

A Novel and Efficient System for the Generation of Neural Progenitor Cells from Human Pluripotent Stem Cells using STEMdiff™ Neural Induction Medium and STEMDIFF™ Neural Rosette Selection Reagent

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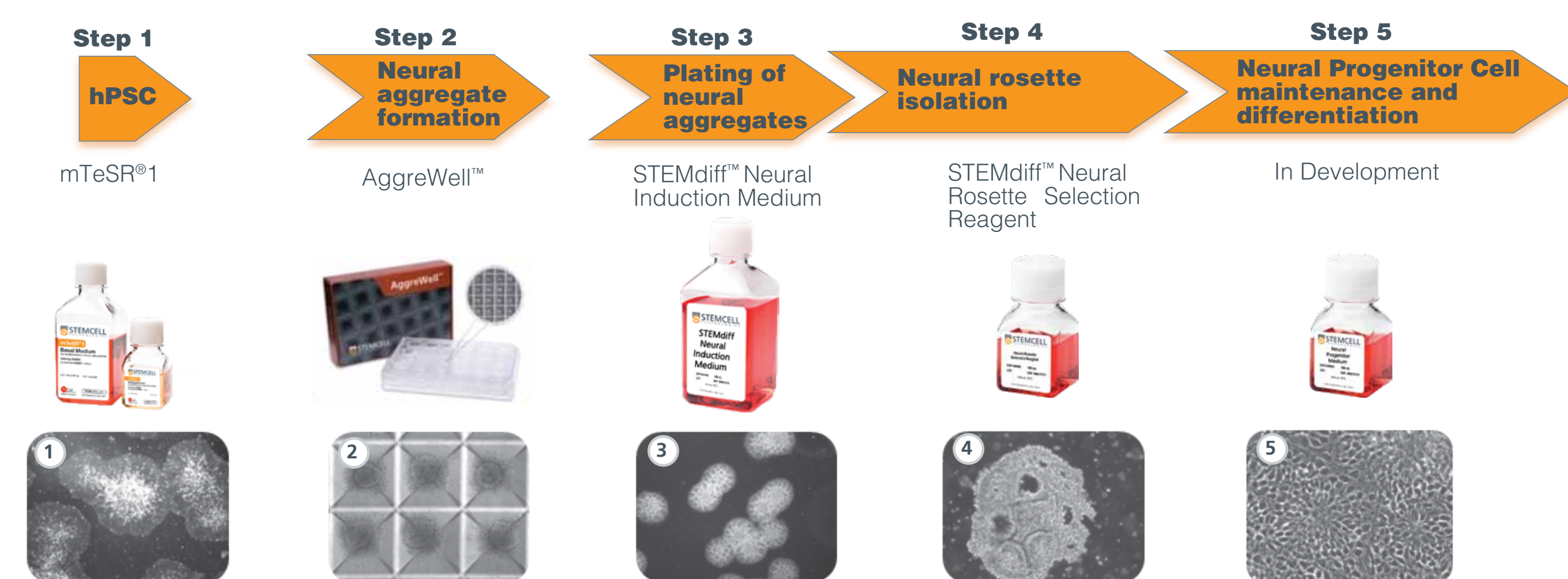
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Introduction

Induction of neuroectoderm from human pluripotent stem cells (hPSCs) is the first step in differentiation protocols used to produce neural progenitor cells (NPC) and more specialized cell types of the central nervous system such as neurons, astrocytes, and oligodendrocytes. In many neural induction protocols, the formation of aggregates or embryoid bodies (EBs) from undifferentiated hPSCs is the first step employed to direct these cells towards a neural fate. However, commonly used EB-formation protocols often result in non-uniformly shaped EBs of varying sizes which can give rise to inconsistent results in neural differentiation. Once formed, EBs are typically plated onto an adherent substrate, where the appearance of neural rosettes is an accepted morphological criterion indicative of early neural progenitor induction. Due to the variability in neural rosette induction, with other cell types originating from all 3 germ layers present, these neural rosettes need to be selected and isolated for subculture. Commonly used manual selection techniques are operator dependent, leading to variable purity of subsequent NPC isolation. In order to standardize protocols for neural induction of hPSCs, we have developed a system consisting of: AggreWell™, STEMdiff™ Neural Induction Medium and STEMdiff™ Neural Rosette Selection Reagent. AggreWell™800 is used to generate uniform-sized aggregates. STEMdiff™ Neural Induction Medium is used for neural induction within aggregates of hPSCs generated in AggreWell™. STEMdiff™ Neural Rosette Selection Reagent was developed for the selection of neural rosette structures from non-NPC rosette outgrowths. Our results show that 90-100% of the neural aggregates cultured in STEMdiff™ Neural Induction Medium generated neural rosettes which contained Pax6, Sox1, and Nestin positive NPCs. The NPCs were sub-cultured for multiple passages and differentiated into mature neurons and astrocytes as determined by the expression of TUJ1 and GFAP, respectively. This work describes a highly efficient protocol for the induction of neural rosettes and isolation of neural progenitor cells from hPSCs and may help to standardize neural differentiation protocols.

Methods

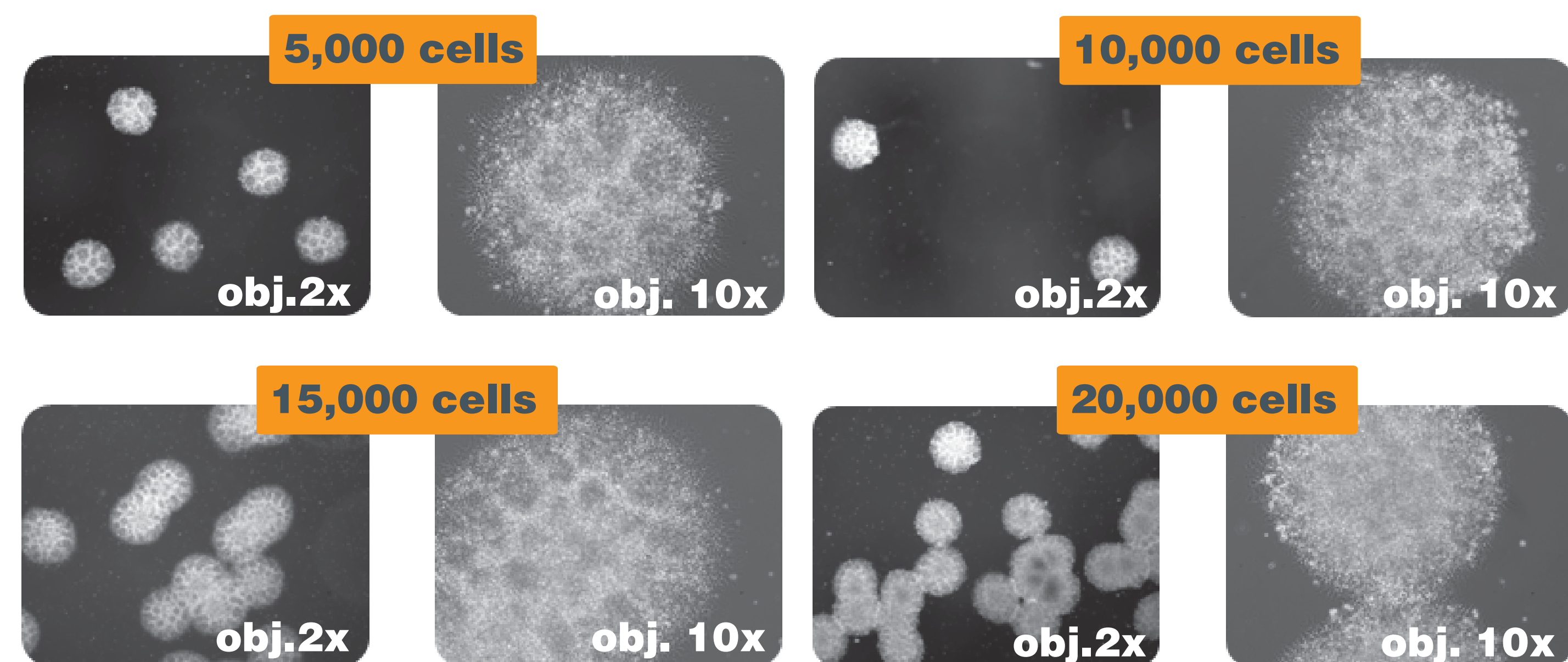
A NOVEL SYSTEM FOR NEURAL INDUCTION



Step 1: hPSCs are maintained in mTeSR[®]1. **Step 2:** AggreWell™800 plates are used to form uniform, size-controlled neural aggregates in combination with STEMdiff™ Neural Induction Medium. **Step 3:** After 5 days, neural aggregates are harvested from the AggreWell™800 plate and seeded onto PLO/L coated plates in STEMdiff™ Neural Induction Medium to give rise to colonies containing neural rosette structures. **Step 4:** Neural rosettes containing putative NPCs are selectively detached from the adherent culture after 7 days, using STEMdiff™ Neural Rosette Selection Reagent. After gentle trituration, these large rosette-containing "clusters" are re-plated onto PLO/L coated plates. This is denoted as Passage 1. When cultures reach 80-90% confluence, they are dissociated using ACCUTASE™ and re-plated onto PLO/L coated plates in Neural Progenitor Cell Medium (in development) (Passage 2). **Step 5:** NPCs are propagated in Neural Progenitor Cell Medium for multiple passages or differentiated into neurons and glial cells.

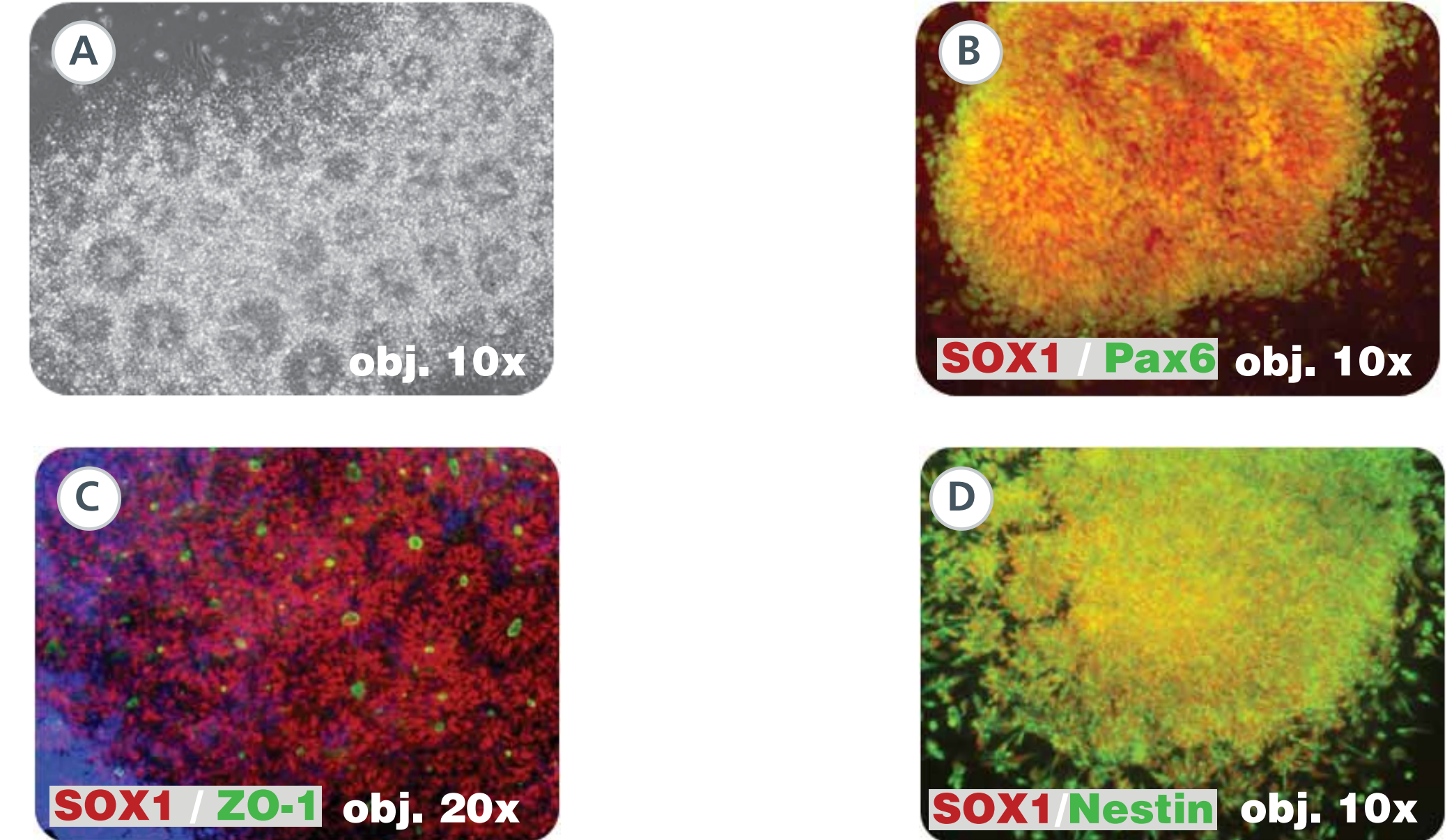
Results

STEPS 2 & 3 - NEURAL AGGREGATE FORMATION AND ATTACHMENT OF NEURAL AGGREGATES: Neural rosette structures appear in more than 90% of colonies from neural aggregates generated in AggreWell™800 and STEMdiff™ Neural Induction Medium



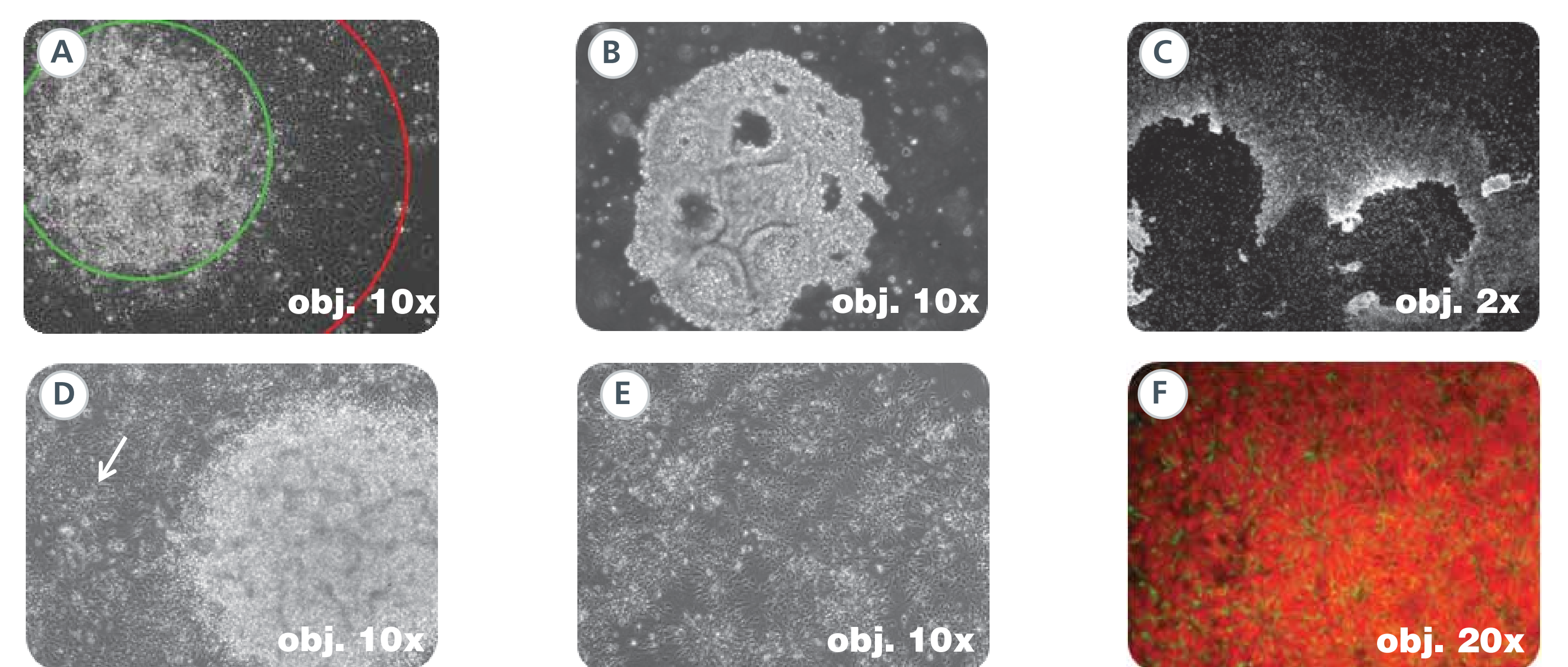
Neural aggregates were generated in AggreWell™800 plates at 5,000, 10,000, 15,000 and 20,000 cells/neural aggregate. After 5 days, neural aggregates were released from the AggreWell™800 plates and plated onto PLO/L coated dishes for attachment. Shown here are neural aggregates of different cell numbers, 1 day after attachment on PLO/L coated dishes. Attached neural aggregates of all sizes contain high numbers of neural rosettes.

STEP 3 - CHARACTERIZATION OF NEURAL AGGREGATES: Neural progenitor cells are present within neural rosette structures



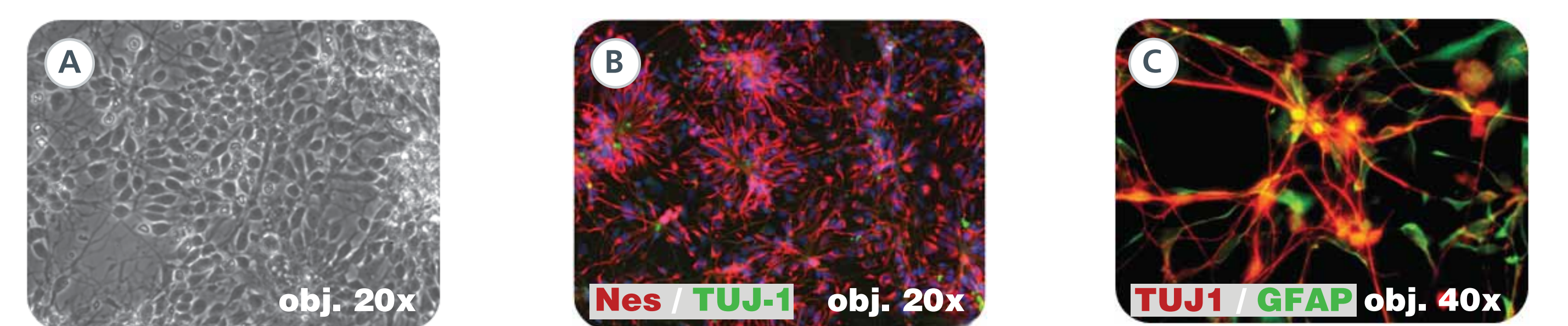
(A) Neural rosette structures observed within neural aggregates of 10000 cells/neural aggregate generated in AggreWell™800 with STEMdiff™ Neural Induction Medium at day 5 after plating onto PLO/L coated plates. **(B)** Immunocytochemical (ICC) staining reveals that cells within the neural rosettes co-express Pax6 (red) and Sox1 (green), two markers for early NPCs. **(C)** The lumens of rosettes are marked by the expression of ZO-1 (green), shown here also stained for Sox1 (red). **(D)** Cells also co-express the neural markers Sox1 (red) and Nestin (green), confirming the presence of bona fide NPCs within neural rosettes.

STEP 4 - SELECTION OF NEURAL ROSETTE STRUCTURES USING STEMDIFF™ NEURAL ROSETTE SELECTION REAGENT: Enriched neural progenitor cell populations can be derived from rosette structures



(A) Neural rosette structures are clearly observed at day 7 with "flat" cells (Zhang et al., 2001; Curchoe et al., 2010) emerging at the periphery of attached neural aggregates (the space between the two indicated circles). These are believed to interfere with the downstream propagation and differentiation of NPCs and hence must be left behind before sub-culturing NPC-containing neural rosettes. **(B)** A detached neural rosette clusters after treatment with STEMdiff™ Neural Rosette Selection Reagent. **(C)** "Flat" cells remained attached to the cell culture dish after treatment with STEMdiff™ Neural Rosette Selection Reagent. **(D)** Isolated neural rosettes from (C) after attachment (day 2 Passage 1). **(E)** When cultures from (D) reach 80-90% confluence they were dissociated into single cells and sub-cultured in Neural Progenitor Cell Medium (in development). NPCs are shown at day 2 Passage 2. **(F)** NPC cultures from (E) co-express Sox1 (red) and Nestin (green).

STEP 5 - NPC MAINTENANCE AND DIFFERENTIATION: Neural progenitor cells differentiate into neurons and astrocytes



NPCs can be propagated in Neural Progenitor Cell Medium (in development) for multiple passages or can be seeded in Differentiation Medium (in development) to induce terminal neuronal and glial differentiation. **(A)** Brightfield image of NPC culture at day 4 Passage 5. **(B)** ICC staining of passage 3 NPCs identifying the NPC marker Nestin (red) and the neuronal marker TUJ1 (green). In the presence of Neural Progenitor Cell Medium, a population of proliferative NPCs is maintained as evident by the low percentage of neurons detected (< 10%). **(C)** Astrocytes, identified by GFAP (green) expression and neurons, identified by TUJ1 (red) expression are present in differentiation cultures indicating that NPCs have multi-lineage potential.

Conclusion

We have developed a novel system aimed at standardizing the first steps of neural differentiation from hPSCs: the induction of neural rosettes and the selection of neural progenitor cells (NPC). This system includes using AggreWell™800 in combination with STEMdiff™ Neural Induction Medium for highly efficient induction of neural rosettes and STEMdiff™ Neural Rosette Selection Reagent for the subsequent selection of NPC-containing neural rosettes. Isolated NPCs can be propagated for several passages or differentiated into neurons and astrocytes. Development is on-going to further characterize these NPCs and to examine their potential to be regionalized using developmental cues, as well as their ability to differentiate into specific subtypes of neurons and glial cells.