

Development of a multi-scale and multi-modality imaging system to characterize tumours and their microenvironment in vivo.

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ABSTRACT

In vivo high-resolution imaging of tumor development is possible through dorsal skinfold chamber implantable on mice model. However, current intravital imaging systems are weakly tolerated along time by mice and do not allow multimodality imaging. Our project aims to develop a new chamber for: 1- long-term micro/macrosopic visualization of tumor (vascular and cellular compartments) and tissue microenvironment; and 2- multimodality imaging (photonic, MRI and sonography).

Our new experimental device was patented in March 2014 and was primarily assessed on 75 mouse engrafted with 4T1-Luc tumor cell line, and validated in confocal and multiphoton imaging after staining the mice vasculature using Dextran 155KDa-TRITC or Dextran 2000kDa-FITC. Simultaneously, a universal stage was designed for optimal removal of respiratory and cardiac artifacts during microscopy assays. Experimental results from optical, ultrasound (B-mode and pulse subtraction mode) and MRI imaging (anatomic sequences) showed that our patented design, unlike commercial devices, improves longitudinal monitoring over several weeks (35 days on average against 12 for the commercial chamber) and allows for a better characterization of the early and late tissue alterations due to tumour development. We also demonstrated the compatibility for multimodality imaging and the increase of mice survival was by a factor of 2.9, with our new skinfold chamber.

Current developments include: 1- defining new procedures for multi-labelling of cells and tissue (screening of fluorescent molecules and imaging protocols); 2- developing ultrasound and MRI imaging procedures with specific probes; 3- correlating optical/ultrasound/MRI data for a complete mapping of tumour development and microenvironment.

Keywords : intravital imaging, skinfold chamber, multi-modality imaging, cancer, parametric characterization

1. INTRODUCTION

In situ observation of early changes in vasculature and its microenvironment at cell level is an essential step in understanding and characterization of tumour physiopathology and development of new treatments. This direct observation is achieved by implementation of dorsal skin fold chambers in vivo. Simultaneous combination of fluorescent vital markers can be used to observe various biological processes [1]. Thus, intravital microscopy has proven its worth for several years in many applications such as: 1 molecular imaging (enzyme activity, gene expression) [2]; 2- cell imaging (cell movements and interactions) [3]; 3- anatomical imaging (the vascular network architecture) (4); 4- functional imaging (circulatory hemodynamics) [5]; 5- evaluating therapies [6,7]. Coupled to image processing algorithms, visual information is completed by mapping quantitative parameters such as density, architecture, the flow resistance and vascular permeability in the context of hemodynamic studies e.g.

Commercial (APJ Trading®) or home made [8,9] devices are currently available but they are only tolerated by animals over 15 days on average before the appearance of skin lesions requiring animal sacrifice. As a result, exploration of some

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slow-growing tumour models cannot be achieved. In addition, late changes occurring in tumor development can not be characterized as some drugs whose efficiency has been proven only for advanced tumoral stages. Our aim was to develop an intravital imaging system allowing long term follow up over several weeks and compatible for multimodality analysis allowing for photonic, MRI, and ultrasound imaging.

2. METHODOLOGY

New dorsal chamber design

The patented model consists in two plates and circular rings manufactured in a white or black PVC material (white version presented on figure 1). Each plate has an upper part with a 12 mm circular opening in their center for receiving the glass coverslip required for microscopic observations. These circular openings are surrounded with a set of holes through which passed the sutures in order to fix and secure the two plates on the back of the animal. The lower parts are 45° reclining side panels ensuring the stability of the system on the back of mice. The plates dimensions are 18 mm in width and 22 mm in height.

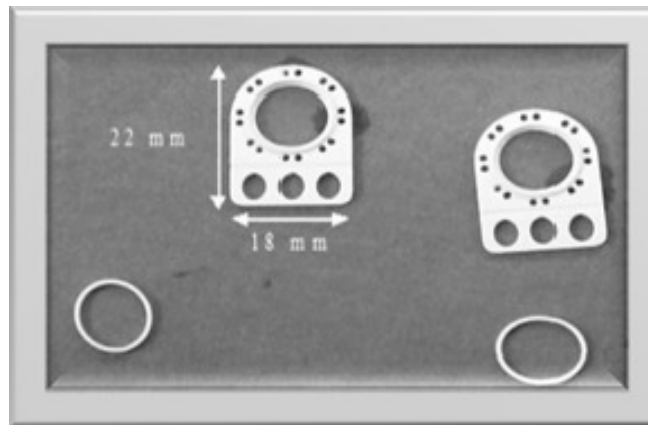


Figure 1: Picture of the twin parts of the new dorsal chamber.

Surgical procedure

All animal experiments and procedures were reviewed and approved by the French Ministry for Animal Care and Use. Nude mice are anesthetized by inhalation of a 1.5-2% isoflurane with 2L/min air flow during all the surgical procedure. After skin disinfection with Betadine and a 100 μ L subcutaneous injection of Lidocaïne, the two plates are placed face to face on either side of a skin fold on the back of the animal. Needles are passed through two adjacent holes to fix the plates together on the skin fold. Thus, eight sutures are realized. On one side, an incision is realized all along the circumference of the circular opening in order to totally remove all skin thicknesses. Tumour cells are then injected in intradermal under a binocular microscope before positioning the coverslip. The coverslip is latter permanently fixed by mechanical pressure exerted by the circular rings which have been clipped forcefully. Finally, a 4mg/kg dose of Tolfedine is subcutaneously administrated to prevent inflammation. Mice are then housed individually in cages in a temperature and humidity controlled environment with free access to food and water.

Tumor cells

First experiments were conducted with the IMR32-GFP-Luc cell line. Because of its too low engraftment rate, the 4T1-Luc or 4T1-Dendra2 mammary cancer cells lines were used in a second time to generate tumor growth in the dorsal chamber. A 20 μ L solution containing 50 000 cells suspended in PBS was injected per mouse. Viability of tumor cells was controlled before and after the surgical procedures by flow cytometry in order to assess the variability in tumor growth between mice surgically operated at the beginning or at the end of the protocol.

Imaging

For all imaging modes, mice were maintained anesthetized by isoflurane inhalation. Hypothermia was prevented by monitoring the temperature in the local environment of the animal.

Photonic imaging

Each mouse was examined twice a week on a fluorescent microscope (AZ100M, Nikon®) and on a multiphoton/confocal microscope (SP8 Leica®) on the same day. As microscopic observations require a high stability of the field of view, a new device has been designed specifically for the dorsal chamber, which can be adapted to any microscope stage. Thus, cardiac and respiratory movement's artefacts have been avoided during microscopic recordings. Two fluorescent dyes at different molecular weight were used for enhancing vasculature: Dextran 155KDa-TRITC or Dextran 2000kDa-FITC. Both have been administrated intravenously through a 100 µL volume at 0.05 mg/µL concentration diluted in PBS. Macroscopic visualization of the entire surface under the 12mm diameter coverslip was first done on the Macroscope in brightfield and fluorescence modes, while focalized regions have been then characterized at the cell level under the confocal. For whole tumour representation, confocal mosaics have been also reconstructed based on scanned regions focused all around the tumour center.

Ultrasonographic imaging

Sonographic examinations have been performed using an Aplio XG (Toshiba®) with a 12 MHz linear probe. Mice were examined in: 1- B mode for anatomical detection of tumour margins; 2- Power Doppler mode (maximum velocity at 3.9 cm/s) for detection of vascular flow in macrovessels on the entire volume by mechanical displacement of the probe; 3- and Pulse Subtraction mode (PS low, IM <0.1) after a bolus injection of 100 µl of Sonovue (Bracco®) in the retroorbital sinus. For the PS mode, vascular signal enhancement produced by microbubbles was recorded in raw data on the maximal longitudinal and transversal section of the chamber.

Magnetic Resonance Imaging

Magnetic resonance imaging was performed on a 7 Tesla NMR scanner (Bruker®). A specific holder has been fabricated by 3D printing, fitting the sample dimensions in order to place it in vertical position at the center of the 7 T magnet. For spin excitation, we used a commercially available volume coil dedicated to whole body mouse imaging. For signal reception, in order to enhance the detection sensitivity, a small home-made surface coil (12 mm in diameter) was used. The surface coil is inactivated during signal transmission. For imaging experiments, the surface coil is placed against the coverslip on the visible side of the tumour and fixed onto the dedicated holder. 2D and 3D TurboRare and Flash anatomical images were acquired for different sequences parameters (pulse intensity, echo and repetition times, and slice thickness) in order to optimize signal detection and thus tumour characterization.

3. DATA, RESULTS

New dorsal chamber validation

Our model was evaluated in terms of: 1- mortality during surgery; 2- time required for its implantation; 3- tolerance (time between day of surgery to the sacrifice of the animal). These results, obtained on 75 mice for our system, were compared to data obtained with a commercial device (APJ trading®) grafted on 17 mice. The results are summarized in table 1.

	Weight	Mortality	Time of surgery	Tolerance
Prototype	0.92 g	9 %	20 min	35 days (min: 13; max: 79)
Titane device	2.14 g	23 %	67 min	12 days (min: 6; max: 20)

Table 1: Comparison between data obtained with our chamber model compared to commercial devices

As seen in table 1, the prototype is significantly lighter than the commercial one which explains in part the increased mice tolerance over time. Our chamber remains straight on the back of the animal all over the protocol whereas the titane one falls rapidly on one side inducing skin lesions and thus animal sacrifice. With our model, tumour necrosis development was possible in most cases and led to animal euthanasia whereas with other model the sacrifice of the animal was required because of reactions due to the chamber itself. As a consequence also, mice regained faster their original weight after surgery. A commercial and MRI compatible system was also tested but rapidly forsaken because of: 1- the constitutive material which was easily eroded by mice; 2- inability to graft tumour cells once the chamber fixed (constitutively solidarity coverslips); 3- degraded quality of optical images.

Imaging

Photonic imaging

Bioluminescence imaging- As bioluminescence is an efficient methodology to rapidly and quantitatively evaluate tumour engraftment and development, tumour detection inside the chamber was assessed with an IVIS50 system (PerkinElmer®). As shown on figure 2a, the white version generated some spontaneous signals overlapping tumour emission while the black version (fig. 2b) consequently considered, did not.

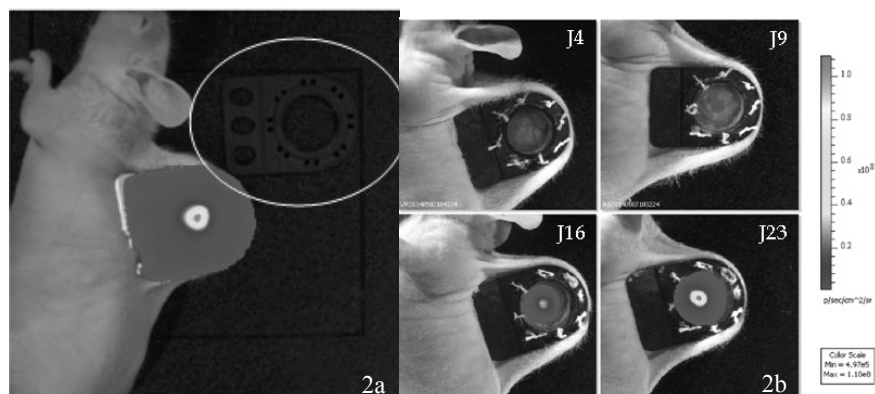


Figure 2: Bioluminescence evaluation of tumor development within the white (2a) and black (2b) version of our dorsal chamber. On Fig. 2a, a black plate can be seen inside the circle showing the absence of residual signals generated by the chamber material.

Fluorescence imaging- For whole tumour visualization, images were firstly acquired on the microscope with an 1x objective at 1.5 zoom. The images quality is at least equivalent to that obtained with the commercial model that we used as a reference to validate our prototype in photonic. It was so possible to follow up tumour development over time in an anatomical and functional point of view.

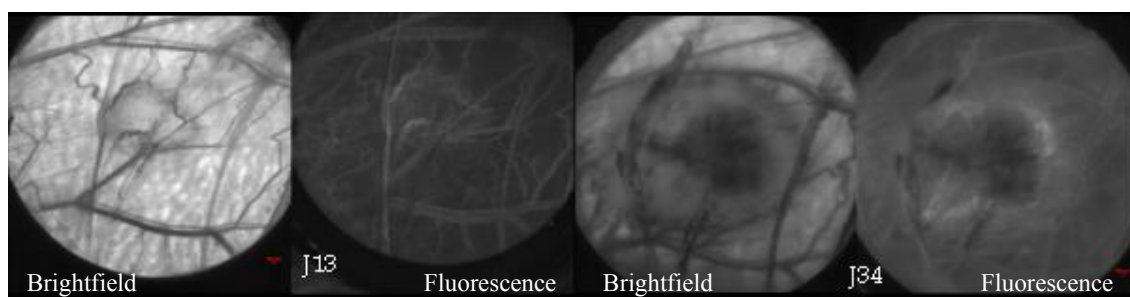


Figure 3: Examples of macroscopic images obtained in brightfield (left) and fluorescence after Dextran155kDa- TRITC injection (right) at two different stages of tumour development (Day 13 (J13) and Day 34 (J34)).

To access cellular events, mice were then examined under the confocal microscope Leica SP8. All images were acquired with a 5x objective with an optical aperture of 0.15 and a working distance of 13.7 mm. Examples of confocal images are given in figure 4 after reconstruction of mosaics from scanned areas as illustrated in Figure 5. Vascular rearrangement inside the tumour but also at the periphery within the healthy tissue can be clearly evaluated, as necrosis also, on the basis of these images. Moreover, tumour cells can be individualized along the vessels which is crucial when considering metastasis processes (cf. fig.5).

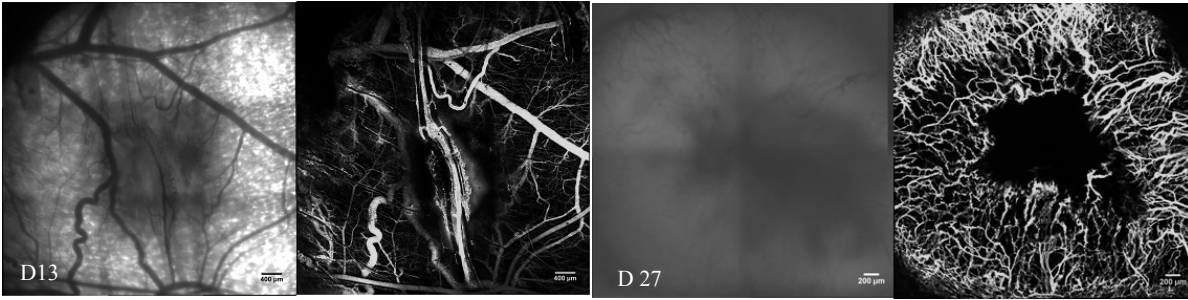


Figure 4: Examples of confocal images obtained in brightfield (left) and fluorescence (right) at two different stages of tumour development: Day 13 and D27 post tumour cells engraftment.

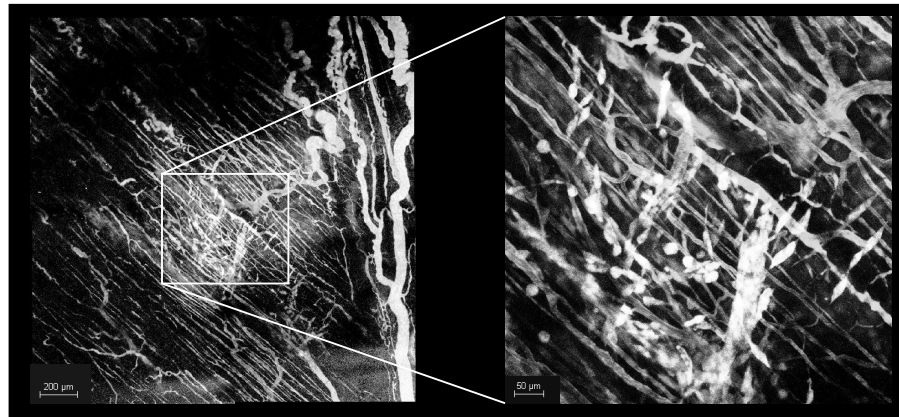


Figure 5: Confocal images of a 4T1-Dendra2 tumour, 9 days post engraftment. Vessel signal was enhanced by an injection of Dextran155kDa- TRITC, appearing in dark gray while tumour cells can be identified in light gray.

Ultrasonographic imaging

As multimodality needs long and repetitive anaesthesia with significant consequences on physiology and potentially death, main efforts have been concentrated on developing and optimizing photonic protocols. As a consequence, sonographic explorations were conducted on some few mice without longitudinal follow up during tumour development. First investigations allowed preliminary conclusions illustrated by figures 6 and 7: 1-Transversal sections are more polluted than longitudinal ones by multiple reflexions of ultrasound signals on the two coverslips only separated by a millimetric gap whereas longitudinal sections allow increased exploration depth; 2- as for Doppler or PS mode, vascularization was poorly detected probably due to low circulating flows and attenuation of ultrasound by material. Raw data are currently quantified in order to exhort some potentially ultrasound signal enhancement (cf. figure 7) inside the chamber even though this was not visually detected.



Figure 6: Anatomical B-mode images according to the transversal (left) and longitudinal (right) section.

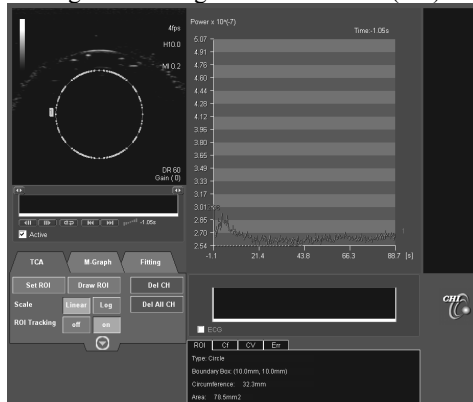


Figure 7: Functional image recorded in Pulse Subtraction mode.

MRI imaging

Before in vivo experiments, preliminary tests were performed on included prototypes in agar gels demonstrating an absence of MRI signals perturbation (images not shown). As MRI experiments were first conducted to optimize sequences, no longitudinal evaluation of tumour development was conducted for instance. Only one-shot acquisitions were done per mouse. For both TurboRare or Flash sequences, acquisition times for whole tumour scanning were acceptable considering animal physiology (around 20 minutes). Tumour delimitation versus healthy tissues and vascular recognition seemed to be more pronounced on TurboRare sequences (Figure 8). This last point has to be confirmed on multiple scans and especially for different tumour stages e.g. with different heterogeneities. Preliminary angiographic tests were done but no conclusive result was actually obtained probably due to low flow rates and inappropriate vessels orientations inside the MRI scanner.

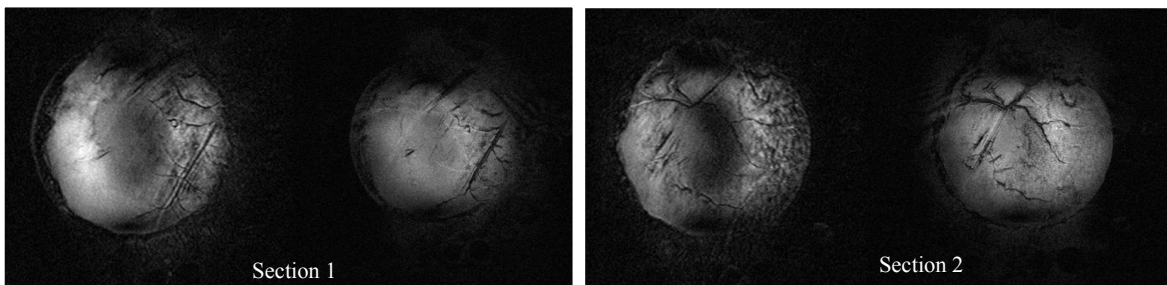


Figure 8: Examples of MRI images obtained with 3D-TurboRare (left) and 3D-Flash (right) sequences on the same tumour at two different sections. TurboRare parameters: Fov 16*16*1, 60 mm; Matrice: 256*256*10; 62*62*160 microns; TR/Te-eff/te=3000/72/9. Flash parameters: Fov 25*25*1, 60 mm; Matrice: 512*512*16; Resolution: 49*49*100 microns; TR/te=50/5,56 angle: 12°.

4. CONCLUSIONS

We have developed and validated a new prototype allowing long-term follow up for tumour characterization applicable to multiple imaging modalities. MRI and sonography acquisition protocols still need to be improved to enhance detection sensitivity of tissue and vasculature. Angiogenesis has been widely and efficiently approached in photonic but need to be completed by cellular markers for mapping different biological process during tumour growth. Qualitative information will then be strengthened by quantitative parameters from algorithms currently under development.

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