The Clonal Fate of Live Cells

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Tracking of individual transplanted cells and their clonal progeny is vital to advancing basic science, translational studies, and clinical applications of cell therapy.[1,2] Technologies for real-time in vivo monitoring of multiple cell populations, such non-invasive three-dimensional visualization of single cells, are still at their infancy. However, in the March issue of *Molecular Therapy*, two independent studies present substantially improved multiplex fluorescent barcoding strategies to mark and visualize clonal cell subpopulations.[3,4]

A commonly used method to visualize and identify specific cells is by flow cytometry, which utilizes lasers to detect cells stained with fluorescent chemical compounds (fluorophores). For example, cell surface biomolecules can be stained either by direct fluorophore binding or by attaching fluorophores to immune conjugates that specifically bind the target biomolecules.[5–7] The potential for clinical application of such technologies to track cells is ever increasing as the spectrum of available fluorescent dyes and powerful imaging platforms continues to grow.[8] The new studies in *Molecular Therapy* build on two approaches to color code cells so that they can be identified by flow cytometry. Fluorescent cell barcoding (FCB) represents high-throughput flow cytometry where distinct cell populations are "barcoded" by labeling with a unique color code (signature of fluorescence intensities), then pooled, stained with fluorophore-antibody complexes, and analyzed by flow cytometry. In fluorescent genetic barcoding (FGB), marking of cells is complemented by insertion of retroviral vectors, each containing a DNA barcode sequence corresponding to a transgene that expresses a specific fluorescence protein. Although FGB allows the study of live cells, one has to keep in mind that the process can alter the fate of cell populations due to insertional mutagenesis and extended culture times.

Maetzig and colleagues[3] built on FCB and FGB and introduced three generations of lentiviral vector transfer systems for the expression of fluorescent reporter dyes (lentiviral vectors can efficiently transduce a wide range of dividing and non-dividing cells and exhibit less genotoxicity than gammaretroviral vectors). First, the authors used monocistronic vectors that express one of three different fluorochromes (meKO2, YFP, or GFP) at bright and dim intensities, with fluorophore expression regulated by two translational control elements (TCEs). For each color, three distinct levels of expression intensity (high, low, absent) could be detected via flow cytometry. This first generation of FGB allows for up to 26 different fluorescent profiles to be introduced into the cell population. For the second generation, the authors used bicistronic and tricistronic vectors with distinct TCEs and self-cleaving peptides for regulation of the coexpressed fluorophores under the control of a promoter that is resistant to silencing. Finally, to design a platform independent from gene transfer conditions, the authors implemented their third generation of FGB, which is comprised of an optimal combination of six mono- and bicistronic vectors that, in addition, have been marked by a color-specific molecular barcode upstream of the 3′ long terminal repeat (LTR) (Figure 1). Importantly, the TCE is deliberately designed so that the fluorescent protein is expressed at a distinct level (in this case high or absent) that is fairly independent from variable numbers of integrated vector copies present in the barcoded cells. Although the study protocol required 28 days of in vitro culture of barcoded cells, the suitability of this approach for use in primary cells was impressively demonstrated in vivo by cell-clone fate tracking in murine and human hematopoietic transplantation assays as well as functional miRNA expression analysis. However, there are still some limitations that must be considered when using this

system. The reduction in the dimension of the color combinations, from 26 to 6 distinct fluorescent barcodes, was needed to reach reliable results independent from the vector transduction conditions in the in vivo experiments. Also, fluorescence expression may be influenced by the differentiation state of the marked cells. Future scaling of the multiplicity of individual traceable clones can then only be achieved by increasing the color space or, more likely, by the use of random color-specific genetic barcodes introduced into the vector as in Cornils et al.[7]

In contrast, Mohme et al.[4] employed optical barcoding in a strategy that made use of up to six different fluorophores—EBPF2, eGFP, mOrange2, T-Sapphire, Venus, dKatushka—to monitor the fate of clonal subpopulations in vivo (Figure 1). They directly built on their expertise with multicolor cell tracking and extended their earlier simpler labeling strategy, by which they had demonstrated impressive visualization of neuronal cells and their progeny in three dimensions in vivo.[9] Remarkably, the new method allows the tracking of up to 41 individually marked cell populations. This quantitative approach is based on a purely binary strategy, whereby when a color is either present or not introduces a sophisticated digital readout with higher stability for longitudinal tracking of clones. As compared to FGB, the authors demonstrated the feasibility of cell recovery and flow cytometric analysis after tumor formation in a xenotransplantation brain tumor mouse model without harming the cell viability. They also demonstrated the feasibility of the subsequential cell sorting and re-culturing ex vivo. The option to study solid tissue is unique and opens the gate for investigating the heterogeneity within composite tissues, such as tumors. The authors extended their fluorescent

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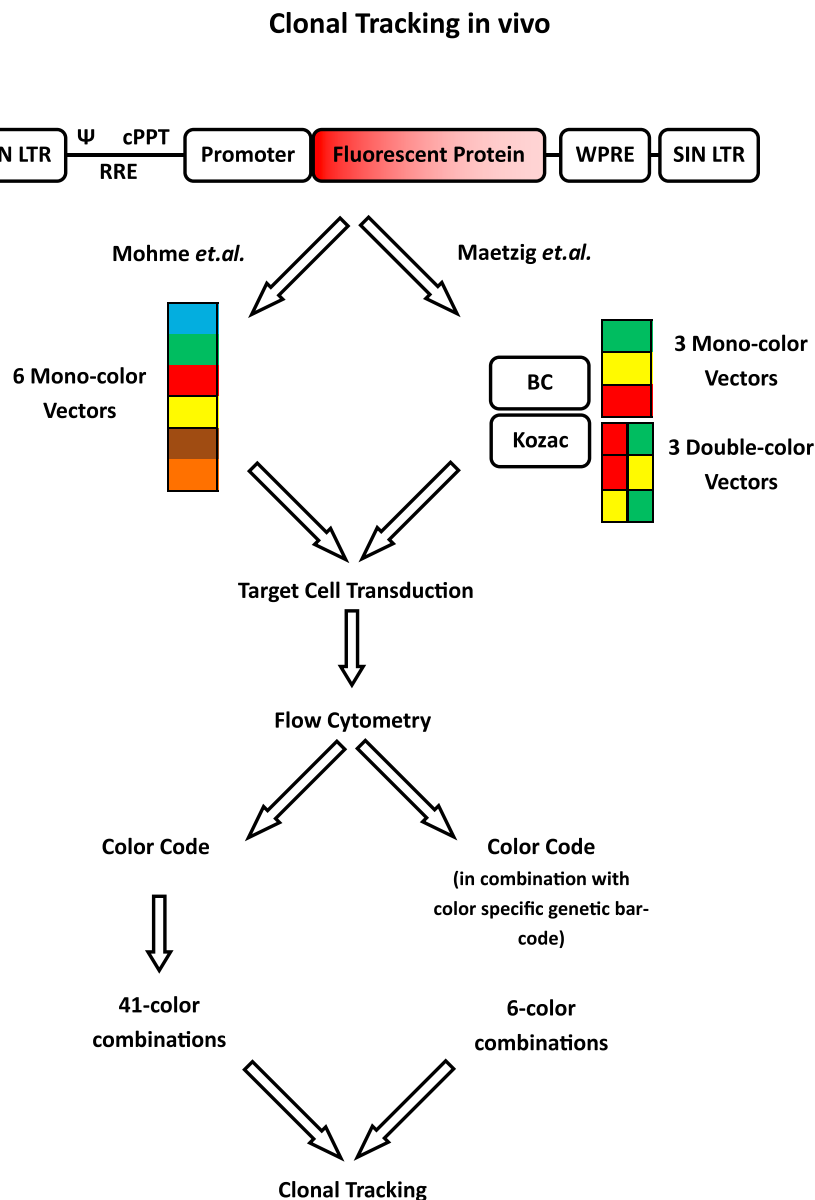**E-mail:** manfred.schmidt@nct-heidelberg.de

Clonal Tracking in vivo

Figure 1. Schematic Comparison of the In Vivo Clonal Tracking Strategies Presented by Maetzig et al.[3] and Mohme et al.[4]

Both fluorescent barcoding strategies rely on a lentiviral vector system expressing the distinct fluorophores. Mohme et al.[4] used a binary codification of fluorescent signal patterns reaching 41 color combinations to track tumorgenic cells and its progeny. Maetzig et al.[3] implemented a series of three different optical barcoding platforms. Only the third generation vector system (shown in this figure) with six color combinations and genetic barcodes was applied for the in vivo hematopoietic tracking experiment. BC, genetic barcode, Kozak, translational control element.

optical barcoding strategy by combining high-throughput readout through flow cytometry with accurate quantification and increased specificity. Even if deregulated gene expression influenced expression of the fluorescent markers, especially in differentiated cell populations, the on/off characteristic of the expression detection makes this approach more resilient to epigenetic alteration.[10–12] However, with up to six

different fluorophores, the untangling and quantification of the clonal mix requires an accurate gating strategy to preclude wrong-call optical barcoding.

In conclusion, both studies further extend the potential of fluorescent barcoding. Importantly, these investigations translate previous experiments to an in vivo setting, thereby significantly expanding the application profile for fluorescence-based analysis of cell transplants. To further improve this technology, one has to keep in mind that even subtle genotoxic effects might also influence the fate of the marked cell populations if multiple vector genomes are integrated into one cellular genome. Future steps in clonal tracking may combine fluorescent optical barcoding and cell-specific expression markers. One could possibly further boost the quantity and quality of visualized marked clones by driving the expression of the fluorescent proteins from differentiation marker-associated promoters. Implementation of a system in which cell- and tissue-specific promoters mediate a color shift may also improve performance. Finally, using a genetic barcoding system that incorporates combinations of unique molecular identifiers may improve the accuracy of clonal quantification and assure independence from normal intracellular gene expression regulation.

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