● **PERSPECTIVE**

In vivo bioluminescence imaging to elucidate stem cell graft differentiation

Neurological disorders including neurodegeneration (e.g., Alzheimer's disease and Parkinson's disease) and acute injuries (e.g., stroke and traumatic brain injury) are the leading cause group of disability-adjusted life years and the second leading cause group of deaths. Different to other tissues, the adult brain retains only a very limited repair potential. Adult neurogenesis, the lifelong generation of new neurons, declines with age and in degenerative diseases, such as Alzheimer's disease. Nevertheless, independently of age, the proliferation and migration of endogenous stem cells is stimulated after brain injuries and might be related to recovery processes (Adamczak et al., 2017). The limited number of endogenous stem cells during adulthood is one of the major limitations for an efficient regeneration of the injury affected brain regions. Therefore, the transplantation of neural stem or progenitor cells (NSCs/NPCs) is extensively studied in mouse models and applied in first clinical trials with the aim to replace dysfunctional or lost neural cells and thus to restore brain function. Long-term survival and differentiation of engrafted NSCs/NPCs, synaptic integration, and projections to distant brain regions, as well as behavioral improvements are promising observations in numerous pre-clinical studies (Grade and Gotz, 2017). Transplanted stem cells initiate a complex series of potentially pro-regenerative processes with an individual time profile as it was shown by *in vivo* molecular imaging (Green et al., 2019).

In the past decade, we have contributed to the optimization and improvement of bioluminescence imaging (BLI) as one important *in vivo* imaging tool to visualize stem cells in the mouse brain. BLI offers the unique ability to investigate gene activity, dynamic processes as well as the effects of therapeutic interventions. BLI is based on the enzymatic production of light that can be measured by a photosensitive charge-coupled device camera. The most efficient enzyme for neuroimaging is the firefly luciferase (FLuc), which requires the presence of oxygen and the energy transfer molecule adenosine triphosphate and, therefore, allows the exclusive detection of vital cells. We have introduced an improved imaging protocol which allows the quantitative correlation between emitted photons and the number of FLuc expressing cells with a minimal detection limit of 1500–3000 Fluc$^+$ stem cells implanted in the mouse brain (Aswendt et al., 2013; Vogel et al., 2018).

The major limitation of that approach is the expression of Fluc under control of a constitutive promoter neglecting important aspects such as differentiation. Differentiation of transplanted cells is usually analyzed *post-mortem* using immunohistochemistry. However, that represents only a snap-shot of the overall time profile of differentiation. Thus, we here developed a method to image neural differentiation quantitatively using bioluminescence.

Imaging stem cell differentiation: Neural stem cell differentiation is considered an important mechanism of brain plasticity. Beside their ability to self-renew, NSCs/NPCs are capable to differentiate into mature neural cells - neurons, astrocytes, and oligodendrocytes. During their commitment into lineage-specific cells, promoter activities and gene expression profiles are constantly changing. These dynamic changes can be visualized by genetic targeting using cell-specific promoter sequences upstream of imaging reporters. To assess the time profile of the neuronal differentiation capacities of human NSCs, we engineered human NSCs to express FLuc under the neuronal progenitor cell-specific human promoter doublecortin and the neuron-specific promoter human synapsin 1 (Tennstaedt et al., 2015). For both cell-specific promoters a time-dependent upregulation was observed. The onset of the doublecortin promoter activity started at 4 days post-engraftment, persisted until 33 days, and decreased thereafter. Contrary to this, the synapsin 1 promoter activity showed a late onset starting at three months after implantation, which is in line with the gene expression dynamics in developing neurons in culture.

In our recently published study, we used human induced pluripotent stem cell (iPSC)-derived NPCs, which were reprogrammed from fetal neural tissue and differentiated into neural cells using small molecules. The dynamics of engrafted human NPCs to differentiate into all three mature neural cells types as well as to proliferate and to migrate towards distant brain regions over a time period of 12 weeks were monitored by BLI (Vogel et al., 2019). NPCs were genetically modified to express FLuc as well as the enhanced green fluorescent protein under control of the constitutive promoter (elongation factor 1 alpha) or cell-specific promoters to monitor graft-dependent differentiation into neurons (microtubule-associated protein 2), astrocytes (glial fibrillary acidic protein, GFAP) or oligodendrocytes (proteolipid protein, PLP). Interestingly, via longitudinal *in vivo* BLI, we observed a consistent proliferation for the

first 6 weeks upon engraftment of NPCs followed by a steady increase of the GFAP-dependent FLuc activity, whereas microtubule-associated protein 2- and PLP-dependent FLuc expression was comparatively low. We confirmed our non-invasive *in vivo* observations by post-mortem histological analysis revealing high numbers of double-positive cells for the human cell marker human nuclei and the astrocyte marker GFAP. Only a few cells positive for the mature neuronal marker Neuronal Nuclei (NeuN) were detected within the border zone of the human NPC grafts and no double-positive cells for Human Nuclei and the oligodendrocyte marker myelin oligodendrocyte glycoprotein were observed. Nevertheless, we could prove the low number of mature neurons identified within the grafts as functionally active neurons capable of generating action potentials and wide-branched neurite outgrows. In addition to our non-invasive longitudinal fate tracking analysis, we conducted at the end of the observation period whole-brain tissue clearing combined with light-sheet fluorescence microscopy (LSFM), followed by three-dimensional image reconstitution for detailed information about the graft expansion within the host brain tissue. Based on the promoter-driven enhanced green fluorescent protein expression, cells were identified via LSFM within cortical areas with single cells projecting and migrating long distance outside of the graft (**Figure 1**). Unfortunately, such single cell events are still under the detection limit of BLI, even when the currently optimal protocol for firefly luciferase-based BLI is being used (Aswendt et al., 2013; Vogel et al., 2018).

In our experiments, the human iPSC-derived NPCs differentiated *in vivo* spontaneously into astroglial cells. A matter of ongoing debate is to which extent differentiation of engrafted cells is directly linked to functional improvements seen in (pre-) clinical studies. In a stroke study by Tornero et al. (2013), pre-differentiated ("fated") cells showed less proliferation and more efficient differentiation into neurons with morphological and functional properties and higher axonal projection density compared with non-fated cells. Under *in vitro* conditions, neuronal differentiation can be stimulated by supplementation of lineage-specific mediators such as growth factors or small molecules which specifically activate signaling pathways. However, under *in vivo* conditions, the availability of such lineage-specific mediators is limited leading to a unilateral lineage commitment. One novel approach to improve survival and differentiation is tissue-based transplantation using cerebral organoids, which contain a large set of NPCs and differentiated neurons in a structured organization (Daviaud et al., 2018).

Future perspectives: Bioluminescence-based molecular imaging is a sensitive approach to reliably detect changes in gene expression profiles under true *in vivo* conditions. However, most of the conducted studies use engineered cells expressing imaging reporter under one cell-specific promoter representing only one developmental stage. To make precise statements about the interaction of several factors, multicistronic approaches are needed. The field of luciferases and substrates is constantly evolving and we expect to see more sensitive and far-red shifted luciferases to further improve *in vivo* BLI sensitivity. Recent developments to improve the quantum yield of red-shifted luciferases were a driving force for dual-color BLI which we used to detect two cell types at the same time (Aswendt et al., 2019). Dual-color BLI using for example green and red-shifted luciferase mutants provides new imaging approaches such as the monitoring of cell graft vitality and differentiation in the same imaging session. A further increase in sensitivity can be achieved by the administration of substrate analogues of the luciferin leading to higher quantum yields and thus allowing lower injection doses. The combination of bioluminescence reporters with light-sensitive acceptor molecules such as channelrhodopsins will allow to manipulate neuronal activity in a gene-related manner thus providing a powerful tool for the further improvement of stem cell-based approaches (Berglund et al., 2016).

Beside the neural cell populations, brain resident immune cells play a crucial role for the maintenance of brain homeostasis. Microglia, the macrophages of the central nervous system, are capable to phagocyte cell debris and to release trophic factors to sustain the immune response and to stimulate endogenous neurogenesis. Neurodegeneration as well as brain injuries are associated with a substantial accumulation of microglial cells surround the lesion site. Understanding the dynamics of the microglia-specific immune response, especially in respect to the polarity of the microglial cells (pro-inflammatory M1 *versus* anti-inflammatory M2 phenotype), will provide additional information about the effectiveness of a potential therapeutic intervention. We recently developed a bioluminescence-based imaging strategy to follow microglia polarity upon stroke over time in the living animal using lentiviral vectors targeting pro- and anti-inflammatory microglia (Collmann et al., 2019). A combination of this microglia approach with the fate mapping approach using multiple FLuc variants will provide further information about the interplay between neuroinflammation and stem cells during disease.

Conclusion: Recent developments in the use of engineered luciferases

Vogel S, Hoehn M, Aswendt M (2020) In vivo bioluminescence imaging to elucidate stem cell graft differentiation.
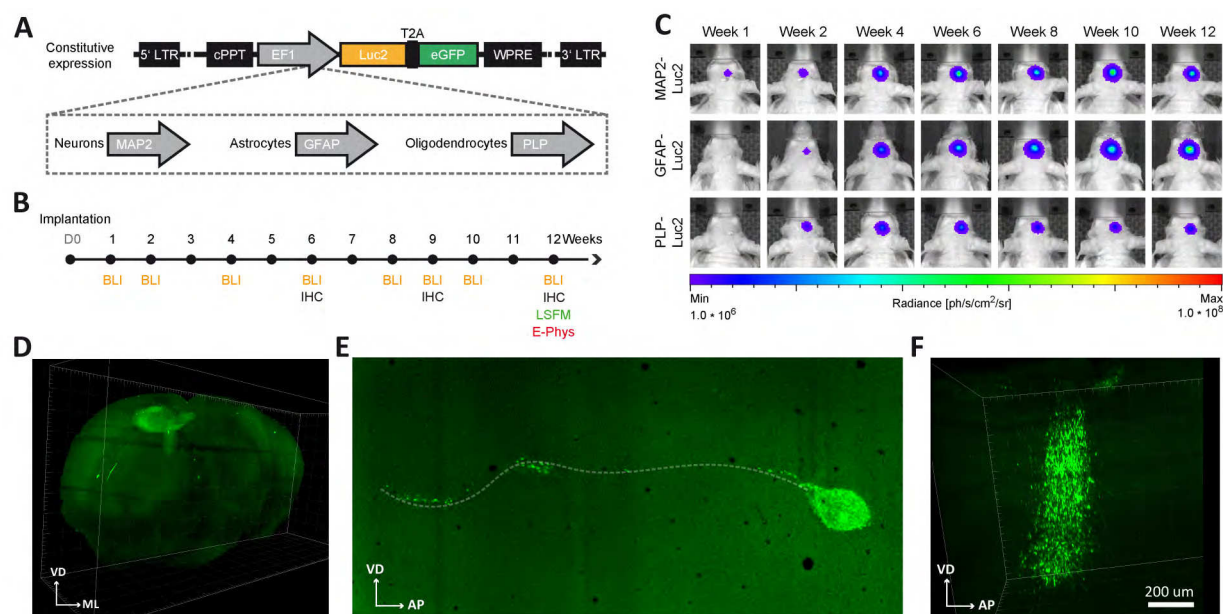*Neural Regen Res 15(1):61-62. doi:10.4103/1673-5374.264449*

Figure 1 Bioluminescence-based fate mapping approach.
(A) Plasmid design of the bicistronic gene expression cassette (Fluc Luc2 and fluorescent protein eGFP) under control of the strong constitutive promoter EF1. Cell-specific expression was achieved by switching EF1 with MAP2 (neurons), GFAP (astrocytes), and PLP (oligodendrocytes), respectively. Lentiviral transduction was used to engineer human iPSC-derived NPCs. (B) Timeline of repetitive BLI during a 12-week observation period after transplantation of NPCs into the brain of NMRI-Nu/Nu mice. (C) Representative top-view BLI/photograph overlays for the different NPCs targeting neuronal and glial differentiation. (D) Three-dimensional reconstruction of a representative NPC cell graft in the cortex obtained with LSFM. (E and F) Single cells project and migrate over long distances, while the majority of cells remains close to the transplantation site. Figure is adapted with permission from Elsevier: Vogel et al., 2019. AP: Anterior-posterior; BLI: bioluminescence imaging; eGFP: enhanced green fluorescent protein; FLuc: firefly luciferase; GFAP: glial fibrillary acidic protein; IHC: immunohistochemistry; iPSC: induced pluripotent stem cell; LSFM: light-sheet fluorescence microscopy; NPCs: neural progenitor cells; PLP: proteolipid protein; VD: ventral-dorsal.

for optical imaging of stem cells in the living mouse have advanced the longitudinal monitoring of viability and differentiation. Our fate mapping approach provides a powerful tool to compare strategies for a more direct commitment of the engrafted NSCs/NPCs and enables a correlation of the differentiation stage with the functional improvement in animal models of neurological disorders.

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