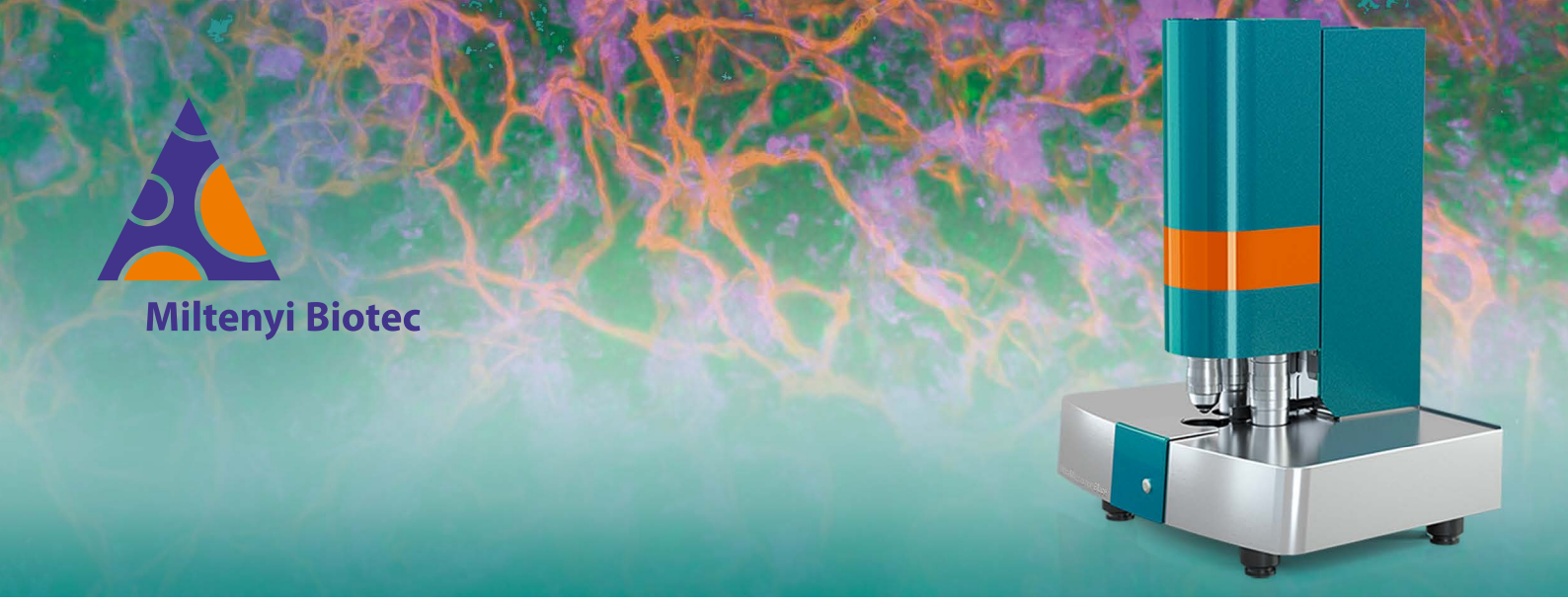

3D imaging and analysis of CAR T cells in solid tumors by UltraMicroscope light sheet systems

Background

Profound analysis of the heterogeneous tumor physiology and distribution of anti-tumor CAR T cells in the tumor microenvironment (TME) requires comprehensive 3D imaging data. Commonly used techniques of classic histology and 2D microscopy are unable to meet this necessity. In this proof-of-concept study we present the strong capacity of 3D fluorescence microscopy with the UltraMicroscope for the analysis of CAR T cells in solid tumors ranging from millimeters to centimeters in size. This light sheet imaging system in combination with tissue clearing allows 3D visualization and quantification of multiple tumor parameters and CAR T cell infiltration at a cellular and even subcellular level.

Light sheet microscopy uses decoupled optical pathways for fluorescence excitation (horizontal) and detection (vertical) to provide optical sectioning. A laser light sheet perpendicular to the axis of observation is used to excite a single plane of a labeled sample (fig. 1). Illumination of only a thin layer of the z-axis results in high-quality 3D imaging of entire organs with only low and strictly localized photodamage and bleaching effects. Moving the sample along the z-axis through the light sheet produces a stack of images that can be combined to visualize large biological samples in 3D with excellent resolution.[1,2,3]

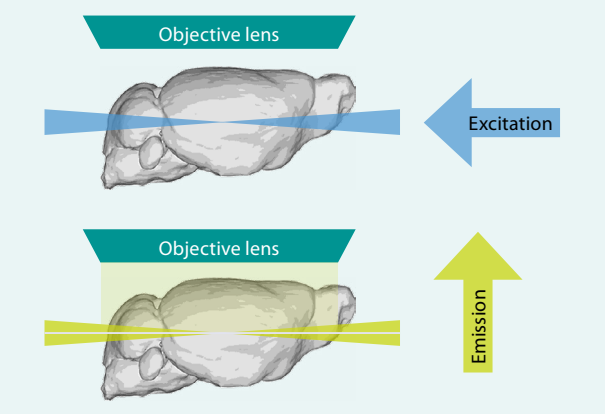

Figure 1: Samples are illuminated from the side by a focused light sheet while the fluorescence light is detected by a sCMOS camera perpendicular to the illumination plane. 3D image stacks are generated by moving the sample through the light sheet.

Materials and methods

3D light sheet imaging

The 3D light sheet imaging workflow was based on three steps (fig. 2), using specific fluorochrome-conjugated antibodies, clearing reagents, and an UltraMicroscope instrument. The UltraMicroscope family of instruments includes the UltraMicroscope II and the fully automated UltraMicroscope Blaze™.

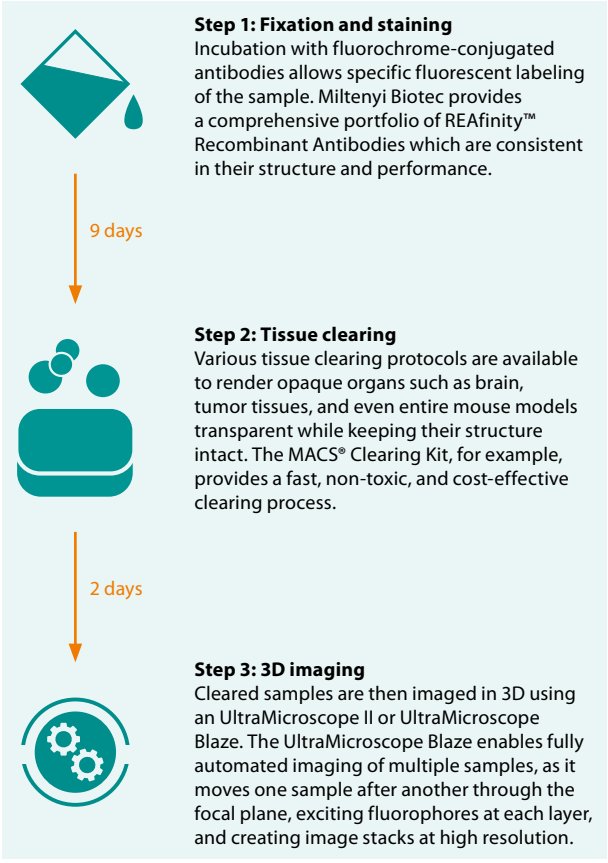

Figure 2: Large-scale 3D light sheet fluorescence imaging workflow.

Pancreas xenograft generation and imaging

Pancreas xenografts were generated by subcutaneous injections of GFP-expressing tumor cells into NOD scid gamma mice (NSG mice) obtained from Charles River Laboratories. After 14 days of engraftment, the animals were treated intravenously (i.v.) with 4×10^6 LNGFR$^+$ CD66c-CAR T cells (n = 2) or 4×10^6 LNGFR$^-$ mock T cells (n = 2). Twelve days after treatment, both groups were injected i.v. with 10 mg/kg of a CD271 (LNGFR) antibody (conjugated to Vio® R667) for detection of CD66c-CAR T cells. After 6 hours, 100 µg rhodamine-labeled lectin (50 µL) (Vector Laboratories) were injected i.v. for detection of the vasculature. After 5 minutes of incubation, the animals were sacrificed, tumors were excised and cleared according to the protocol of the MACS Clearing Kit. The cleared specimens were then imaged with the UltraMicroscope II. Samples were scanned with a diameter of up to 5 mm at a voxel step size of 4 µm. Excitation and emission wavelengths of the fluorophores were as follows: Vio R667: excitation max. 647 nm, emission max. 670 nm; rhodamine-lectin: excitation max. 550 nm, emission max. 575 nm; GFP: excitation max. 395 nm, emission max. 488 nm.

LEARN MORE

For more information, find our application protocol "Immunostaining and clearing of primary human tumors and patient-derived xenografts for 3D imaging analysis" on

▸ **miltenyibiotec.com/clearing-tumors**

Results

Light sheet imaging with prior tissue clearing enabled an extensive 3D analysis of large pancreatic carcinoma xenografts. 3D data from rhodamine-lectin–labeled samples showed chaotic, irregular, and highly branched vascular structures that are typical of an angiogenic vasculature within a tumor with a high level of heterogeneity. Compared to the mock-treated xenograft the CAR T cell–treated tumor appeared to have a greater vascularization level (fig. 3A).

Moreover, the CD271 (LNGFR)-Vio R667 signals indicated that CAR T cells did not migrate deeply into the tumor (fig. 3B, left). Instead, CAR T cells accumulated in the tumor periphery, which confirms previous findings using 2D fluorescence microscopy and the reported lack of a therapeutic effect.[4,5]

CD271 (LNGFR)-Vio R667 signals in mock-treated tumors were found only near necrotic areas (fig. 3B, right), thus indicating leakage of the labeling agent due to the enhanced permeability and retention (EPR) effect. Necrosis was evaluated based on the lack of GFP expression (not shown) and the extent of vascularization. Lack of labeling in the tumor periphery indicates specificity of the CD271 (LNGFR)-Vio R667 antibody.

Figure 4 shows a 3D rendered image of another pancreatic xenograft. Staining of vasculature and infiltrating CAR T cells was performed as indicated for figure 3. Expression of GFP in tumor cells is shown additionally.

Thanks to the wealth of comprehensive and detailed 3D data, it is now possible to quantify CAR T cell numbers, their distribution within the tumor regions, and their distance from the closest vessel (figs. 3, 4, and video).

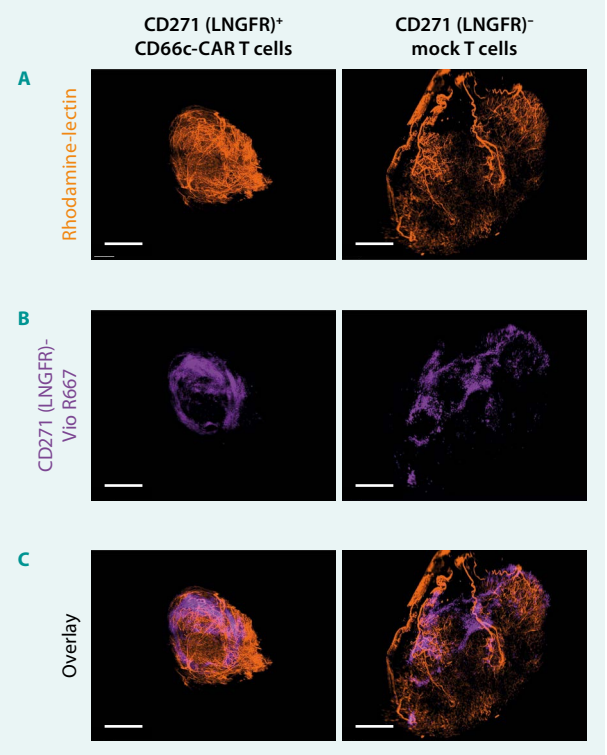

Figure 3: 3D imaging of CD66c-CAR T cells and vasculature in a pancreatic carcinoma xenograft using the UltraMicroscope II. (A) Tumor vasculature network, including feeding vessels, labeled with rhodamine-lectin. (B) CD66c-CAR T cells labeled with a CD271 (LNGFR)-Vio R667 antibody. (C) Overlay. Scale bar: 500 µm.

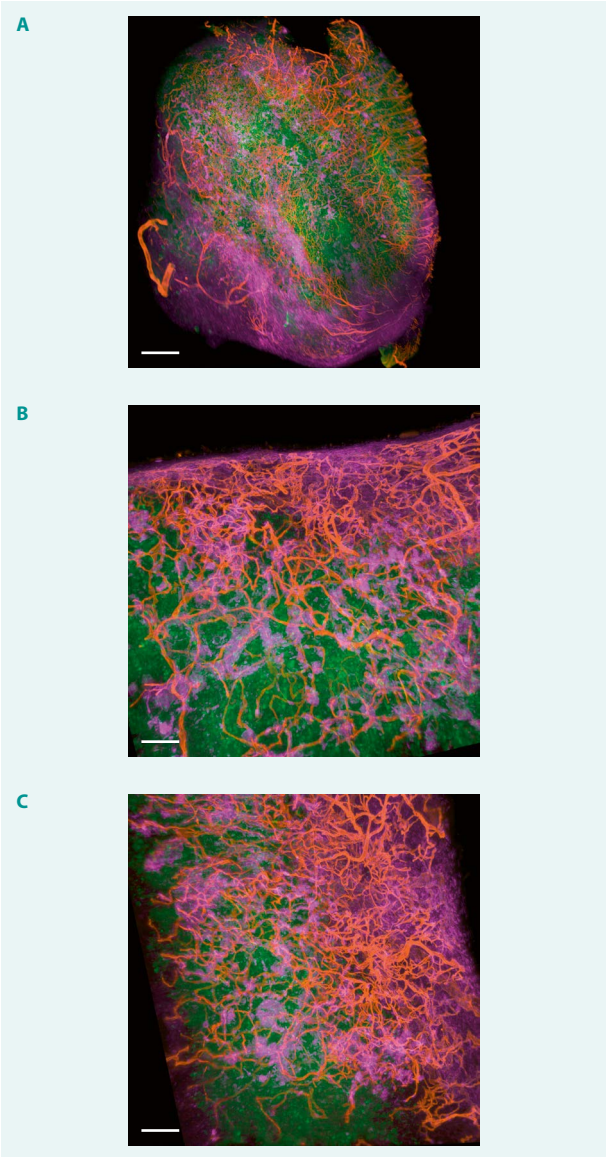

Figure 4: 3D rendering of a pancreatic carcinoma xenograft. GFP-expressing tumor cells are shown in green, infiltrating CAR T cells in purple, and the vasculature in orange. Imaging was performed using an UltraMicroscope II. Staining was performed as indicated for figure 3. (A) Overview of the xenograft; scale bar 400 µm. (B, C) Detailed view; scale bar 100 µm.

VIDEO

Watch our video showing a 3D rendered "flight" through a pancreatic carcinoma xenograft. Imaging was performed using an UltraMicroscope.

▸ **miltenyibiotec.com/pancreas-carcinoma-video**

Conclusion

- Large-scale light sheet fluorescence imaging with the UltraMicroscope enables high-resolution 3D visualization of solid tumors and their microenvironment.
- This platform allows quantification of multiple tumor parameters and CAR T cell infiltration at subcellular resolution.
- Comprehensive 3D analysis enables the evaluation of potential cellular therapies in organs and large tissues in immuno-oncology research.
- Higher throughput of multiple samples can be achieved thanks to the batch measurement of the fully automated UltraMicroscope Blaze.

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