cells with electrophysiological
assays, which provides a relatively rigorous yet rapid means of measuring the fidelity of
reprogramming. Thus, the rapid, efficient, and user-friendly nature of this iN cell reprogramming
protocol make it a valuable addition to the rapidly expanding toolkit of cellular reprogramming
systems (Vierbuchen, 2011 #12).

**Comparison with other methods:** Since our initial reports (Vierbuchen, 2010 #12)(Pang, 2011 #13),
multiple other mouse and human induced neuron papers have been published (see summary in Table
1). These studies demonstrated that by varying the neuronal transcription factors used for
reprogramming it is possible to generate dopaminergic (Pfisterer, 2011 #14)(Caiazzo, 2011 #15)(Kim,
2011 #31), GABAergic (Yoo, 2011 #17), and spinal motor-like iN cells (Son, 2011 #18). These
results suggest that future modifications to the transcription factor cocktails used for reprogramming
could allow for the generation of other specific neuronal cell types.

It has also been known for some time that cells expressing astrocytic markers from the early
postnatal brain can be reprogrammed into functional neuron-like cells. For example, forced
expression of the transcription factors *pax6* (Heins, 2002 #32), *ascl1/neurog2* (also known as *ngn2*)
(Berninger, 2007 #23) or *dlx2* (Heinrich, 2010 #35) can convert neonatal cortical astroglia into
neuron-like cells with relatively high efficiency. Furthermore, forced expression of *ngn2* has been
shown to be sufficient to convert postnatal cortical astroglia (from mouse or rat) into glutamateergic
neurons capable of forming synaptic networks (Heinrich, 2010 #35)(Heinrich, 2011 #36)(Blum, 2011
#37). While useful as models of nuclear reprogramming, these methods suffer from their reliance on
astrocytes, which are difficult to acquire from adult mice and humans and would thus be difficult to
apply to human patient cells.

From a practical perspective, the generation of human iN cells from fibroblast cultures derived
from patients suffering from neurological diseases could be used to establish *in vitro* models of these
devastating afflictions (Dolmetsch, 2011 #27). Currently, the only alternative method for studying live
neural cells from adult human tissue involves iPS cell reprogramming followed by subsequent
differentiation into neural lineages. While this approach has the potential advantage that iPS cell-
derived neurons recapitulate embryonic neuronal development from pluripotent cells (albeit *in vitro*)
the iPS cell approach suffers from line to line variability, high initial input of labor and resources, poor
reproducibility of some neural differentiation protocols, and heterogeneity of the resulting neural cells
(Saha, 2009 #57). By skipping the iPS cell intermediate, direct reprogramming of fibroblasts to
neurons circumvents some of these issues, and thus could provide a valuable complement to current
approaches in the field (Hansen, 2011 #58). Importantly, both approaches have recently been
suggested to be able to recapitulate relevant disease-specific features *in vitro* (Brennand, 2011
#28)(Marchetto, 2010 #29)(Qiang, 2011 #19)(Son, 2011 #18) and to provide functional improvement
following transplantation of neuronal cells into a mouse model of Parkinson’s disease (Wernig, 2008
#53)(Kim, 2011 #31).

**Limitations of the protocol:** Given the nature of lentiviral gene delivery, a heterogeneous mixture of
cells with variable numbers of viral integrations will be produced. As with iPS cell methodologies, it
is likely that protocols for producing more homogeneous iN cell populations will be developed in the
future (Wernig, 2008 #53). Despite the relatively high efficiency of conversion, it is difficult to
produce large numbers of iN cells, as they cannot be expanded once generated. For studies that require
large numbers of cells, differentiation of neurons from ES or iPS cells will be more feasible. As with
other *in vitro* generated neurons, iN cells potentially exhibit specific molecular and functional
differences compared to their *in vivo* counterparts. Thus, it is important to implement stringent
functional assays and molecular criteria to assess the quality of the cells produced with this protocol.

   Compared to mouse, human iN cell generation is less efficient and human iN cells take longer
to mature, both in terms of their intrinsic membrane properties (i.e. action potentials and sodium
currents) and synaptic formation when co-cultured with mouse cortical neurons (7 days for mouse iN
cells vs. 3-5 weeks in human). Even after extended culture periods the synaptic responses remain small
suggesting human iN cells are still fairly immature. This is also observed with human ES cell-derived
neuronal cultures, which are known to require long periods of time to form synapses and tend to

   The vast majority of cells produced using this protocol exhibit an excitatory phenotype (i.e.
glutamatergic) based on their expression of vesicular glutamate transporters (vGlut1 and 2) and, for
mouse iN cells, the exclusive generation of AMPA and NMDA receptor-mediated EPSCs (Fig. 2e-g).
However, as mentioned previously, varying the transcription factors used for reprogramming can
generate other types of neurons. It remains to be determined whether these cells exhibit a specific
regional identity corresponding to a population of neurons found *in vivo*.

*Experimental design*

**Lentivirus production and plasmids:** We utilize a doxycycline-inducible lentiviral expression system
to express reprogramming factors. Complementary DNAs for *Ascl1, Brn2, Myt1l, and NeuroD1* were
cloned into a TetO-FUW lentiviral backbone (Wernig, 2008, #38) to generate TetO-FUW-*Ascl1*
(Addgene, plasmid 27150), TetO-FUW-*Brn2* (Addgene, plasmid 27151), TetO-FUW-*Myt1l* (Addgene,
plasmid 27152), TetO-FUW-Neurod1 (Addgene, plasmid 30129). This expression system makes use of the recombinant tetracycline trans-activator (rtTA) protein, expressed via an FUW lentiviral vector containing the human ubiquitin C promoter, that binds to the TetO promoter in a doxycycline-dependent manner (FUW-M2rtTA, Addgene, plasmid 20342)(Gossen, 1995, #43). Thus, for proper expression of the viral transgenes the recipient cells need to be infected with both the TetO-FUW lentiviral particles containing the reprogramming factors and FUW-M2rtTA and cultured in media containing doxycycline. We use the 3rd generation viral packaging system developed by the Trono laboratory; pRSV-rev (Addgene, plasmid 12253), pMDLg/pRRE (Addgene, plasmid 12251) and pMD2.G (Addgene, plasmid 12259).

In contrast to commonly used “helper” cells for lentiviral production, the transient transfection of the viral backbone and packaging plasmids results in very high expression rates and reliably leads to high titer viral supernatants. To transfect 293T cells for lentiviral production we typically use the calcium-phosphate precipitation method, described extensively before (Tiscornia, 2006 #45). When properly optimized it is an efficient, reproducible and economic transfection method. Commercially available lipofection agents such as Fugene 6 can also be used, and generally require less optimization but are more expensive. Depending on the application, diluted viral supernatant can be used to infect cells or the viral particles can be further concentrated by ultracentrifugation of the viral supernatant. Ideally, the infection efficiency should be evaluated using antibodies against each expressed transgene.

It is important to note that the size of the cDNA insert in the lentiviral backbone will affect the levels of viral production. For example, the TetO-FUW lentiviral vector containing Myt1l cDNA (3.5kb) will, on average, produce lower amounts of virus per plate than TetO-FUW Ascl1 (700 bp). To account for this, we find that adding 2-2.5 fold more Myt1l viral supernatant for each infection provides more comparable infection rates. Thus, for a typical infection, the ratio of lentiviral supernatant would be 1:1:1.5: 2.0 (Ascl1:Brn2:RtTA:Myt1l). We increase the amount of rtTA in order to ensure that there is a sufficient amount to transactivate the TetO promoters driving the reprogramming factors.

**Fibroblast Derivation and Culture (Box 1):** We utilize a protocol for the isolation of mouse embryonic fibroblasts (MEFs) that helps to ensure that pre-existing cells with neuronal potential in the fibroblast cultures do not confound the results of reprogramming experiments. For a more detailed protocol for isolating MEFs see (Nagy, 2003 #54). While it is relatively easy to assure that no tissue from the central nervous system is included in each preparation of embryonic fibroblasts, it is much more difficult to remove neural crest progenitor cells via dissection. Therefore we discard most of the embryo and only use fore- and hindlimbs to generate MEFs. To assess the contribution of neural crest cells to our fibroblast cultures, we have carefully characterized MEF lines generated in this manner with a panel of markers of central and peripheral nervous system stem cells and conclude that the contamination of neural crest cells is marginal in these cultures (Vierbuchen 2010 #12). We only use fibroblasts that have been passaged 3-4 times for reprogramming to further minimize any potential contribution of neural crest tissues. For experiments that require co-culture of iN cells with mouse cortical neurons or glial cells, we utilize TauEGFP reporter mice that express EGFP under the control
of the Tau promoter (Tucker, 2001 #46). This allows for unambiguous detection of iN cells derived from fibroblasts in cultures of cortical neurons derived from wild type mice.

**Mouse tail tip fibroblast derivation and culture (Box 2):**

To generate connective tissue fibroblast cultures from postnatal mice we use the distal portion of the tail. The resulting fibroblast cultures arguably represent a heterogeneous population, comprised of cells from cartilage, bone, muscle, tendons, dermis, peripheral nerves and blood vessels. Again, we have carefully characterized these cultures using an array of antibodies recognizing various characteristic neural and neural crest antigens and we concluded that the fraction of neural crest-derived cells is very low if at all present.

**Lentiviral Infection of fibroblasts and culture of iN cells (Protocol steps 1-X):** As with methods for induction of pluripotent stem cells from fibroblasts, high infection rates are critical to generate iN cells efficiently. We add polybrene to all of our infections in order to maximize infection efficiency. The lentiviral production process varies between batches, and thus we advise infecting fibroblasts with several concentrations (over a range of 3-5 fold) of viral supernatant or concentrated lentiviral particles in order to ensure that the optimal lentiviral titer is achieved. For more reproducible results, a large batch of virus can be generated, aliquoted, and titred. Given that lentiviral supernatant is HEK293T conditioned media, it is critical to dilute the viral supernatant when adding it to fibroblasts with at least an equal volume of fresh media (i.e. at least a 1:1 ratio of fresh MEF media to total viral supernatant). However, if the viral production process is effective, a significantly smaller amount of total viral supernatant will be required (ratios as low as 1:15). For a typical infection, the total viral supernatant would consist of TetO-FUW-Ascl1, TetO-FUW-Brn2, TetO-FUW-rTA and TetO-FUW-Myt1l in a 1:1:1.5:2 ratio.

Both concentrated viral particles and supernatant are effective for generating iN cells, however, it is easier to store and titer concentrated lentivirus. A Tet-O-EGFP plasmid should be included in every experiment as a proxy for measuring the lentiviral titer, and can be an important tool for determining whether the lentiviral production was successful. Tet-O-EGFP viral supernatant can be added in the same amount as one of the transcription factors in order to estimate what percentage of the cells received each individual viral transgene. Infection rates can also be confirmed by fixing infected cells and staining them using antibodies that recognize Ascl1 and Brn2. Under these conditions, we find that optimal efficiency is achieved when approximately 65-85% of the cells are EGFP positive. The TetO-EGFP virus is also useful for visualizing the axons and dendrites extending from the iN cells, which can be difficult to visualize under phase contrast amongst fibroblasts (Fig. 3a). For experimental controls, we infect cells with M2-rTA virus alone, or in combination with TetO-EGFP. Under these conditions no cells with neuronal properties are generated.

After lentiviral infection, fibroblasts are kept in MEF media containing doxycycline for 24-48 hours before being switched into basic neuronal media (N3) with doxycycline for the duration of the experiment (Fig. 1). It is important at this stage to keep the fibroblasts from becoming too confluent, as this will lead to excessive cell death when the cells are switched into serum-free neuronal media.
Plating and infecting cells on the same day can help to prevent over-proliferation, and allows for better control of the starting cell population for efficiency calculations (see steps XX-YY). The optimal cell density after culture in MEF media for 24-48 hours should be 70-90% confluency in order to provide a suitable feeder layer for iN cells. The duration of culture in MEF media can be adjusted as needed to achieve desired level of confluency before switching the cells to serum-free neural media.

Each different type of fibroblast behaves slightly differently during the reprogramming process. MEFs acclimate to the serum-free neuronal culture medium better than TTFs, which tend to become sparse after 7-10 days in neuronal media. The optimal number of plated cells might need to be increased slightly for some lines of tail-tip fibroblasts, as they tend to grow more slowly than embryonic fibroblasts. In principle human fibroblast cultures are treated similarly to mouse fibroblasts. The major difference being that the infection rates are higher in human fibroblasts and thus less virus, typically unconcentrated supernatant, can be used. Therefore, the cell densities at the time point of infection are very critical and will need to be optimized for every human fibroblast line as there are subtle differences in growth behavior between lines.

**Mature iN cell cultures for functional studies (protocol steps):** In order to generate iN cells capable of forming synaptic connections, it is necessary to co-culture developing iN cells with primary astrocytes or astrocyte conditioned media (Wu, 2007 #40)(Johnson, 2007 #39)(Kucukdereli, 2011 #47)(Ullian 2004, #48)(Pfrieger, 1997 #49). Astrocytes produce neurotrophic factors and help support the growth and maturation of synapses in cultured neurons (Eroglu, 2010 #50). For iN cell maturation, we typically plate astrocytes onto pre-established iN cell cultures 3-7 days after the addition of doxycycline. It takes an additional 2-3 weeks of co-culture for iN cells to form functional synapses.

If using MEFs derived from a neuronal reporter strain, such as TauEGFP, developing iN cells can be purified by FACS and re-plated onto established glial cultures. We have found the results obtained from both methods to be qualitatively similar. Additionally, it is essential to add neuronal growth media containing serum and other factors beneficial for neurons to the cultures for 5-7 days before recordings of synaptic activity are performed (Maximov, 2007 #51). However, neuronal growth medium contains serum, which will cause non-reprogrammed fibroblasts and astrocytes to proliferate. Adding a proliferation inhibitor such as cytosine arabinose (Ara-C) can help to stop the fibroblasts from overproliferating, but it may also cause some cytotoxicity in neurons (see steps XX).

For human iN cell experiments, FACS purification is slightly more difficult, requiring either the use of a fluorescent reporter construct to label maturing iN cells or EGFP infection followed by dissociation onto EGFP-negative mouse cortical cultures (see step XX). We use a construct with the synapsin promoter driving expression of tdTomato (Synapsin-tdTomato, see reagents) that is preferentially (although not exclusively) active in iN cells undergoing reprogramming (10-14 days after infection). We typically co-infect human fibroblasts with EGFP to help visualize neurites and to approximate the infection levels of the four reprogramming factors. This also provides a means for discriminating human iN cells from mouse cortical neurons or glia when co-cultured for synapse formation assays. We have also used selection methods to remove uninfected cells and thus enrich for iN cells without FACS (T. V., N. Y., M.W. unpublished data)(Yoo, 2011 #17). However, separating iN cells from fibroblasts by FACS allows you to generate denser cultures of iN cells that are required
for synapse formation. We have also had success using papain to dissociate cultures of fibroblasts undergoing reprogramming (at d12-14 after infection) and replating them onto cultures of mouse cortical neurons or glia grown in growth media with Ara-C (see step XX). After dissociation or FACS purification, human iN cells require another 4-5 weeks to receive synaptic inputs from mouse cortical neurons.

**Reagents and Equipment List**

- Sterile PBS, pH 7.4 (Invitrogen, cat. no. 10010-023)
- Cosmic Calf Serum (Thermo Scientific, cat. no. SH3008704)
- MEM non-essential amino acid solution 100× (Invitrogen, cat. no. 11140-050)
- Penicillin/Streptomycin 100× (Invitrogen, cat. no. 15140-122)
- Sodium Pyruvate 100× (Invitrogen, cat. no. 11360-070)
- 2-mercaptoethanol (Sigma, cat. no. M7522) (Caution)
* DNAse (Sigma, cat. no. DN25)
* Trypsin (Sigma, cat. no. T4674)
* Sodium Selenite (Sigma, cat. no. S5262)
* Progesterone (Sigma, cat. no. P8783)
* Putrescine (Sigma, cat. no. P-5780)
* Insulin (Sigma, cat. no. I-6634)
* Transferrin (Sigma, cat. no. T-1147) or apo-Transferrin (Sigma, cat. no. T2252)
* Freezing media (reagent preparation)
  - DMSO (Fischer, cat. no. 22363548)
* Polyornithine (Sigma, cat. no. P3655)
* 70% ethanol (v/v)
* Sterile water (Invitrogen, cat. no. 15230-162)
* Growth Media
  - Minimal essential medium (1×) (Invitrogen, cat. no. 51200-038)
  - Glucose (Sigma, cat. no. G8270)
  - Transferrin (Calbiochem, cat. no. 616420)
  - Fetal Bovine Serum (Hyclone, cat. no. SH30071.03)
  - NaHCO₃ (Sigma, cat. no. S5761)
  - L-glutamine (Invitrogen, cat. no. 25030164)
  - B27 supplement (Invitrogen, cat. no. 17504-044)
* iN Flow buffer (reagent preparation)
  - Neurobasal media (Invitrogen, cat. no. 21103-049)
  - MgCl₂ (Sigma, cat. no. M8266)

* Recombinant human GDNF (R&D systems, cat. no. 212-GD)
* Recombinant human NT-3 (R&D systems, cat. no. 267-N3)
* Recombinant human BDNF (R&D systems, cat. no. 248-BD)
* Recombinant human CNTF (R&D systems, cat. no. 257-NT)
* Cytosine β-D-arabinofuranoside hydrochloride (Ara-C) (Sigma, cat. no. C1768)
* Doxycycline hyclate (Sigma, cat. no. D9891)
* Hexadimethrine bromide (Polybrene) (Sigma, cat. no. H9268)
* Papain (Worthington Biochemical, cat. no. LS003127)
  - papain activating solution components (reagent preparation)
**Wernig lab**

**Generation of iN cells from mouse and human fibroblasts**

<table>
<thead>
<tr>
<th>Reagent/Equipment</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2× BBS</strong> (reagent preparation)</td>
<td>BES (Sigma, cat. no. B4554)</td>
</tr>
<tr>
<td><strong>10× CaCl₂</strong> (Sigma, cat. no. 06991) (reagent preparation)</td>
<td></td>
</tr>
<tr>
<td><strong>HBSS</strong> (Invitrogen, cat. no. 14175-095)</td>
<td></td>
</tr>
<tr>
<td><strong>EDTA</strong> (Sigma, cat. no. E5134)</td>
<td></td>
</tr>
<tr>
<td><strong>BD Matrigel Basement Membrane Matrix</strong> (Fisher, cat. no. CB40234 Ea)</td>
<td></td>
</tr>
<tr>
<td><strong>Tet-O-FUW-Ascl1</strong> (Addgene, plasmid 27150)</td>
<td></td>
</tr>
<tr>
<td><strong>Tet-O-FUW-Brn2</strong> (Addgene, plasmid 27151)</td>
<td></td>
</tr>
<tr>
<td><strong>Tet-O-FUW-Myt1I</strong> (Addgene, plasmid 27152)</td>
<td></td>
</tr>
<tr>
<td><strong>Tet-O-FUW-EGFP</strong> (Addgene, plasmid 30130)</td>
<td></td>
</tr>
<tr>
<td><strong>Tet-O-FUW-Neurod1</strong> (Addgene, plasmid 30129)</td>
<td></td>
</tr>
<tr>
<td><strong>FUW-M2rtTA</strong> (Addgene, plasmid 20342)</td>
<td></td>
</tr>
<tr>
<td><strong>pRSV-rev</strong> (Addgene, plasmid 12253)</td>
<td></td>
</tr>
<tr>
<td><strong>pMDLg/pRRE</strong> (Addgene, plasmid 12251)</td>
<td></td>
</tr>
<tr>
<td><strong>pMD2.G</strong> (Addgene, plasmid 12259)</td>
<td></td>
</tr>
<tr>
<td><strong>HEK293T cells</strong> (ATCC, cat. no. CRL-11268)</td>
<td></td>
</tr>
<tr>
<td><strong>TauEGFP homozygous knockin mouse</strong> (Jackson Labs, Stock no. 004779)</td>
<td></td>
</tr>
</tbody>
</table>

- Dissecting forceps (WPI, Dumont #1)
- Dissecting scissors (WPI, cat. no. 503242)
- Curved tip scissors (WPI, cat no. 555524s)
- Fine pincers for removing meninges (WPI, Dumont #3)
- Flame source
- Dissection microscope

- 0.45 micron PVDF filters (Millipore, cat. no. SLHV033RS)
- 0.40 micron mesh filters (VWR, cat. no. 89080-322)
- 500 ml bottle top filter PES, 0.22 uM (VWR, cat. no. 28199-970)
- 10 mL syringes (VWR, cat. no. 66064-766)
- Sterile 500 ml media bottles
- 15 cm sterile tissue culture dishes (VWR, cat. no. 82050-598)
- 10 cm sterile tissue culture dishes (VWR, cat. no. 25382-166)
- 6 cm sterile tissue culture dishes (VWR, cat. no. 25382-100)
- 5 mL sterile serological pipettes (VWR, cat. no. 53300-421)
- 10 mL sterile serological pipettes (VWR, cat. no. 53300-523)
- 25 mL sterile serological pipettes (VWR, cat. no. 53300-567)
- Cryovials (VWR, cat. no. 66008-935)
- 37 degree water bath
- Hemocytometer
- Coverslips (GmbH & Co KG, Cat. no. 01105209) (see equipment setup)
- 2mL screw cap tubes (USA Scientific, cat. no. 1420-8700)
- 15 ml falcon tubes (VWR, cat. no. 21008-936)
- 50 ml Falcon tubes (VWR, cat. no. 21008-940)
- 5-ml BD Falcon Round-Bottom Tubes (VWR, cat. no. 60819-295)
- Bench top vortex
- Beckman Optima L-80 XP Ultracentrifuge (or similar model)
Generation of iN cells from mouse and human fibroblasts

Wernig lab

-Polyallomer ultracentrifuge tubes (32 mL) (Beckman Coulter, cat. no. 355642)
-Neuronal Class III Beta-Tubulin (Tuj1, Rabbit monoclonal) (Covance, cat. no. MRB-435P).
-Mouse anti-Map2 (Sigma, cat. no. M4403)
-Mouse anti-NeuN, clone A60 (Millipore, cat. no. MAB377)
-Rabbit anti-Ascl1 (Abcam, cat. no. ab74065)
-Goat anti-Brn2 (C-20 clone) (Santa Cruz Biotechnology, cat. no. sc-6029)
-Rabbit anti-synapsin (Clone E028, provided by TC Sudhof)

Reagent Setup:

Optimizing HEK293T transfection with 2× BBS-CaCl2: Use TetO-EGFP DNA with lentiviral packaging plasmids as test DNA. Make identical transfection reactions and test 2.5 M CaCl2 amounts from 40 µl to 120 µl in increments of 10 µl. Make sure to adjust the volume of water accordingly for each reaction so that water + DNA+ CaCl2 = 500µl. Perform transfections as described below, one 10 cm plate for each mix. 16 hours later, check EGFP levels of each plate using fluorescence microscope. In order to efficiently make lentivirus, transfection efficiency should be at least 70%. A second test transfection can be done using smaller increments of 2.5 M CaCl2 concentration (5 µl) in order to further refine the amount required for optimal transfection efficiency. It is critical to optimize the transfection using the exact same conditions that will be used for the lentiviral production. Changes in the amount of total DNA, the tissue culture plate size, the amount of media on the cells, cell density, or the timing of the media change before the transfection can dramatically alter results.

MEF Media: To make 500 mL: 435 mL DMEM, 50 mL CCS (final concentration 10% vol/vol), 5 mL 100× Pen/Strep, 5 mL 100× Sodium Pyruvate, 5 mL 100× MEM NEAA, 4 µL of 2-Mercaptoethanol. Sterile filter with 0.22 µM bottle top filter into sterile media bottle. Store at 4°C for up to one month.

N3 media (mouse iN): To make 500 mL: 490 mL DMEM/F12 (Invitrogen 11320-033), (optional: 5 mL Pen/Strep), 2 mL insulin stock, 1 mL Apo-transferrin stock, 500 µL progesterone stock, 500 µL putrescine stock, 30 µL sodium selenite stock

N3+NTF media (human iN): N3 media supplemented with CNTF (20ng/ml), BDNF (10ng/ml), NT3 (10ng/ml) and GDNF (20ng/ml). Growth factor stock solution should be added to N3 media immediately before use.

Supplemented Minimal essential media: To make 500 mL: 482 mL of MEM (Invitrogen): 12.5 mL 20% glucose stock (0.5% w/v), 1.25 mL NaHCO3 stock, 1 mL apo-Transferrin stock, 4 mL insulin stock. Filter with a 0.22 µM filter and store at 4°C for up to one month.

Plating media: To make 100 mL: 89 mL supplemented MEM, 10 mL FBS (10% v/v), 1 mL 0.2M glutamine solution (is this a prepared stock?), 400 µL insulin stock. Filter with a 0.22 µM filter and store at 4°C for up to one month.

Growth Media (no AraC): To make 100 mL: 93 mL supplemented MEM, 5 mL FBS (5% v/v), 250 µL glutamine solution, 2 mL B-27 supplement. Filter with a 0.22 µM filter and store at 4°C for up to one month.

iN Flow Buffer: 500mL Neurobasal media with 10mM MgCl2, 5mM EDTA and 2.5 g glucose. Adjust pH to 7.4 with NaOH. Sterile filter and store at 4°C for 1-2 months.

GDNF: To make the stock, dissolve 10 µg GDNF in 100 µL sterile 0.1% BSA/PBS. Keep 10 µL aliquots in -80°C.

BDNF: To make the stock, dissolve 5 µg BDNF in 200 µL sterile 0.1% BSA/PBS. Keep 20 µL aliquots in -80°C.
NT-3: To make the stock, dissolve 5 µg NT3 in 100 µL sterile 0.1% BSA/PBS. Keep 10 µL aliquots in -80°C.

CNTF: To make the stock, dissolve 10 µg CNTF in 100 µL sterile 0.1% BSA/PBS. Keep 10 µL aliquots in -80°C.

0.25% Trypsin: Dilute 2.5% trypsin stock with HBSS-EDTA for 0.25% stock solution. Store aliquots at -20 (long term) or 4°C for up to one month.

Papain activating solution: 5 mL HBSS, 80 µl Papain, 5 µl EDTA (0.5 µM), 5 µl CaCl2 (1 µM). Filter with a 0.22 µM filter and store at 4°C for up to one year.

2× Freezing media: To make 100 mL: 40 mL DMEM, 40 mL CCS, 20 mL DMSO. Sterile filter and store at 4°C for up to one month. Dilute 1:1 with MEF media to freeze down cells.

2× BBS solution: (50 mM BES, 280 mM NaCl, 1.5 mM Na2HPO4): Add H2O (double distilled) up to 900 mL. Dissolve, titrate to pH 6.95 with 1M NaOH and bring volume to 1 liter. Sterile filter, aliquot and store at 4°C.

2.5 M CaCl2: Dissolve in sterile water. Aliquot into 1.5-ml screw cap tubes and store at −20°C.

Polyornithine: To make 100× stock, dilute 100 mg in 67 mL water. Filter with 0.22 µM filter and store at −20°C in 5 mL aliquots. Dilute 100× stock in sterile water and filter into sterile bottle. Store at 4 degrees for up to 3 months.

Doxycycline: 2 mg/mL dissolved in water (1000× stock). Sterilize with a 0.22 µM filter and store at -20°C. Protect from light.

Hexadimethrine bromide (Polybrene): 8 mg/ml dissolved in water (1000× stock). Filter through a 0.22 µM filter. Store at -20°C.

DNase: 10 mg/ml in sterile water, adjust pH to 7.3, filter with 0.22 µM syringe filter, aliquot and store at −20°C.

Apo-Transferrin (Sigma T2036 or T1147): Dissolve 500 mg in 20 mL water and store at −80°C in 1 mL aliquots.

Insulin (Sigma I6634): Dissolve 500 mg in 80ml 10 mM NaOH, and store at −80°C in 2 mL aliquots.

Sodium Selenite (Sigma S5261): Hygroscopic. Keep dry and seal well with parafilm. Dissolve 86.5 mg in 10 mL water, dilute further 1:100 in water (e.g. 100µl in 10ml) and store at −80°C in 1 mL aliquots. Thawed aliquots are stable at 4 C for at least up to one month.

Progesterone (Sigma P8783): Dissolve 32 mg in 50 mL ethanol, dilute 1:100 in ethanol, and store at −80°C in 0.5 mL aliquots.

Putrescine (P5780): Dissolve 0.16 g in 10 mL water and store at −80°C in 0.5 mL aliquots.

Glucose: For 20% stock: Dissolve 20g glucose in 100 mL sterile water. Filter with a 0.22 µM filter and store at 4°C for 3-6 months.

NaHCO3 Stock (for MEM): For 8% stock: Dissolve 1 g NaHCO3 in 12.5 mL sterile water. Filter with a 0.22 µM filter and store at 4°C for up to one week.

Cytosine arabinose (Ara-C): Dissolve 12 mg Ara-C in 50 mL sterile water. Filter with 0.22 µM filter. Aliquot and store at -20°C.

Matrigel (BD): Prepare fresh before use. Dilute 1:50 in DMEM and keep on ice.

Fetal bovine serum (Hyclone): Heat inactivate for 30 min at 57°C before use. Filter with 0.22 µM filter and store at 4°C for up to one month.

Preparation of polyornithine coated plates: Apply enough poly-ornithine to coat the entire surface area of a sterile tissue culture plate. Place in sterile 37°C incubator for 120 minutes. Remove poly-ornithine and rinse once with HBSS to remove residual polyornithine before use.

Preparation of Matrigel coated coverslips: Thaw matrigel on ice. Coat treated coverslips with 80-100 µL and incubate at 37°C for 60 minutes. Remove 90% of the liquid before plating cells.
**Equipment Setup**

Preparation of coverslips for cortical neuron culture: Perform the following washes:

1) 100% Xylene 30 min
2) 100% Acetone 30 min ×2
3) 100% Ethanol 30 min ×3,
4) 70% Ethanol 30 min ×3
5) Dry coverslips by separating them and placing them on clean paper towels in a laminar flow hood or fume hood. Collect clean dry coverslips in a glass petri dish and autoclave

**Timing:**

*Preparation of non-neural mouse embryonic fibroblasts
*Preparation of perinatal tail tip fibroblasts
*Preparation of primary human foreskin fibroblasts:
*Glial cell isolation
*Preparation of mouse cortical cultures
*Lentiviral production using HEK293T cells
*Preparing fibroblasts for lentiviral infection and culturing iN cells
*Generating mature iN cultures

---

**Box 1: Preparation of non-neural mouse embryonic fibroblasts**

Timing: 2-3 hours

**Important:** All procedures with live animals should adhere to relevant institutional guidelines for animal welfare.

**Important:** Perform all subsequent procedures in sterile tissue-culture hood.

Fill a petri dish with 70% ethanol and submerge surgical instruments. Regularly sterilize instruments while working to avoid contamination.

1) Remove E13.5-E14.5 embryos from uterine horns and place in a 10cm tissue culture dish filled with HBSS.

2) Wash twice with 20 mL HBSS and place in a fresh 10 cm tissue culture dish

3) Under a dissection microscope remove the arms and legs of the embryo with surgical scissors and pincers. Be careful to not include any tissue above the shoulder and hip joints. Place limbs in a few drops of HBSS in a 15cm tissue culture dish.

4) Place extremities from 3-4 mice on a 15cm tissue culture dish. Using curved scissors, thoroughly mince until homogenous (approximately 2-3 minutes). Thorough disruption of the tissue is essential for generating a single cell suspension.
5) Add 1 mL of 0.25% trypsin solution and incubate for 15 minutes at 37°C. Make sure to shake the plate once or twice during the incubation.

6) Briefly triturate dissociated tissue using a 10 mL pipette.

7) Place cells in a 37°C incubator

8) When cells become confluent (typically 2-4 days later), remove MEF media, rinse once with HBSS, and add 3 mL of 0.25% trypsin. Incubate for 2-3 minutes in 37°C incubator. Quench trypsin by adding 3 mL MEF media. Transfer detached cells into a 15 mL falcon tube.

9) Spin down cell solution at 200 rcf for 3-5 minutes. Remove supernatant and resuspend cell pellet in MEF media.

10) Add cells to 15cm tissue culture dishes containing 20 mL of MEF media. One 15cm plate can be replated onto 3-4 15 cm plates (i.e. a 1:3 or 1:4 split).

11) When cells become confluent remove MEF media, rinse once with HBSS, and add 3 mL of 0.25% trypsin. Incubate for 3 minutes in 37°C incubator. Quench trypsin by adding 3 mL MEF media.

12) Spin down cell suspension at 200 rcf for 3 minutes. Remove supernatant and resuspend cell pellet in 1.5 mL MEF media per 15cm plate of cells. Add an equal volume of 2× freezing media and mix well.

13) Quickly aliquot cell suspension into cryotubes, 1 mL/tube (equivalent of 1/3 of cells from a 15 cm plate). Immediately place cryotubes in an insulated container to promote slow freezing and store at -80°C overnight.

14) Transfer cells to liquid nitrogen for long-term storage.

**Box 2: Preparation of Perinatal Tail Tip Fibroblasts**

**Timing:** 2 hours

1) Using sharp scissors, remove the distal third of the tail from 3-5 day old mouse pups. Place tails into a 10 cm tissue culture dish.

2) Thoroughly rinse tail pieces in 70% ethanol in a sterile tissue culture dish for 10-20 seconds (any longer and cells will start to die).

3) Rinse tail pieces twice in 10-15 mL of PBS or HBSS to remove residual ethanol. Transfer tails into a fresh tissue culture dish with HBSS or PBS.

4) Transfer 2-3 tails into a 10 cm tissue culture dish. Put approximately 50-70µl of 0.25% trypsin on the tails. Use the curved scissors to mince the tail thoroughly (3-5 minutes per batch of tails). It is important that the tail is uniformly dissociated to achieve a paste like consistency. If there is too much liquid it will be difficult to thoroughly chop the tails.
5) Add 300-500 µL of trypsin solution onto the tails and incubate for 10-12 minutes in a 37°C incubator.

6) Spread the tail paste evenly around a tissue culture plate, creating as many single pieces of tissue as possible. Tail fibroblasts will migrate out of these chunks.

7) Let the dissociated tails dry for 5-7 minutes, so that the chunks begin to adhere to the plate. Add MEF media dropwise to cover the surface of the tissue chunks, but not any more than is necessary. It is essential not add the media too quickly. If the chunks float, it is unlikely that they will adhere to the plate. Gently place dish into 37°C incubator and avoid moving for at least 24 hours.

**CRITICAL STEP!**

8) Allow cells to grow until the plate is confluent. If cells are growing as isolated islands, they can be removed with trypsin and plated onto the same plate so that they cover the surface of the plate evenly. Timing: 3-7 days.

9) When cells become confluent, trypsinize and split cells 1:3. After splitting, allow the cells to become confluent and freeze for later use (see Box 1, steps 11-14). Transfer cells to liquid nitrogen for long-term storage.

**Box 3: Preparation of Primary Human foreskin fibroblasts:**

1) Briefly rinse the foreskin sample in 70% ethanol (no more than 10-20 seconds). To remove residual ethanol, thoroughly wash sample with sterile PBS or HBSS.

2) Place sample on the lid of a 100-mm tissue culture dish and spread it out with the epidermal side down. Remove the subcutaneous tissue by scraping the dermal side using two pairs of forceps.

3) Place the dermal portion in a 100-mm tissue culture dish and cut it into small (2- to 3-mm) squares using a surgical scalpel.

4) Place the skin pieces in the center of a 100-mm tissue culture dish. Place a sterile 22-mm glass coverslip gently over the skin specimens.

5) Add MEF media gently and place the cells in tissue-culture incubator. Cells will slowly migrate out of chunks over the course of the first week.

6) Maintain the culture until the cells become dense enough to become contact-inhibited. Cells usually grow as distinct islands and do not cover the entire plate. To redistribute cells evenly, dissociate cells with 0.125% trypsin and replate them back onto the same plate and let them grow until they become confluent.

7) Split cells with 0.125% trypsin 1:3 or 1:4 and allow them to become confluent again. Freeze down cells (see Box 1 steps 11-14)
Box 4: Glial Cell Isolation

1) Anesthetize postnatal day 4-5 pups on ice. Remove heads from pups with surgical scissors and place in a 10 cm tissue culture dish (2-3 brains for each 10 cm tissue culture dish). To prepare a significant amount of cells at least 4-6 pups are required.
2) Wash heads three times in HBSS, transfer into a fresh 10 cm plate.
3) Remove mouse heads one at a time and place in a 10 cm dish filled with HBSS. Remove brain from skull and then remove the meninges using fine tweezers and dissect the cortices from the brain. Place the isolated cortices in a fresh 10 cm dish containing 3 ml of HBSS.
4) Using flattened scissors cut cortical tissue into pieces (approx 1 mm square). Transfer liquid to a 15mL falcon tube containing HBSS. Rinse plates with 1 mL of HBSS to get all remaining pieces of tissue and add to the falcon tube.
5) Add 500 µl of 2.5% trypsin and 500 µl of DNase solution to the falcon tube and place in the 37°C water bath for 15 min. Mix every few minutes.
6) With the debris settled at the bottom, transfer the supernatant to a 50mL falcon tube containing 1.5 mL of serum.
7) Add 4mL of HBSS, 500 µl 2.5%Trypsin, and 500 µl DNase to the tissue remaining in the 15 mL falcon tube. Place in a 37°C water bath for 15 minutes, mixing every few minutes.

TROUBLESHOOTING

8) With tissue settled at the bottom of the tube, transfer supernatant (~4mL) to the same 50mL falcon tube used earlier.
9) Use a pipette to triturate the remaining tissue. Transfer through a 0.40 µM cell strainer into the 50 mL falcon tube with previously collected supernatant.
10) Centrifuge the 50 mL falcon tube containing the dissociated glial cells at 200 rcf for 5 min at room temperature.
11) Remove supernatant and resuspend cells in MEF media and plate on a 15cm tissue culture plate (2-3 cortices per 10cm dish).
12) Replace media daily until cells become confluent
13) Split cells 1:2 with 0.25% trypsin
14) Freeze down glial cells after the replated cells become confluent again (i.e. passage 1) (see box 1, steps 11-14)

TROUBLESHOOTING

NOTE: If desired, glial cells can be passaged onto iN cultures without freezing, however, they should be expanded for one additional passage in order to remove any remaining neurons from the cultures.

Box 5: Preparation of mouse cortical cultures

NOTE: This step is only required for determining whether mouse cortical neurons form synapses on iN cells.
NOTE: It is not possible to freeze down cortical cultures and thus this step must be tightly coordinated with the infection of fibroblasts with the reprogramming factors in order for the cortical cultures to be ready at the same time as the iN cells.

NOTE: Prepare matrigel coated, pre-treated coverslips prior to dissection (see reagent preparation).

**Dissection**

1) Aliquot 10-15 mL of cold HBSS into a 15 mL falcon tube and place on ice

2) Prepare papain solution and warm to 37°C in water bath.

3) Decapitate P0 mouse. One mouse will provide enough material for 12 coverslips of cortical neurons

4) Pin the head down using blunt forceps through the eye sockets.

5) Using microscissors or a scalpel, gently cut along the midline, starting at the cerebellum to around the eye sockets

6) Gently open the skull using forceps to expose the brain

7) Use a spatula (starting from the olfactory bulbs) to scoop the brain out into ice-cold HBSS

8) Use blunt forceps to pin the brain down by the cerebellum

9) Gently use a microscalpel to cut around the midbrain to free both cortices

10) Remove all meninges from each hemisphere with fine forceps

11) While gently holding the cortex, use fine forceps (#5 or #55 Dumont) to cut out the hippocampus

12) Remove the lower ¼ of the cortex with a microscalpel (contains unwanted cell types)

13) Put each hippocampus-free cortex into 15ml conical tube with ice-cold HBSS.

**Trituration**

14) Carefully aspirate HBSS from 15 mL falcon tube containing cortices

15) Filter papain solution into tube containing cortices with a 0.22 μM filter attached to a syringe

16) Incubate for 20 minutes at 37°C, gently flick tube after 10 minutes tube to ensure that the sample mixes well.
17) Aspirate papain, add 10-12 mL plating media to wash, wait for cortices to settle to the bottom of the tube then aspirate. Repeat.

18) After the second wash, add 3 mL plating media per 12 coverslips of cells and begin to triturate

19) Using a P1000 pipette, triturate tissue until no chunks are visible

20) Place 0.40 µM cell strainer on top of a 50 mL falcon tube and pipette triturated solution through

21) Aspirate excess Matrigel from cover slips immediately before plating.

22) Add 11 mL plating media and distribute 1mL/well of a 24 well plate

23) 24 hrs after initial plating, remove 800 µL of media and replace with Growth media (no Ara-C)

24) 48-72 hrs after plating (DIV2-3, when glial density is ~60-70%) remove 500 µL media and replace with 500 µL fresh growth media with AraC (final concentration of 2 µM AraC)

25) Replace the media on DIV7 and 14 with fresh growth media containing AraC.

NOTE: When adding media, always add slowly down the side of the well.

Box 6: Lentiviral Production using HEK293T cells

IMPORTANT: All steps must be performed in a tissue culture hood using sterile technique

Caution: Infectious viral particles will be produced following transfection. BSL-2/2+ level safety precautions are essential for the following steps.

Timing: 5-7 days

1) 16-24 hours prior to transfection, plate 4.0 X 10⁶ HEK293T cells in 6 mL of MEF media on polyornithine coated 10cm plates.

2) Remove media, replace with 9 mL of fresh, pre-warmed MEF media. This should be done 0-2 hours before adding transfection mixture.

3) For every 10 cm plate of virus produced, prepare 10 µg of lentiviral plasmid (e.g. Tet-O-FUW Ascl1), 5 µg of pMDLg/pRRE, 2.5 µg of pRSV-rev, and 2.5 µg of pMD2.G in sterile water, for a total volume 500-(CaCl₂ volume) µl in a 2 mL screw cap tube.

4) Add predetermined amount of 2.5M CaCl₂ dropwise to water/DNA mixture (see reagent set-up). The final volume of this solution should be 500 µl. Vortex for 10 seconds.

5) Immediately add 500 µL of 2× BBS to the transfection mixture. Vortex for 10 seconds.
6) Let solution stand for 30-60 minutes at room temperature.

7) Lightly mix solution by pipetting up and down and add 1 ml dropwise to each plate of HEK293T cells.

8) 16-20 hours after transfection, replace media with 4ml of fresh MEF media. Check EGFP fluorescence of Tet-O-EGFP plate to ensure that the transfection worked properly.

NOTE: For every batch of lentiviral production include at least one plate transfected with TetO-EGFP. EGFP fluorescence will be visible 10-16 hours after transfection. If less than 60% of cells are EGFP-positive after 16 hours it is likely that lentiviral production will be sub-optimal, and thus transfection should be repeated.

TROUBLESHOOTING

9) Harvest viral supernatant 24 hours later (40-44 hours after transfection). You can replace the viral supernatant and add another 4 mL of media and do a second viral collection 12 hours later, although this is not recommended if you are infecting cells directly with the supernatant.

10) Filter viral supernatant into a falcon tube using a 0.45µM filter to remove HEK293T cells. 

Alternate step: Lentiviral supernatant can be centrifuged at 1000g for 5 minutes (to pellet any remaining HEK293T cells) and transferred into a fresh Falcon tube, being careful not to disturb the cell pellet. Supernatant can be stored at 4°C for up to 10 days.

Concentration of virus

11) Pellet filtered viral supernatant in a polyallomer ultracentrifuge tube with an ultracentrifuge at 25,000 rcf for 90 minutes at 4°C.

12) Add 75-100 µl of DMEM (Cat. no. 12430) containing polybrene (8 µg/µl) to viral pellet and allow pellet to resuspend slowly overnight at 4°C (don’t pipette up and down).

NOTE: Viral pellet will not be visible, although their will be pelleted proteins and other components from the MEF media

13) Gently resuspend pellet by lightly shaking the tubes and pipetting up and down once or twice and aliquot into screw cap tubes. Virus can be kept at 4°C for up to two weeks, or -80°C for long-term storage. Freezing will cause significant loss of activity. Repeated freeze/thaws are not recommended.

Preparing fibroblasts for lentiviral Infection

1) Generate frozen stocks of relevant fibroblast populations for reprogramming (see Box 1-3)

2) For experiments requiring synaptic recordings, it is necessary to generate both mouse cortical neurons and mouse glial cultures (see Box 4-5). Cortical cultures need to be prepared fresh for every experiment, usually 6-8 days before infection of fibroblast cultures for reprogramming. Glial cultures
can be generated in advance and frozen for use at any time. They should be thawed and passaged once before use, which requires thawing one vial of cells 5-7 days prior to d7 after initiation of fibroblast reprogramming.

3) Prepare fresh lentiviral supernatant (see Box 6) for use within 10 days or a stock of frozen, concentrated lentiviral supernatant for use as needed

4) Remove a vial of passage 1 MEFs from liquid nitrogen storage.

5) Thaw cells in a 37°C water bath. As soon as cell solution has thawed, transfer cells into a falcon tube containing 5 mL fresh media.

**CRITICAL STEP!** Freezing media contains DMSO, which can be toxic to cells at room temperature. It is essential to transfer cells to fresh media as quickly as possible.

6) Spin cells for 3 minutes at 200 rcf. Remove supernatant.

7) Resuspend cells in MEF media. Plate onto tissue culture dish

8) Once cells become 80-90% confluent (approximately 2-4 days) they can be split and replated for infection.

9) For infection, plate MEF or TTF cells (passage 3) on a 6cm dish, use approximately 5 X10⁵ cells and 3 mL of MEF media. Poly-ornithine coated plates (see reagent preparation) can be used to help iN cells remain attached after prolonged culture, although it is not required.

10) Mouse iN: 7-24 hours later, prepare viral supernatant for infection. Mix viral supernatants for Ascl1:Brn2:RtTA: Myt1l in a 1:1:1.5:2 ratio.

11) Remove media from fibroblasts and add MEF media with polybrene. The final concentration of polybrene after MEF media is mixed with lentiviral supernatant should be 8 µg/µl.

12) Add viral supernatant to each well in desired concentrations. Adjust the volume of MEF media so that the total volume of media is constant for each condition.

13) 12-16 hours later: remove viral media and replace with fresh MEF media containing doxycycline (2 µg/µl).

**NOTE:** If TetO-EGFP infection was performed, EGFP fluorescence will be visible 8-16 hours after the addition of doxycycline.

14) Mouse fibroblasts: After 24-48 hours, switch cells into N3 media containing doxycycline (2 µg/µL final concentration)
Human fibroblasts: After 24-48 hours, switch cells into N3+NTF media containing doxycycline (2 µg/mL final concentration)

**CRITICAL STEP!** Do not allow fibroblasts to become overly confluent. Switch to N3 media after 24 hours if cells are too dense.

15) Every 2-3 days for the duration of the culture period replace ½ of N3 media with pre-warmed, fresh N3 media containing doxycycline.

**TROUBLESHOOTING**

**Generating mature iN cell cultures**

**Adding glia directly onto iN cells:**

Follow steps 1-15 to infect fibroblasts.

Thaw passage 1 glia and plate approximately at the same time as fibroblast infection. Allow cells to grow for 3-5 days (or until confluent).

Detach glia using 0.25% trypsin, spin down for 3 minutes at 200 rcf, remove supernatant and resuspend in N3 media. Count cells using a hemocytometer.

Plate 60-150,000 glia on infected fibroblast cultures 3-7 days after addition of doxycycline to the infected fibroblasts. The appropriate number of glia to add will depend on the density of infected fibroblasts.

**FACS sorting iN cells onto glia:**

Follow steps 1-15 to infect MEFs or TTFs.

Thaw passage 1 glia and plate at the time of infection. Allow cells to grow for 3-5 days.

Detach glia using 0.25% trypsin, spin down for 3 minutes at 200 rcf, remove supernatant and resuspend in N3 media.

12-24 hours prior to isolating iN cells by FACS: Plate glial cells on matrigel coated coverslips in a 24 well plate so that they are approximately 50-60% confluent.

7-10 days after the addition of doxycycline: Detach iN cultures from plates using 0.25% trypsin.

Wash twice with HBSS. Thoroughly resuspend cells in flow buffer (see reagent preparation) for FACS isolation.

Pass cell suspension through a 70 µM filter to remove chunks.

Transfer cells in flow buffer to a FACS tube and keep on ice in preparation for sorting.
Sort iN cells into Neurobasal+5% serum (approximately 500 µL). Final volume will depend on the amount of liquid that cells are resuspended in for sorting.

Spin down cells at

TROUBLESHOOTING

Remove media from glial cells. Replace media with N3+Dox. Add approximately 80-140,000 FACS sorted EGFP-positive events to each well of glia. Plate multiple wells in order to allow for multiple synaptic measurements.

Replace ½ of N3+Dox media every 3-4 days.

NOTE: If cells become too confluent, iN cells will suffer. Use Ara-C in the growth media to prevent excessive proliferation

After approximately one week, remove all media and replace with growth media with Ara-C (2 µg/mL) and without doxycycline.

Culture cells in growth media for at least 5 days before attempting to measure synaptic activity. Change ½ of the media every 5 days.

TROUBLESHOOTING

Papain dissociation of human iN cells

Infect human fibroblasts with BAMN and EGFP (see steps XX-YY)

Prepare cortical cultures (see box 5). Given that cortical cultures require newborn mice, this step can be difficult to time properly. Ideally, the experiment should be set up such that cortical cultures are 7 DIV when infected human fibroblasts are 14 days post doxycycline.

Prepare papain solution (see reagent preparation)

Dissociate human iN cells at 14 days post doxycycline. Wash cells once with HBSS and add 2 mL of papain solution to each 6 cm dish of human iN cells. Let set for 2-3 minutes.

Visually confirm that cells are lifting off of the plate. Tap the side of the plate lightly and wait for an additional minute to promote further detachment if necessary.

Collect the detached cells and put them into a 15 mL Falcon tube. Add 10 mL of plating medium (contains 10% FBS) to inactivate papain.

Spin 200 rcf for 10 minutes. Aspirate supernatant and add 10 mL plating medium to wash once.

Spin at 200 rcf for 5 minutes and resuspend cells in 0.5 to 1 mL plating medium and use 200 ul pipette tip to gently triturate the cells.
Add cell suspension to mouse cortical cultures (7 DIV, see Box 5) or mouse glial cultures plated on matrigel coated coverslips (See Box 4).

**CRITICAL STEP!:** It is critical that the culture media contains Ara-C (2 μg/μl) to ensure that fibroblasts do not proliferate extensively after replating.

**NOTE:** The appropriate amount of cells to plate will depend on the efficiency of iN cell induction, the density of glial or cortical cultures and the viability of iN cells following dissociation and thus should be determined empirically.

**Anticipated results (mouse):**
In a successful experiment, approximately 10-20% of mouse embryonic fibroblasts can be stably reprogrammed into iN cells over the course of 5-12 days (Fig. 2A) (see table for experimental results summary). Embryonic fibroblasts reprogram more quickly and more efficiently than tail-tip fibroblasts. In MEFs, activation of immature neuronal markers, such as beta-III-tubulin and TauEGFP, can occur as early as 48-72 hours after the addition of doxycycline. Neuronal gene activation coincides with the first notable morphological changes in fibroblasts, as a subset of reprogramming fibroblasts tend to become spherical and extend short processes by d3-5. Neurites are readily visible by d5-8, and continue to become more elaborate as reprogramming proceeds. While initially dispersed evenly throughout the plate, iN cells tend to migrate together and form densely packed clusters of approximately 3-15 cells by d12-15 (see Fig. 2A for an example). These morphological changes parallel the acquisition of neuronal functions (Fig. 1a, Fig 2c-d). The average resting membrane potential of iN cells is approximately -30mV by d7 and decreases steadily to ~ -55mV by d19. Induced neurons fire single action potentials following current injection as early as d6 and fire repetitive trains of action potentials by d12 (Fig 2C). Following 7 days of co-culture with mouse cortical neurons and glia, FACS-purified iN cells can receive excitatory (NMDA- and AMPA-receptor mediated) and inhibitory synaptic inputs and exhibit short-term synaptic plasticity. Furthermore, when FACS-purified iN cells are co-cultured with primary mouse glia in neuronal growth media they form synapses with one another after 10-18 days (Fig. 2e-g). Both AMPA and NMDA receptor mediated excitatory post-synaptic potentials are evident in iN cell cultures, and can be distinguished by pharmacological isolation (using NBQX or D-AP5) or by varying the holding potential while recording synaptic responses following extracellular stimulation (Fig. 2e-f).

**Anticipated results (human):** See Fig. 1, 3
In a successful experiment, approximately 2-4% of the starting fibroblast population can be reprogrammed into iN cells over the course of 12-24 days (Fig 3a-b). There are not significant differences in reprogramming efficiencies between fetal and early postnatal fibroblasts, however, the efficiency of neuronal induction in adult fibroblast lines decreases significantly. The initial activation of immature neuronal markers generally occurs 9-12 days after doxycycline addition, with robust staining for MAP2, β-III-tubulin and NeuN antigens by day 20 (Fig. 3c-e). Neurites extending from developing human iN cells tend to be more difficult to discern using phase-contrast microscopy, as the non-reprogrammed fibroblasts have a thin, elongated morphology and form denser sheets of cells. Co-infection with EGFP virus makes it easier to see the morphology of single cells. Human iN cells also...
generally remain spread across the plate, rather than forming tight clusters like mouse iN cells (compare Fig. 2A with Fig. 3b).

Human iN cells also tend to be delayed in terms of functional maturation compared to mouse iN cells. For example, mouse iN cells fire repetitive action potentials by day 12, whereas human iN cells require 20 days or more to mature to a similar functional state. Human iN cells also take substantially longer to receive synaptic input from mouse cortical neurons (7 days vs. 4-5 weeks). Future studies will be required to optimize the culture conditions that promote more efficient reprogramming and more complete maturation of human iN cells in vitro.

### Table 1: Summary of published protocols for iN cell generation

<table>
<thead>
<tr>
<th>Starting Cell type</th>
<th>Target cell type</th>
<th>Reprogramming factors</th>
<th>Additional factors added</th>
<th>Evidence of functional maturity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEF, TTF, HFF, HPF</td>
<td>Glutamatergic neurons</td>
<td>Brn2, Ascl1, Myt1l, NeuroD1**</td>
<td>Synaptic formation (iN-iN), mouse neuron-iN**</td>
<td>(Vierbuchen, 2010 #12)</td>
<td>(Pang, 2010 #13)</td>
</tr>
<tr>
<td>MEF, TTF, HPF</td>
<td>Dopaminergic neurons</td>
<td>Ascl1, Nurr1, Lmx1a</td>
<td>Dopamine release (RP-HPLC)</td>
<td>(Caizzo, 2011 #15)</td>
<td></td>
</tr>
<tr>
<td>HFF, HPF</td>
<td>Dopaminergic Neurons</td>
<td>Brn2, Ascl1, Myt1l, Lmx1a, Foxa2</td>
<td>Action potential generation</td>
<td>(Pfisterer, 2011 #14)</td>
<td></td>
</tr>
<tr>
<td>TTF</td>
<td>Dopaminergic neurons</td>
<td>Ascl1, Pitx3, Lmx1a, Nurr1, Foxa2, EN1</td>
<td>Sonic hedgehog, FGF8</td>
<td>(Kim, 2011 #31)</td>
<td></td>
</tr>
<tr>
<td>HFF, HPF</td>
<td>Glutamatergic neurons, GABAergic neurons</td>
<td>Ascl1, Myt1l, NeuroD2, miR-9/9*, miR-124</td>
<td>bFGF, valproic acid</td>
<td>Synapse formation (iN-iN)</td>
<td>(Yoo, 2011 #17)</td>
</tr>
<tr>
<td>MEF, TTF, HFF</td>
<td>Spinal motor neurons</td>
<td>Brn2, Ascl1, Myt1l, Lhx3, Isl1, Ngn2, Mnx1 (Hb9), NeuroD1**</td>
<td>Formation of functional neuromuscular junctions, exhibited disease-specific phenotype in vitro, in vivo integration into chick</td>
<td>(Son, 2011 #18)</td>
<td></td>
</tr>
<tr>
<td>HPF</td>
<td>Glutamatergic neurons</td>
<td>Brn2, Myt1l, miR-124</td>
<td>bFGF, noggin</td>
<td>XXXXXXXXX</td>
<td>(Ambasudhan, 2011 #20)</td>
</tr>
<tr>
<td>Mouse hepatocytes</td>
<td>Glutamatergic neurons</td>
<td>Brn2, Ascl1, Myt1l</td>
<td>Synaptic formation (mouse neuron-iN)</td>
<td>(Marro, 2011 #24)</td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:** MEF (mouse embryonic fibroblasts), TTF (mouse tail-tip fibroblasts), HFF (human fetal fibroblasts), HPF (human postnatal fibroblasts), bFGF (basic fibroblast growth factor), RP-HPLC (reversed-phase high performance liquid chromatography). **Denotes gene used only for human fibroblast reprogramming.
### Table 2: Troubleshooting

<table>
<thead>
<tr>
<th>Step</th>
<th>Problem</th>
<th>Possible Reasons</th>
<th>Possible Solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Box 4: glia preps</td>
<td>Glia prep yields insufficient cells or cells die after plating</td>
<td>- Tissue over/under digested</td>
<td>- Check the concentrations of trypsin and DNAse. Repeat dissociation and frequently check cells in microscope to monitor dissociation</td>
</tr>
<tr>
<td>Box 4: glia preps</td>
<td>Fibroblasts outgrow glial cells</td>
<td>Meninges and other tissue from skull not fully removed</td>
<td>Be careful to fully remove meninges from cortices</td>
</tr>
<tr>
<td>Box 6: Virus prep</td>
<td>Transfection efficiency is low</td>
<td>- pH of transfection solution is off - 293T cells are too sparse or too confluent - transfection performed with old media on cells</td>
<td>- Re-optimize CaCl₂ and 2X BBS solution (see reagent prep) - repeat transfection with fewer 293T cells - Change media immediately before transfection</td>
</tr>
<tr>
<td>Viral titer is poor despite good transfection efficiency</td>
<td>- Incorrect or damaged lentiviral plasmids - Incorrect or damaged packaging plasmids</td>
<td>- Double check that all plasmids are correct - do not use HEK293Ts for too long. Replace regularly with cells from a master stock.</td>
<td></td>
</tr>
<tr>
<td>Low reprogramming efficiency (but little cell death)</td>
<td>- Viral titer is too low - Cells are too dense</td>
<td>- Check infection efficiency by IF - thaw new MEFs and repeat infection with more virus - plate fewer cells for infection or switch cells to N3 media sooner after initial addition of doxycycline</td>
<td></td>
</tr>
<tr>
<td>Low reprogramming efficiency despite high infection rate</td>
<td>- Fibroblast cultures are unhealthy - Lentiviral plasmids are degraded - N3 media is prepared incorrectly or has lost activity - Doxycycline has lost activity or stock is prepared incorrectly - Cells are too dense</td>
<td>- Prepare fresh fibroblasts from primary tissue - sequence lentiviral cDNAs and test digest plasmids - repeat experiment with new media - aliquot N3 media to prevent repeated temperature changes. Never use one batch for more than a month - prepare new stock of doxycycline. Make small aliquots, protect from light, and make dox media fresh for every media exchange</td>
<td></td>
</tr>
<tr>
<td>Excessive cell death during reprogramming</td>
<td>- Fibroblasts are too dense - Fibroblast line has been passaged excessively - Viral titer is too high - N3 media is incorrect or too old - Polybrene concentration is too high</td>
<td>- Use lower passage fibroblasts - Reduce the amount of virus</td>
<td></td>
</tr>
<tr>
<td>Synapse formation Steps XX-YY</td>
<td>Human iN cells die during reprogramming</td>
<td>- Fibroblasts are too dense - Viral titer is too high - Neurotrophic factors (NTFs) are incorrect or degraded</td>
<td>- Plate fewer cells - Include multiple concentrations of virus in every experiment - Make small aliquots of NTFs and make media + NTFs fresh before every media exchange</td>
</tr>
<tr>
<td>Cell death after sorting</td>
<td>- Nozzle on sorter is too small or pressure is too high - Glial/cortical cultures are too dense - Dissociation method was too harsh - Ara-C concentration is too high</td>
<td>- Nozzle size on sorter should be at least 100µM and pressure should be no more than XX - Plate fewer glia/neurons on each coverslip - Use papain dissociation - Only use Ara-C if absolutely necessary</td>
<td></td>
</tr>
<tr>
<td>No synapse formation</td>
<td>- Growth media components are sub-optimal - iN cell reprogramming is incomplete - Glial cultures are too dense - Glial cells are sub-optimal</td>
<td>- Do not substitute media components, use exactly the components listed in the protocol - Sort iN cells from a plate with lower transgene expression - Plate fewer glia per coverslip - Prepare new glial cultures - Supplement cultures with neurotrophic factors</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1: Experimental outline
a) Important landmarks during conversion of mouse fibroblasts to iN cells. b) Conversion of human fibroblasts to iN cells. Note the differences in timing of reprogramming and functional maturation.
Figure 2: Summary of anticipated results for mouse fibroblast-neuron conversion

a) Tuj1 staining of MEF-iN cells 12 days after induction. Note that iN cells have migrated together to form clusters of 3-15 cells (arrowhead).

b) FACS purified BAM MEF-iN cells plated onto glia, fixed 22 days after addition of doxycycline. MAP2 (green), Synapsin1 (red). Note the punctate staining pattern of synapsin closely apposed to MAP2-positive dendrites.

c) Representative traces of MEF-iN cells membrane potential in response to step depolarization by current injection 12 days after addition of doxycycline (lower panel). Membrane potential was current clamped at approximately -65 mV.

d) Whole cell currents recorded in voltage clamp mode from d12 iN cells; cells were held at -70mV; step depolarization from -90 to +50mV at 10mV intervals was delivered (lower panel). The inset shows Na+ currents.

e) Synaptic responses recorded from MEF-iN cells purified by FACS at d7 and co-cultured with glia for 14-21 days. Representative traces of evoked synaptic responses recorded from TTF-iN cells purified by FACS at d7 and co-cultured with glia for 14-21 days. $V_{h}$, holding potential. At a $V_{h}$ of -70mV, AMPAR-mediated EPSCs were recorded; at a $V_{h}$ of +60mV, NMDAR-mediated EPSCs were revealed.

f) Spontaneous excitatory post-synaptic currents (EPSCs) recorded from MEF-iN cells cultured on a glial monolayer.
Figure 3: Summary of results from a typical human iN cell experiment

a) Human postnatal dermal fibroblasts infected with BAMN+EGFP XX days after XX and stained with Tuj1 (b). c) Human iN cells co-express Tuj1 and NeuN, Neurofilament (d) and MAP2 (e). f) Representative recordings of HPF-iN cells membrane potential in response to step depolarization by current injection 28 days after addition of doxycycline (lower panel). Membrane potential was current clamped at approximately -63 mV. g) Representative traces of whole cell currents in response to voltage depolarization 28 days after addition of doxycycline. Both fast activating and inactivating currents are present. Treatment with tetrodotoxin (TTX) effectively blocks the observed sodium currents.