Journal Club

Stretching the timescale of intravital imaging in tumors

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Abbreviations: SHG, second harmonic generation; MIW, mammary imaging window; 3D, three dimensions; 4D, four dimensions

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Since the time it was pioneered in 1992, intravital imaging of tumors at cellular resolution has offered us the extremely important opportunity of “seeing biology.” However, until now, most studies were monitoring tumor cell behavior in the same animal over short times, requiring the combining of acquired data into a hypothesis via statistical analysis. In the last year, different groups have independently developed techniques to extend the timescale of intravital imaging to several days. This improvement allows one to address the connection between tumor cell behavior and the microenvironment which surrounds them. We can now assess dynamics of the cell-cell interactions in tumors, analyze tumor cell fate and changes in the tumor extracellular matrix which accompany tumor progression.

Intravital imaging of tumors at cellular resolution offers insight into the physiology of cells in vivo in real time. The first published study which included injectable dyes to monitor tumor metastasis inside the embryo was done by the group of Groom. Some years later, Farina,2 and then Naumov,3 and co-workers, published a study which included injectable dyes to monitor tumor growth. The first study which offered partial data on slow processes, these technical improvements allow us to visualize long-term results of multiple cell-cell and cell-extracellular matrix interactions in vivo.

Some low-resolution studies have proposed a reversible flap9 on the tumor tissue implanted several days earlier. However, visualized areas were not the same at each of the timepoints. Also, as skin flaps were opened repeatedly, they were potentially influencing the microenvironment by surgery-related immune/inflammatory responses. In addition, several groups have been using a dorsal skinfold chamber10 in which the tumor is grown ectopically, in the space between the skin and glass coverslip on the back of the mouse. This preparation could be used for either low resolution measurements over several days, or short-term measurements at cellular resolution.

In the last few months, several studies have included techniques which extend the time-scale of intravital imaging in tumors from hours to days (Table 1). In contrast to the previous techniques which offered partial data on slow processes, these technical improvements allow us to visualize long-term results of multiple cell-cell and cell-extracellular matrix interactions in vivo.

Segall-Condeelis groups11,12 have developed a technique to visualize and quantify invasion and intravasation of single tumor cells in orthotopic mammary tumors. They designed a mammary imaging window (MIW), which enables imaging the tumor in serial imaging sessions. Moreover, to properly position the animal on the microscope and keep the animal orientation the same over several sessions, they use a stereotactic imaging box.11 Due to cell replication and motility, angiogenesis and consequent changes in tissue shape, a registration landmark is essential in order to recognize the region of interest in each of the imaging sessions. In Kedrin et al.12 a photoswitchable protein Dendra2 was used as a tumor cell marker, making it possible to differentiate between total tumor cells (green) and chosen cells of interest (red). By photomarking and visualizing selected populations of cells within the tumor, team quantified and compared the metastatic behavior of cells in different tumor microenvironments within the same tumor. The number of imaging sessions which visualize a specific group of cells in areas surrounding major blood vessels is limited by high cell motility and intravasation. However, in areas where only microvessels are present (Fig. 1), this technique can monitor cell invasion of the surrounding environment for up to seven days.

Similarly, in Perentes et al.,13 Boucher-Jain groups use serial imaging sessions made possible via dorsal chamber implantation...
and intravital multiphoton microscopy to study the mechanism of collagen fiber remodeling by tumor-associated fibroblasts. The internal landmark used in order to recognize and image the same microenvironment in several imaging sessions is collagen itself. Fibers are visualized by second harmonic generation (SHG), without any additional labeling. Since the resulting images are misaligned due to different animal orientations and tissue changes over serial imaging sessions, additional registration approach based on fluorescence intensity (TurboReg) was applied during data post-processing. Images taken over nine-day periods were aligned based on similar bulk distribution of collagen fibers. Further, individual fibers were analyzed for a decrease in length and an increase in area overlap with surrounding GFP-fibroblasts.

Werb and co-workers have used a different method when comparing the dynamics of stromal cells in different microenvironments of breast carcinoma, as presented in Egeblad et al. In order to optically access the tumor, they used an improved version of the ‘skin-flap’ technique. This allows work on transgenic mouse models, such as MMTV-PyMT, which can have several tumor stages present in one animal. Imaging was done over a single session that extends up to 27 h by carefully controlling temperature, anesthesia and animal hydration. While the use of spinning disc confocal microscopy limits imaging depth to ~2 cell diameters deep into the tissue, large areas of the tumor can be imaged and with high speed. The high speed of acquisition results from simultaneous illumination of ~1,000 rotating pinholes at a time and using cameras as detectors. This means that the limiting factor in the speed of data acquisition is the brightness of cells inside the tumor. As the excitation is achieved via single photon events, the implementation of additional laser lines is much cheaper and fairly straightforward. Moreover, by using a motorized stage which is controlled by software, several fields of view can be combined into mosaic images of a larger area. The final output of this set up is a 4D movie which contains up to four-channels, three z-sections, 45 timepoints per hour and compiles five fields into a mosaic view.

Finally, Dunphy et al. recently proposed an interesting microcartography approach in which fluorescent beads are inserted inside the dorsal skinfold chamber as reference points. Based on the visualization of beads, coordinates of the region of interest are recalculated in each of the series of imaging sessions.

We can now map the fate of tumor cells over days or monitor changes in the extracellular matrix inside the tumor as the tumor grows and progresses. These improvements allow assessment of the dynamics of cell-cell and cell-matrix interactions inside the tumor. Visualization and quantification of these interactions, the more precise definitions of microenvironments and the identification of 43

Table 1  Comparison of new intravital techniques for long-term imaging

| Technique                      | MIW + photoswitching<sup>12</sup> | Dorsal skinfold + SHG recognition<sup>13</sup> | Extended skinflap<sup>15</sup> |
|--------------------------------|-----------------------------------|-----------------------------------------------|--------------------------------|
| Orthotopic tumors              | Yes                               | No                                            | Yes                            |
| Long-term anesthesia needed     | No                                | No                                            | Yes                            |
| Multiple imaging sessions available | Yes                           | Yes                                            | No                             |
| Microscopy                     | Confocal and multiphoton          | Multiphoton                                   | Spinning disc confocal         |
| Depth of imaging               | ~120 μm<sup>12</sup>              | ~100 μm<sup>13</sup>                          | <70 μm<sup>15</sup>           |
| Detectors                      | PMT (1 for each channel)          | PMT (1 for each channel)                      | Camera                         |
| Number of channels             | 4                                 | 2                                             | 4                              |

Figure 1. Photoconverted regions which are not in the vicinity of major blood vessels show a relatively slow dispersion of cells throughout a seven day period. Images are the result of serial intravital imaging sessions (0–168 h after photoswitching) of mammary tumor cells which express cytoplasmic Dendra2. Fluorescence intensity at each time point was normalized to 0 h level. Photo converted region (red) is 150x 150μm at 0h.
stromal cells essential to tumor progression are all within reach. In addition, the analysis of mechanisms of drug action on single cells in real time in vivo, is now an achievable goal.

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References

1. MacDonald C, Schmidt EE, Morris VL, Chambers AF, Groom AC. Intravital videomicroscopy of the chorioallantoic membrane microcirculation: a model system for studying metastasis. Microvasc Res 1992; 4:185-99.

2. Farina KL, Wyckoff JB, Rivera J, Lee H, Segall JE, Condeelis JS, et al. Cell motility of tumor cells visualized in living intact primary tumors using green fluorescent protein. Cancer Res 1998; 58:2528-32.

3. Naumov GN, Wilson SM, MacDonald IC, Schmidt EE, Morris VL, Groom AC, et al. Cellular expression of green fluorescent protein, coupled with high-resolution in vivo videomicroscopy, to monitor steps in tumor metastasis. J Cell Sci 1992; 112:1835-42.

4. Brown EB, Campbell RB, Tsuchi Y, Xu L, Carmeliet P, Fukushima D, et al. In vivo measurement of gene expression, angiogenesis and physiological function in tumors using multiphoton laser scanning microscopy. Nature Med 2001; 7:864-8.

5. Wang W, Wyckoff JB, Frohlich VC, Oleynikov Y, Huttelmaier S, Zavadil J, et al. Single Cell Behavior in Metastatic Primary Mammary Tumors Correlated with Gene Expression Patterns Revealed by Molecular Profiling. Cancer Res 2002; 62:6278-88.

6. Jain RK, Munn LL, Fukushima D. Dissecting tumor pathophysiology using intravital microscopy. Nat Rev Cancer 2002; 2:266-76.

7. Sidani M, Wyckoff JW, Xue C, Segall JE, Condeelis J. Probing the microenvironment of mammary tumors using multiphoton microscopy. J Mammary Gland Biol Neoplasia 2006; 11:151-63.

8. Hoffman RM. The multiple uses of fluorescent proteins to visualize cancer in vivo. Nat Rev Cancer 2005; 5:796-806.

9. Yang M, Baranov E, Wang J-W, Jiang P, Wang X, Sun F-X, et al. Direct external imaging of nascent cancer, tumor progression, angiogenesis and metastasis on internal organs in the fluorescent orthotopic model. Proc Natl Acad Sci USA 2002; 99:8824-9.

10. Lehr HA, Leung M, Menger MD, Nolte D, Mersmer K. Dorsal skinfold chamber technique for intravital microscopy in nude mice. Am J Pathol 1993; 143:1055-62.

11. Gligorijevic B, Kedrin D, Segall JE, Condeelis J, van Rheenen J, Dendra2 Photoswitching through the Mammary Imaging Window. J. Vis. Exp. 2009; 28. doi: 10.3791/1278.

12. Kedrin D, Gligorijevic B, Wyckoff J, Verkuilba VV, Condeelis J, Segall JE, et al. Intravital imaging of metastatic behavior through a mammary imaging window. Nat Methods 2008; 5:1019-21.

13. Perentes JY, McKee TD, Ley CD, Mathiew H, Dawson M, Padera TP, et al. In vivo imaging of extracellular matrix remodeling by tumor-associated fibroblasts. Nat Methods 2009; 6:1019-21.

14. Thevenaz P, Ruttimann UE, Unser M. A pyramid approach to subpixel registration based on intensity. IEEE Trans. Image Process 1998; 7:27-41.

15. Egeblad M, Ewald AJ, Askarud HA, Itruit ML, Weil BM, Bainbridge E, et al. Visualizing stromal cell dynamics in different tumor microenvironments by spinning disk confocal microscopy. Dis Model Mech 2008; 1:155-67.

16. Ahmed F, Wyckoff J, Lim EY, Wang W, Wang Y, Hennighausen L, et al. GFP Expression in the Mammary Gland for Imaging of Mammary Tumor Cells in Transgenic Mice. Cancer res 2002; 62:7166-9.

17. Nakano A. Spinning-disk confocal microscopy: a cutting-edge tool for imaging membrane traffic. Cell Struct Funct 2002; 27:349-55.

18. Dunphy MP, Entenberg D, Toledo-Crow R, Larson SM. In vivo microcartography and subcellular imaging of tumor angiogenesis: A novel platform for translational angiogenesis research. Microvasc Res 2009; 78:51-6.