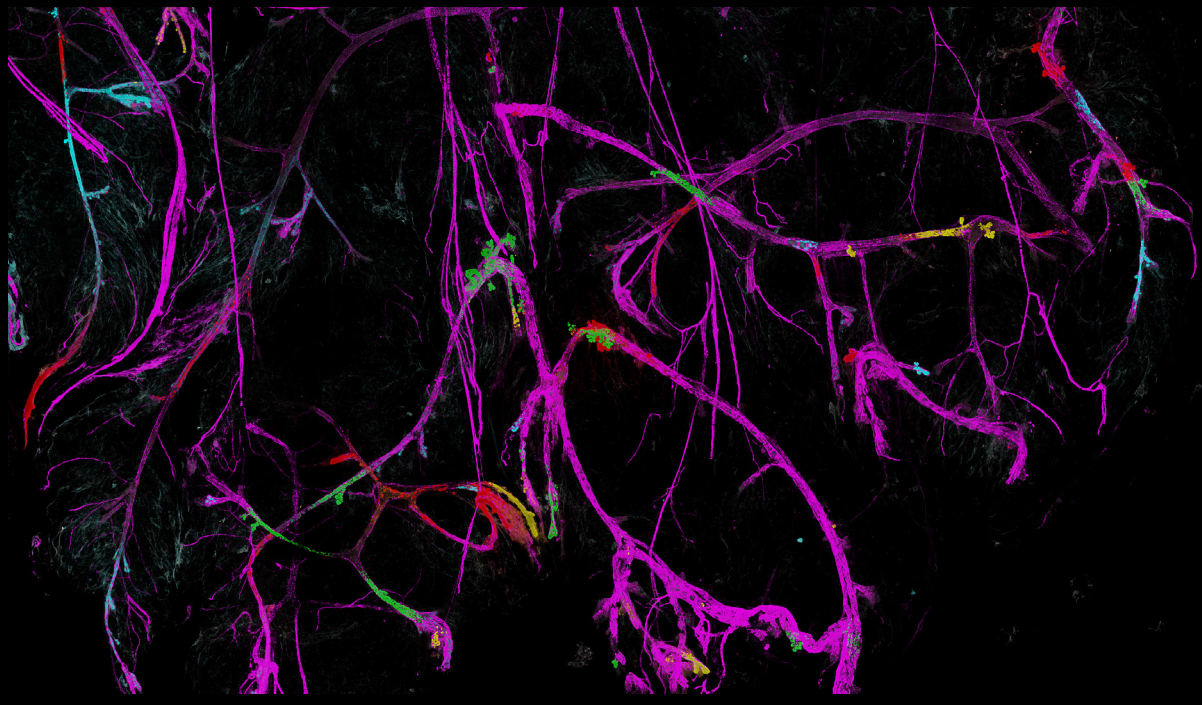
From Eye to Insight

Application Note

IN VIVO MULTI COLOR IMAGING OF A CONFETTI BREAST CANCER MOUSE MODEL

How to gain deep insights into the development of cancer using spectrally tuneable 2-photon microscopy



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Understanding the complexity of cancer spread poses a number of challenges: developing reliable mouse models for cancer metastasis, being able to label the components involved in the cancer process and finding the tools to visualize the labeled components.

This application note reviews a labeling method called lineage tracing that allows the behavior of cancer cells within an organism to be followed. It also focuses on microscopy techniques to visualize labelled cells in a living model organism.

Lineage tracing and the confetti construct

Lineage tracing can be used to follow the progression of cells by using a reporter construct that will only be activated if a specific protein of a (stem) cell is present. One way to accomplish this is by Cre-mediated removal of a stop-codon in the reporter construct. Cre is short for 'cyclization recombination' and describes a group of enzymes that cut and recombine DNA marked with a specific sequence (loxP site).

The precondition required for the use of this method is to have an animal model that has been modified with two vectors: A vector coding for Cre behind a specific promoter and a second vector coding for a reporter, usually a fluorescent protein, after a ubiquitous promoter – one that is expressed in every cell – and a stop sequence flanked by LaxP-sites, a specific DNA-sequence recognized by Cre. Cells that express the specific promoter will then produce Cre. This strategy gives specificity to the labeling technique, as Cre is expressed only in cells with a specific characteristic, e.g., in endothelial stem cells. Next, Cre will recognize the LaxP-sites surrounding 'stop' sequence of the second vector and selectively remove it. Then only the vector with the ubiquitous promoter and the coding sequence for the fluorescent protein are left. Cells, subsequently, produce fluorescent protein which can be visualized using microscopy techniques.

For an overview of the lineage tracing technique, see the following scheme:

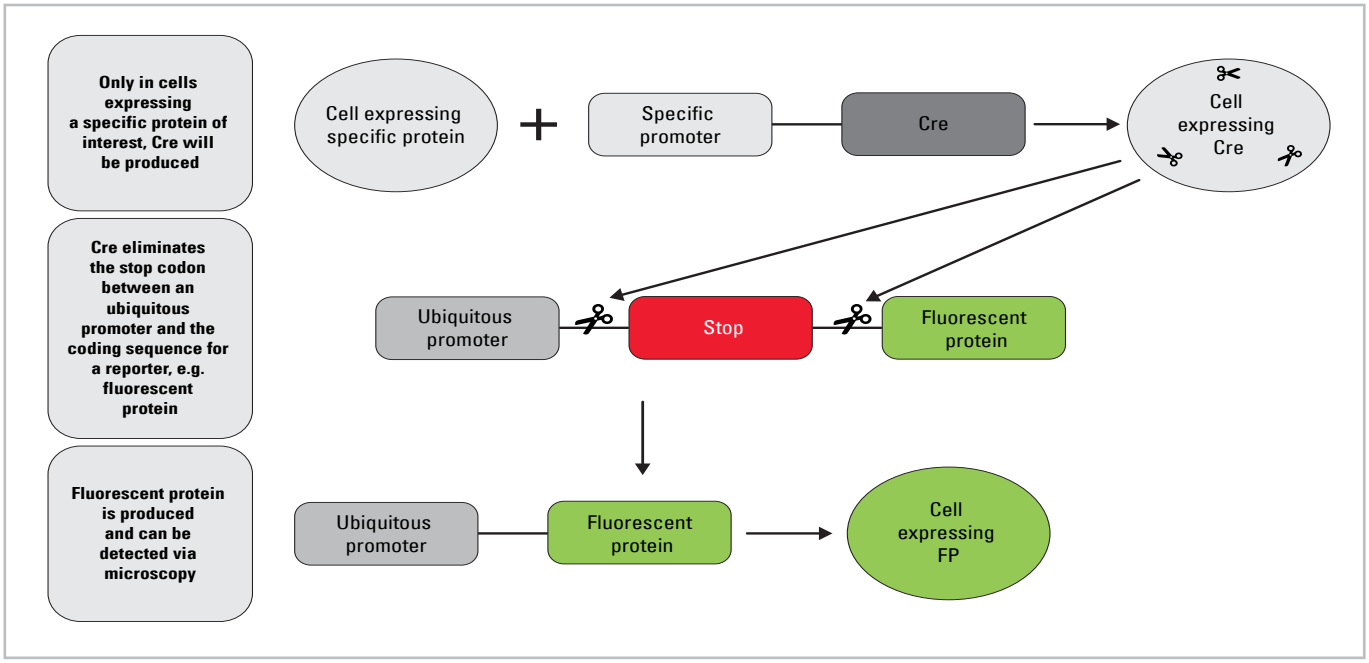

Image title page: Overview of healthy mouse mammary gland tissue (ex vivo) expressing the confetti construct with CFP (cyan), GFP (green), YFP (yellow) and RFP (red) within the epithelium (after tracing by randomly recombined cells). In magenta: myoepithelial cells (outer layer of ducts) expressing smooth muscle actin stained with Alexa 647 and excited with 1250 nm (Thin duct like structures are lymphatic- and bloodvessels (magenta). Image depicts part of the whole mount mammary gland Tile Scan (10 x 8 tiles) acquired in stack sequential mode.

The general concept of lineage tracing has been present in the biologist's toolbox since the 19th century and has been through many improvements and advancements ever since. Especially, the extension to multicolor reporter lines has been a milestone, enabling different stem cell lines to be traced in one animal model. Multicolor reporters facilitate the study of interactions between different cell types or simply the following of more cells at one time.

One widely known variant of lineage tracing is called 'brainbow'[1]. With brainbow, a mix of fluorescent proteins is expressed through multiple rounds of recombination, giving a multitude of resulting combinations that can be spectrally separated. Using this approach, denser labeling is possible, so individual cells can be distinguished by their spectral properties.

In 2010[2], a simpler multicolor lineage tracing variant, called confetti, was introduced. Confetti tracing facilitates random labeling of cells in only four distinct colors: CFP, GFP, YFP and RFP. Whereas 'brainbow' uses a stochastic mix of fluorophores to label each cell, 'confetti' recombination only leads to the expression of one of the four fluorophores per cell. This enables the labeling of more cell types in the same animal and allows to trace the stem cell type they proliferated from. The confetti construct is now commonly used to trace cancer cells as they spread and metastasize. It also enables researchers to determine if cells originate from the same or different stem cells.

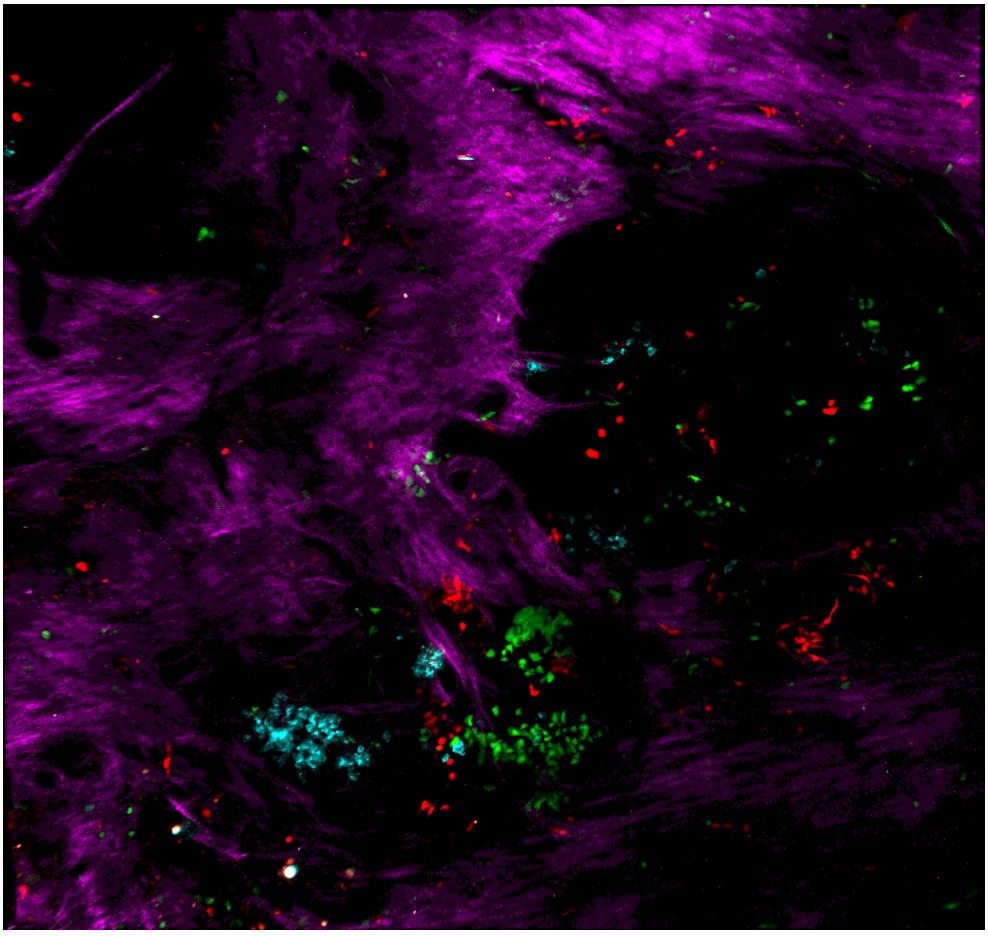

Figure 1: Mammary gland tumor expressing Confetti construct with CFP (cyan), YFP (green), and RFP (red) that labels subsets of tumor cells. GFP is only in nuclei (hardly visible). Magenta: SHG of Collagen I that helps to "contour" the tumor shape. Imaging performed during a late tumor stage and results show the progression of cancer. Black areas, where no confetti is expressed in cells, belong to the tumor as well.

Microscopy techniques suited to image multicolor reporter lines

Once the model is labeled with a fluorescent reporter, a suitable imaging technique is needed to ensure that researchers get the maximum of information out of the precious model. Intravital imaging offers the most physiologically relevant option by implanting "windows" at the location of interest, such as brain, intestines or lungs, in the living animal. By this imaging window the same tumor area can be visualized over multiple weeks, resulting in four-dimensioal information (xyzt) on tumor growth and progression. Then, a deep tissue imaging technique that is compatible with living cells and tissues is crucial. At this point, multiphoton microscopy comes into action.

A suitable multiphoton microscope for this type of experiment needs to be able to penetrate deep into the tissue, ideally beyond 1mm deep depending on the tissue properties. It also has to offer spectral capabilities which can be adapted in the best way for the multicolor sample.
Traditional multiphoton microscopes use fixed filter sets and are, therefore, limited: the combination of usable fluorophores is given by the hardware and neither sequential acquisition of different spectral windows nor flexible adaption are possible. STELLARIS 8 DIVE with 4Tune overcomes this fundamental challenge. 4Tune is a spectrally tunable multiphoton detector located on the non-descanned site[3]. It offers the most sensitive detection of scattered photons from deeper tissue layers along with full spectral flexibility. Together with the intuitive integration into the ImageCompass smart user interface, STELLARIS 8 DIVE is the ideal tool for deep in vivo multicolor imaging.

Find out more about
4Tune – the spectral NDD

https://www.leica-microsystems.com/science-lab/mission-impossible-accomplished-tunable-colors-for-non-descanning-detection/

Find out more about
multiphoton microscopy

https://www.leica-microsystems.com/science-lab/principles-of-multiphoton-microscopy-for-deep-tissue-imaging/

Find out more about
STELLARIS 8 DIVE

https://www.leica-microsystems.com/products/confocal-microscopes/p/stellaris-8-dive/

How to acquire images from the confetti mouse with STELLARIS 8 DIVE

Multi-photon experiments with multiple intravital imaging sessions could be quite complex and a simple way to define the imaging settings would be beneficial. ImageCompass, the user interface of STELLARIS allows a simplified definition of multi-color imaging settings. This simplicity is demonstrated below using an example of confetti imaging in mouse models for breast cancer. A drag and drop mechanism with presets for imaging settings, which include the excitation line, emission spectrum, and detection range, facilitates the setup of the experiment. Alternatively, settings can be defined freely without any presets, giving researchers maximum flexibility.

For multiphoton microscopy, it is important to set up the imaging for specific labelled fluorophores or fluorescent proteins for best signal separation and specificity. Detection windows can be set in a way to reduce spectral crosstalk and, therefore, improve the image quality. This capability is crucial for in vivo imaging in cancer research.

Another big advantage of ImageCompass is that detection windows for the Second Harmonic Generation (SHG) signal can simply be placed "below" the 2-photon excitation line (see figure 2). This can be done with any detector. Thus, the SHG imaging window can be set without any limitations known from the handling of fixed filter sets. SHG gives a valuable additional signal to specific labelled cells that helps to better understand the structural context of the tumor.

In addition, a Third Harmonic Generation (THG) signal can be detected easily together with specific fluorophores (for further reading, see THG application note[4]).

Experimental settings

To visualize the confetti construct (CFP, GFP, YFP and RFP) plus SHG in a mouse model for breast cancer, a first setting was defined with 960 nm to excite GFP, YFP, and RFP. The 4Tune-detector with its 4 non-descanned detectors (NDDs) was used and the detection windows were freely defined: 470 to 485 nm on NDD1 (SHG), 496 to 559 nm on NDD3 (GFP), and 586 to 666 nm on NDD4 (RFP and YFP). YFP was detected in part in NDD3 together with GFP, but also in the RFP-channel and, therefore, YFP-expressing cells will show up as yellow in the overlay image.

In a second setting, 860 nm was selected to excite CFP and the NDD1 (435 - 498 nm) was used for detection. This image acquisition was carried out in a frame sequential mode. Frame sequential is not the fastest way but very suitable for the in vivo imaging of these tumor models as the cell movement speed is moderate.

These settings for the four confetti colors allow researchers to perform multiple intravital imaging sessions through a mammary imaging window and to trace the progeny of each cancer stem cell. Images were acquired with a 25x (HCX, IRAPO, N.A. 0.95, WD Imaging settings optimized for each experimental purpose.

Settings at a glance: Sequential frame by frame

Setting 1: 960 nm to excite GFP, YFP, and RFP.

Detection: HyD NDD1 470 – 485 nm (SHG),
HyD NDD3 496 – 559 nm (GFP),
HyD NDD4 586 – 666 nm (RFP and YFP).

Setting 2: 860 nm was selected to excite CFP

Detection: HyD NDD1 435 - 498 nm

Figure 2: Window of the LAS X ImageCompass user interface showing presets for confocal (C) and multiphoton (M) imaging at the top. Presets can be used in a drag and drop manner. Imaging settings for the confetti mouse tumor (CFP, GFP, YFP and RFP) plus SHG (NDD1) are shown below in a frame sequential mode.

Imaging settings optimized for each experimental purpose

The selected ImageCompass settings can be modified easily and optimized depending on the actual experiment and system configuration. Image acquisition can be set to either achieve best color separation due to the ability to flexibly set any the detection window, highest acquisition speed for in vivo-experiments, or highest resolution (often used for fixed samples). The following example shows the settings for maximizing the acquisition speed.

In a two-multiphoton two laser beam system, a line sequential setting using two different excitation wavelengths would be optimal for in vivo imaging over time and in a volume. In this case, all four NDDs can be used with two settings in a line sequential mode.

Settings at a glance: Sequential Line by Line

Setting 1: 960 nm from MP laser 1 to excite GFP, YFP and RFP

Detection ranges: HyD NDD2 470-520 nm (GFP),
HyD NDD3 521-565 nm (YFP),
HyD NDD4 566-615 nm (RFP).

Setting 2: 860 nm from MP 2 laser for SHG and CFP
HyD NDD1 420-440 nm (SHG),
HyD NDD2 470-520 nm (CFP)

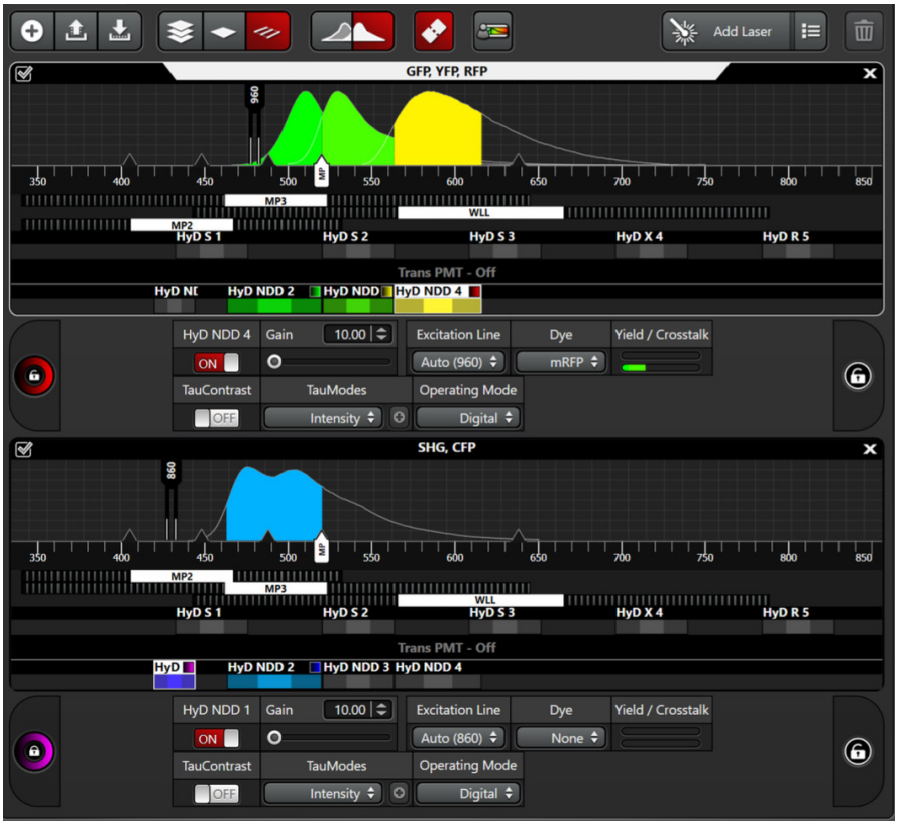

Figure 3: Window of the LAS X ImageCompass user interface showing imaging settings for the confetti mouse tumor (CFP, GFP, YFP and RFP) plus SHG (NDD1). GFP, YFP, and RFP are defined in setting 1 and CFP and SHG are defined in setting 2, both in a line sequential mode for fast image acquisition. For GFP and CFP, the same detector is used (HyD NDD 2), but with specific excitation wavelengths (960 and 860 nm) from the two available MP-lasers.

Mouse models and experiment

Here the visualization of a confetti construct (CFP, GFP, YFP and RFP) in mouse models is described for both breast cancer (Tumor model: MMTV-PyMT;Rosa26-CreERT2;Rosa26-Confetti) and a healthy mammary gland (Healthy mammary gland: Rosa26-CreERT2; Rosa26-Confetti). The mice spontaneously develop breast cancer in this tumor model[5]. After the development of a tumor, a mammary imaging window was implanted, and the mouse was treated with a low dose of Tamoxifen at the same time. The result is the recombination of genetic information as described previously[6]. The expression of R26-CreERT2 is normal in any tumor cell and, stochastically in some cells, confetti will be recombined and fluorophores (CFP, GFP, YFP and RFP) are expressed. Here, Cre is ubiquitously present and kept in the cytoplasm of cells as it is coupled to the ERT2-protein. Only when Tamoxifen is taken up by a cell, it binds to ERT2 and Cre can translocate to the nucleus of the cell and exert its function. For healthy mammary glands, the approach described in C. Scheele et al.[7] was used.

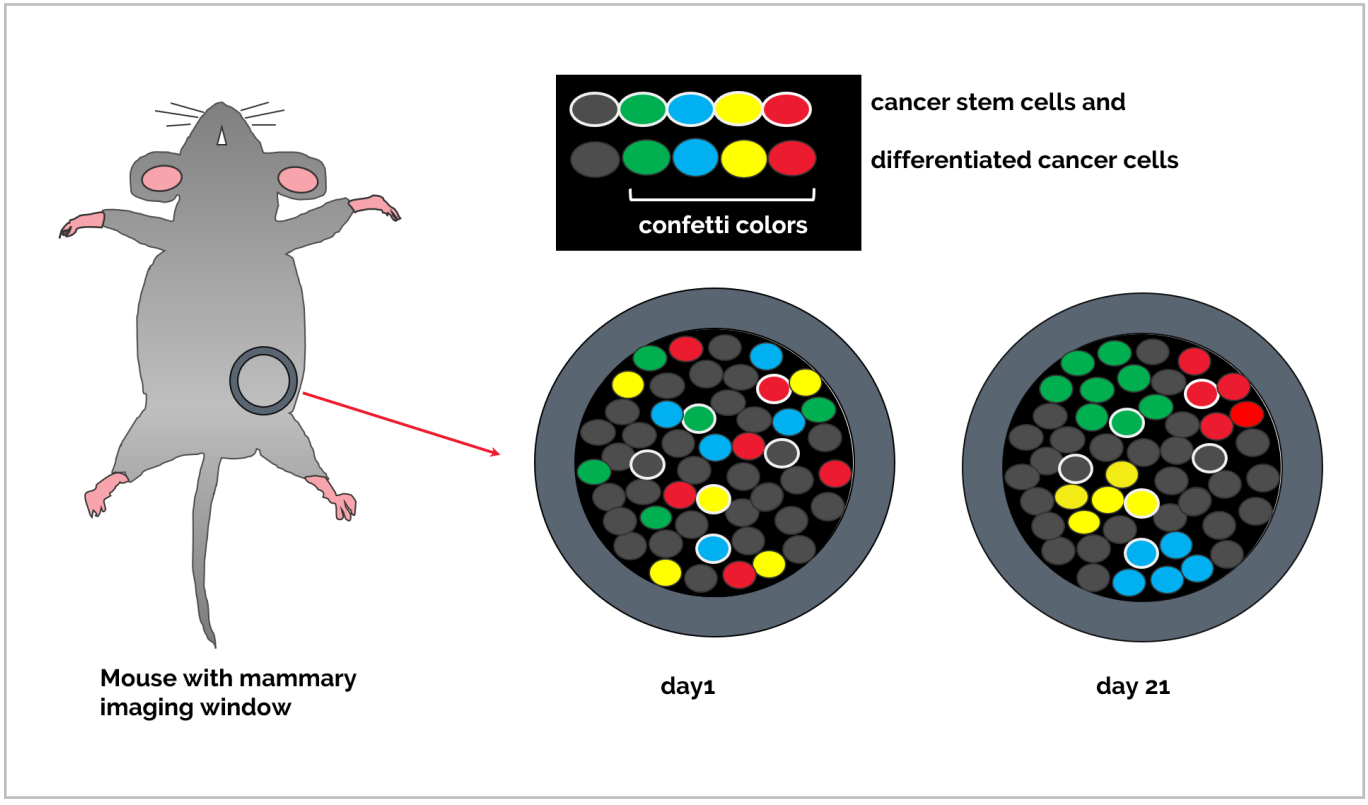

Figure 4: Scheme of the experimental setup for intravital lineage tracing. The confetti colors (CFP, GFP, YFP, and RFP) are randomly expressed in mice with genetic mammary tumors. Multiple intravital imaging sessions were carried out through the mammary imaging window to trace the progeny of each cancer stem cell and study the process of tumor growth and metastasis. Concept adapted from reference 6.

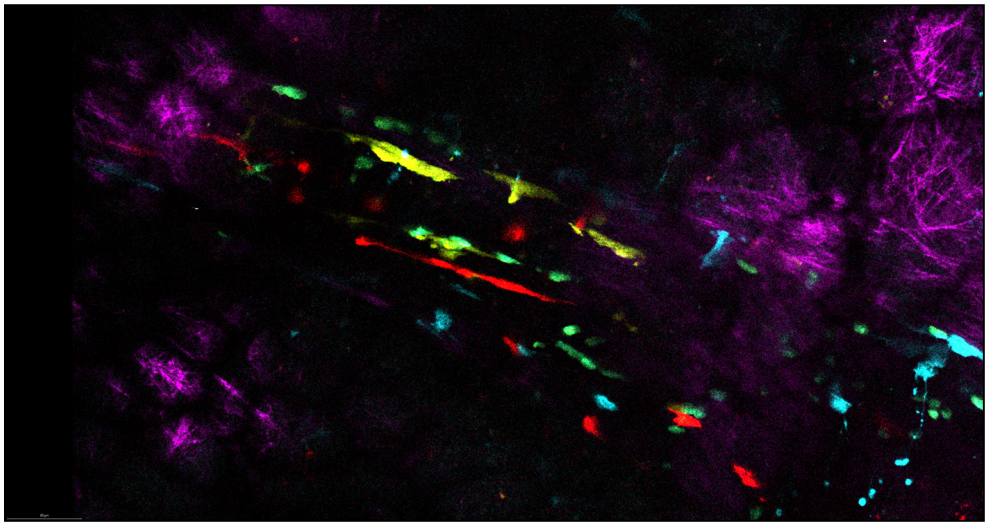

Figure 5: Healthy mammary gland expressing the confetti construct (CFP, GFP, YFP, and RFP) that labels ductular cells. The mammary duct structure is nicely visible. No cluster of cells is present. Magenta indicates SHG.

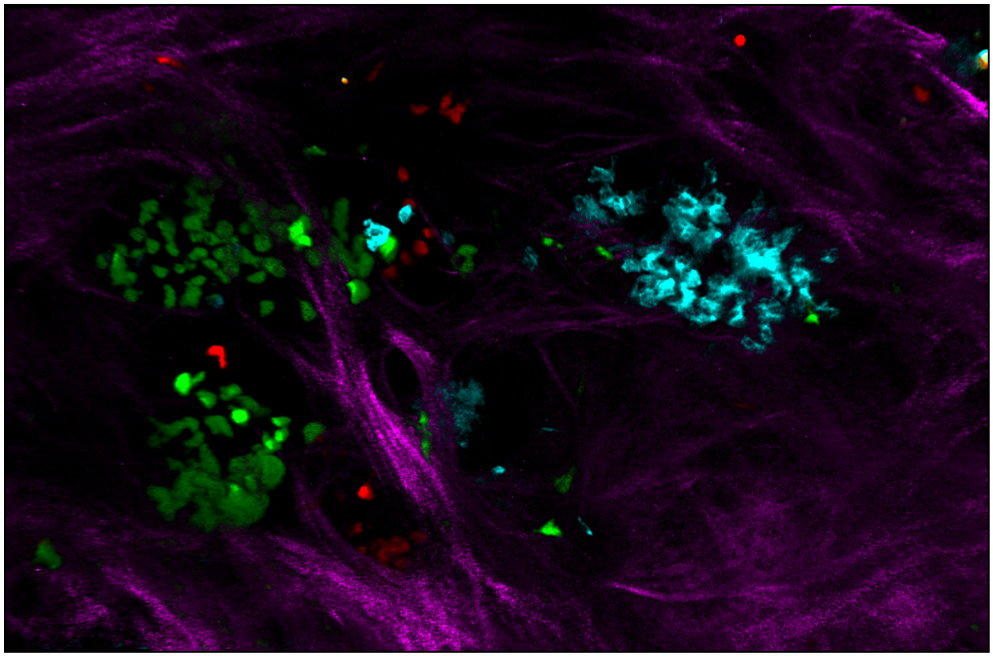

Figure 6: Mammary gland tumor (detail) expressing confetti construct with CFP (cyan), YFP (green) and RFP (red) that labels subsets of tumor cells. GFP only in nuclei. Magenta: SHG (Collagen I) Imaging performed during a late tumor stage and showing the progression of cancer. Black regions belong to the tumor as well and represent areas where no confetti recombination occurred in the cells. At this stage of tumor growth, no structure of mammary ducts is present anymore. Green indicates YFP as GFP is present only in nuclei (not visible).

Summary - A rainbow of possibilities

Lineage tracing in a confetti animal model combined with multiphoton imaging using the STELLARIS 8 DIVE allows researchers to follow the progression of cancer cells. Multiphoton imaging is an adequate technique for intravital imaging and deep penetration into tumor tissue. Imaging of confetti fluorophores is quite demanding and conventional dichroic filters lack the flexibility needed to adapt spectral windows. This lack of flexibility makes them suboptimal for distinguishing all fluorophores in such a complex sample, especially where they spectrally overlap, such as CFP, GFP, and YFP.

4Tune, the spectral non-descanned detector in the STELLARIS 8 DIVE, is flexible and enables an optimal setup of detection ranges for each fluorophore, simplifying complex experiments significantly. In addition, windows as narrow as 10 nm in width – which are optimal for collecting SHG signals – can be defined without the need of a special hardware filter. These windows were used for tumor imaging as well. In this example, SHG helps to reveal the tumor size and shape and its progression without any additional labelling. Thus, it facilitates navigating through healthy and tumorous tissue.

In summary, the flexibility provided by the spectral 4Tune non-descanned detector – which is seamlessly integrated into the user interface ImageCompass - allows researchers to focus on the sample instead of the microscope. The choice of fluorophores is not limited by the hardware and the intuitive setup is fast and allows researchers to make the most of the limited time that the animal model can be kept under anesthesia for imaging. With STELLARIS 8 DIVE and 4Tune a rainbow of possibilities is available for intravital cancer research.

Acknowledgments

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References

[1] Livet, J. et al.; Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system; Nature 2007. https://doi.org/10.1038/nature06293

[2] H. J. Snippert et al.; Intestinal Crypt Homeostasis Results from Neutral Competition between Symmetrically Dividing Lgr5 Stem Cells; Cell 2010. https://doi.org/10.1016/j.cell.2010.09.016

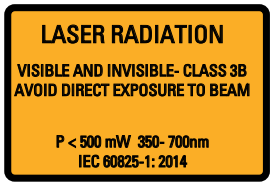
[3] R. Borlinghaus, H. Gugel; Mission Impossible Accomplished: Tunable Colors for Non-descanning Detection; Leica Science Lab 2017. https://www.leica-microsystems.com/science-lab/mission-impossible-accomplished-tunable-colors-for-non-descanning-detection/

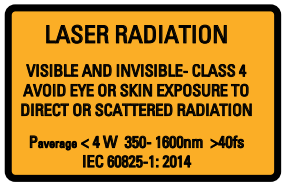
[4] S. Holzmeister, Joel Zindel; Label-Free Blood Flow Quantification; Leica Science Lab 2020. https://www.leica-microsystems.com/science-lab/how-to-quantify-label-free-blood-flow/

[5] E.Y. Lin et al.; Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases; Am J Path 2003. https://doi.org/10.1016/s0002-9440(10)63568-7

[6] A. Zomer et al.; Brief Report: Intravital Imaging of Cancer Stem Cell Plasticity in Mammary Tumors; STEM CELLS 2013 https://doi.org/10.1002/stem.1296

[7] C. Scheele et al.; Identity and dynamics of mammary stem cells during branching morphogenesis; Nature 2017. https://doi.org/10.1038/nature21046





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