Fluorescence lifetime imaging to differentiate bound from unbound ICG-cRGD both *in vitro* and *in vivo*

Paulien L. Stegehuis[a], Martin C. Boonstra[a], Karien E. de Rooij[a,b], François E. Powolny[c], Riccardo Sinisi[c], Harald Homulle[c,d], Claudio Bruschini[c], Edoardo Charbon[c,d], Cornelis J.H. van de Velde[a], Boudewijn P.F. Lelieveldt[a,d], Alexander L. Vahrmeijer[a], Jouke Dijkstra[a], Martijn van de Giessen[a]

[a] Leiden University Medical Center, Leiden, the Netherlands, [b] Percuros B.V., Enschede, the Netherlands, [c] Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland, [d] Delft University of Technology, Delft, the Netherlands.

ABSTRACT

Excision of the whole tumor is crucial, but remains difficult for many tumor types. Fluorescence lifetime imaging could be helpful intraoperative to differentiate normal from tumor tissue. In this study we investigated the difference in fluorescence lifetime imaging of indocyanine green coupled to cyclic RGD free in solution/serum or bound to integrins e.g. in tumors. The U87-MG glioblastoma cell line, expressing high integrin levels, was cultured to use *in vitro* and to induce 4 subcutaneous tumors in a-thymic mice (n=4). Lifetimes of bound and unbound probe were measured with an experimental time-domain single-photon avalanche diode array (time resolution <100ps). *In vivo* measurements were taken 30-60 minutes after intravenous injection, and after 24 hours.

The *in vitro* lifetime of the fluorophores was similar at different concentrations (20, 50 and 100μM) and showed a statistically significant higher lifetime ($p<0.001$) of bound probe compared to unbound probe. *In vivo*, lifetimes of the fluorophores in tumors were significantly higher ($p<0.001$) than at the control site (tail) at 30-60 minutes after probe injection. Lifetimes after 24 hours confirmed tumor-specific binding (also validated by fluorescence intensity images).

Based on the difference in lifetime imaging, it can be concluded that it is feasible to separate between bound and unbound probes *in vivo*.

Keywords: Fluorescence lifetime imaging, ICG-cRGD, U87-MG, *in vitro*, *in vivo*

1. INTRODUCTION

Accurate intraoperative detection and imaging of tumors remains difficult and time consuming. It is important to excise the whole tumor to preclude local recurrence; however, surgeons rely mainly on palpation and visual inspection. Fluorescence intensity imaging using near infrared (NIR) tumor targeting probes is a technique that can guide surgeons and improve discrimination between tumor and normal tissue[1,2]. Unfortunately autofluorescence hampers the detection of fluorescence and can cause misinterpretation, and patients have to be injected a few hours in advance [3]. Therefor we use another fluorescent characteristic in this paper: fluorescence lifetime.

Fluorescence lifetime of a fluorophore is the time a molecule resides in the excited state before returning to the ground state through fluorescence emission. The lifetime depends on the environment and its affinity to other molecules, and is relatively insensitive to fluorophore concentration [4,5]. This affinity to other molecules could be exploited by linking the fluorophore to a peptide which binds to receptors overexpressed in tumor tissue. The optical probe binds in tumor tissue, but not (or to a much lesser extent) in normal tissue, resulting in differences in lifetime which should, ideally, be visible shortly after injection.

In this study we use indocyanine green (ICG), currently one of the two NIR fluorophores approved by the Food and Drug Administration for clinical use, coupled to cyclic RGD (cRGD)[6]. cRGD is a natural occurring peptide that binds to

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a variety of integrins (including $\alpha_v\beta_3$), known cancer biomarkers as they are overexpressed on angiogenic endothelial cells in various tumor types, including glioblastoma.

The purpose of this feasibility study was to investigate whether binding cRGD-ICG to integrins influences fluorescence lifetime of the probe and if so, whether it is measurable with our experimental set-up.

2. MATERIALS & METHODS

2.1 Imaging systems

The fluorescence lifetime imaging setup is described extensively in Powolny et al., 2013[7,8,9]. Briefly, the system measured photon arrival times using Single Photon Synchronous Detection (SPSD). This time-domain setup consisted of a photon sensitive sensor array and a picoseconds pulsed diode laser with a wavelength of 790 nm for sample illumination. The system had a per pixel timing resolution smaller than 100 ps. Two filters (excitation 775/50 nm (center/bandwidth), emission 845/55 nm) and a dichroic mirror (810 nm) delivered excitation illumination to the sample and emission light to the sensor (all from Chroma Technology Corporation, Bellow Falls, USA). The image was formed using a hemispheric lens.

High resolution fluorescence intensity scanning of the *in vitro* well plate experiments was performed with the Odyssey Infrared Imaging System (LI-COR) using the 800 nm channel. The PEARL imaging system (LI-COR) was used for *in vivo* fluorescence intensity imaging. A laser with an excitation wavelength of 785 nm was used. Exposure times are by default optimized during imaging.

2.2 Probe synthesis

In order to target integrin $\alpha_v\beta_3$, monomeric RGD (purity >98%) containing c(RGDfK) structures was conjugated by amide bond using a coupling reagent to the ICG-modified derivative. The excitation wavelength of ICG is 750 to 800 nm and it emits light with a wavelength over 800 nm.

2.3 *In vitro* experiments

The U87-MG glioblastoma cell line was cultured in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS; Greiner Bio One, Kremsmünster, Austria), 100U/ml penicillin and 100µg/ml streptomycin (Gibco). To optimise the FLIM set-up *in vitro*, U87-MG cells were seeded in a black 96-well plate at a density of $94x10^3$ cells/cm^2 and allowed to attach overnight. The following day the cells were incubated with a dose range of 10, 20 or 50 µM of cRGD-ICG in DMEM without phenol red containing 2% FCS at 37 ºC. After 1 hour, half of the wells were washed with DMEM/2% FCS, while in the other half the probe solution remained present during the measurements to enable discrimination between bound and unbound probe. After lifetime measurements the 96-well plate was imaged with the Odyssey scanner.

2.4 *In vivo* experiments

Six till eight week-old a-thymic female mice (CD1-Foxn1nu, Charles River Laboratories, l'Arbresle, France) weighing 25-35 g, were used. Throughout tumor inoculation and the measurement procedures, the animals were anesthetized with 4% isoflurane for induction and with 2% isoflurane for maintenance (flow of 0.5 L/min). All animal experiments were approved for animal health, ethics, and research by the Animal Welfare Committee of the Leiden University Medical Center. To induce subcutaneous tumors, U87-MG cells were injected at four sites on the back (1.5-3 million cells per spot) and tumor growth was monitored using a digital caliper. 30 Nanomol (50 µg) of cRGD-ICG was injected intravenously, when the tumors were fully grown. For the tail, a small additional cRGD-ICG bolus was injected subcutaneous to create a pocket of unbound probe. Fluorescence lifetime was measured 30-60 minutes and 24 hours after injection in two tumors at one side of the mice. Before measuring tumor, the skin above it was removed, to prevent the influence of fluorescence signal from the skin. After each lifetime measurement the mice were imaged with the PEARL imaging system to evaluate NIR signals.

2.5 Signal analysis

In the data analysis, performed with MATLAB (version R2014a), the measurements from "hot" and "dead" pixels were discarded. Subsequently lifetimes were estimated from the 1% of the pixels with the highest photon count. To calculate the fluorescence lifetime, we used a single-exponential model. In the acquisition protocol in this study, the time-curve was sampled at 165 points with 23 ps in between. After deconvolution with the impulse response function of the imaging system fluorescence lifetimes were estimated by iteratively fitting a single exponential function with an offset that accounts for background noise. Fluorescence lifetimes were computed for each selected pixel, as well as their mean, standard deviation and median. More details on the raw data characteristics can be found in an earlier description of the imaging system[8].

2.6 Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed with SPSS software (IBM SPSS statistics version 20). Variance between groups was first assessed via one way analysis of variation (ANOVA). Differences in means between two groups were assessed via the post-hoc Bonferroni test. $P < 0.05$ was considered statistical significant.

3. RESULTS

3.1 *In vitro* experiments

In vitro lifetimes of 20, 50 and 100 μM ICG-cRGD, were measured, in a solution (unbound probe), unwashed (bound and unbound probe) and washed (bound probe). Two measurements of the solution at every concentration were taken, and three measurements were taken of unwashed and washed wells. Figure 1 shows the measured lifetimes as a boxplot graph.

We found a similar lifetime at different concentrations; although there appears to be a trend of decreasing lifetime with increasing concentration, no statistical differences were found. The washed cells showed a significantly higher lifetime (382 ± 7 ps; $p<0.001$) than both the unwashed cells (354 ± 10 ps) and the solution (353 ± 6 ps). The fluorescence intensity images taken with the Odyssey showed a dose-dependent intensity, which was higher for the unwashed cells and solution, than in the washed cells.

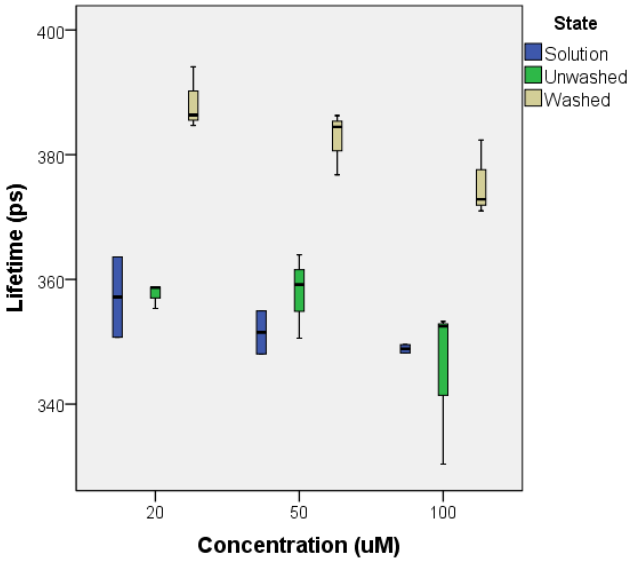

Figure 1. Mean lifetimes of *in vitro* experiments grouped by state. Similar lifetimes were found for unwashed cells and the solutions. Lifetimes of washed cells differed significantly from both other groups.

3.2 *In vivo* experiments

Four mice with 4 tumors each were imaged 3 times at the tail (unbound probe) and 2 tumors (bound probe) 30-60 minutes after injection. After 24 hours, the tail and the other 2 tumors were imaged in the same way. Figure 2 shows an intensity plot and the corresponding lifetime plot. Figure 3 shows the mean lifetimes of the tumors and tails grouped together for all mice at both points in time.

Lifetimes in tumors were significantly higher (409 ± 5 ps; $p<0.001$) (n=24) than in the tail (389 ± 13 ps) (n=12) for measurements shortly after injection. After 24 hours, lifetimes were still significantly higher ($p=0.019$) for tumors (428 ± 86 ps; n=24) than for measurements at the tail (366 ± 11 ps; n=12), although for tumors, the standard deviation became much larger. The lifetime of tail measurements after 30-60 minutes was significantly lower ($p<0.001$) than those after 24 hours. For tumor measurements we found no significant differences over time. The PEARL fluorescence intensity images showed high intensity at the tumor locations, the kidneys and the tail (near the injection spot). After 24 hours, the tumor-to-background ratio was higher than it was 1 hour after injection, due to the wash-out of ICG.

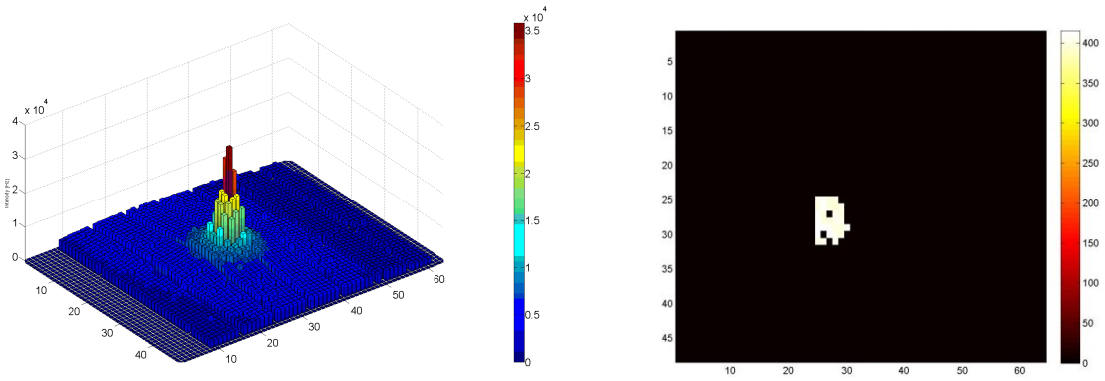

Figure 2. The left image is an intensity plot of a tumor (in photon counts per pixel per second), the right image shows the corresponding lifetimes per pixel (in ps).

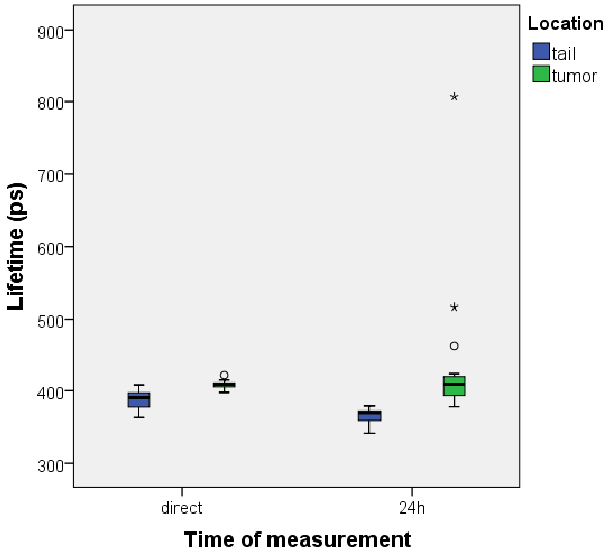

Figure 3. Mean lifetimes of *in vivo* measurements shortly after injection and after 24 hours both at the tail and tumor. The measurements of all mice are grouped together.

4. DISCUSSION AND CONCLUSION

With this pilot study we demonstrated the feasibility of the experimental time-domain single-photon avalanche diode array to separate bound and unbound probes *in vivo* and *in vitro*. In addition we have shown that, within the measured range *in vitro*, the lifetime is not dependent on the concentration of the probe.

In vitro we found that bound probe has a higher lifetime than either the combination of bound and unbound probe and just unbound probe. We did not find a difference in lifetimes between the latter; in the unwashed sample there may have been much more unbound probe present than bound probe. *In vivo* we also observed a higher lifetime of bound probe in the tumor than unbound probe in the tail. The lifetime elongation is reported prior in literature, and one explanation could be the binding to serum albumin[5,10].

The mouse tail was used as a control site, because it does not contain integrins, preventing the probe from binding. The large difference in binding characteristics of tumor and control site was advantageous for this feasibility study. For this technique to be of additional value in the clinic, smaller differences in the ratio between bound and unbound probe need to be differentiated. To gain further evidence for the feasibility of *in vivo* fluorescence lifetime imaging, we are currently working on larger studies, that include different tumor targeting fluorescent agents.

A limitation of our current fluorescence lifetime imaging setup is long acquisition times, in the order of two minutes per sample. This is due to low photon detection efficiency (PDE) of the current sensor and due to an oversampling of the exponential curve. On the sensor side, improvements in design are likely to increase the PDE with at least 2 orders of magnitude. Together with improvements in acquisition strategy (less oversampling) we expect the acquisition time to be reduced below a second in the near future.

Despite current limitations in imaging, this study has shown that *in vivo* differentiation between tumor tissue and other tissue is feasible based on macroscopic fluorescence lifetime imaging. This may be a promising next step in fluorescence guided surgery.

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