

## Application Note

# Fluorescence Lifetime Imaging (FLIM) based analysis of lipid organization in hepatocytes using the MicroTime 200



**Benedikt Krämer, Felix Koberling, PicoQuant GmbH  
Astrid Tannert, Thomas Korte, Andreas Hermann, Humboldt University Berlin,  
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## Introduction

This application note describes the usage of the time-resolved confocal fluorescence microscope MicroTime 200 [1] for Fluorescence Lifetime Imaging (FLIM) studies in order to assess the organization of lipids in cell compartments.

An important function of the liver is the secretion of bile fluid by hepatocytes into canalicular spaces [2]. The bile fluid consists of micelle forming bile salts, phospholipids and cholesterol. The aim of this study was to determine the molecular order of these lipids in the canalicular lumen relative to other cell compartments like the cell membrane.

The differentiation between micellar and bilayer type lipid environments in liver cells (hepatocytes) is possible by characteristic changes in the fluorescence lifetime of the dye Nitrobenzoxadiazole (NBD), which thus can be used to probe local physical properties. Accordingly, we imaged NBD labelled phospholipids (N360 [3]) in living hepatocytes originating from a cultivated cancer cell line (HepG2) which form canalicular vacuoles.

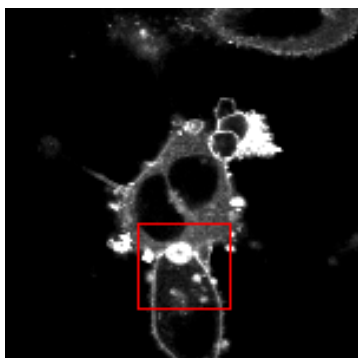
## Experimental Details

For fluorescence excitation a pulsed picosecond diode laser with an output wavelength of 468 nm was passed through a narrow band clean-up filter and reflected with a dichroic beam splitter centered at about 485 nm into a standard inverted microscope base. Here, a high numerical aperture objective (100x, NA 1.3, oil immersion) focused the light to a sub-femtoliter excitation volume inside the sample. To image a region, the sample was raster-scanned with a X,Y,Z - piezo driven device through the excitation focus. The acquisition time was set to 90 seconds. Fluorescence from excited molecules was collected with the same objective, filtered after the dichroic with an additional longpass filter to reject remaining scattered laser light and passed through a small aperture to enable confocal detection. After passing a sample specific longpass filter transmitting wavelengths greater 500 nm, the fluorescence was detected with an avalanche photo diode using the method of time-correlated single photon counting (TCSPC).

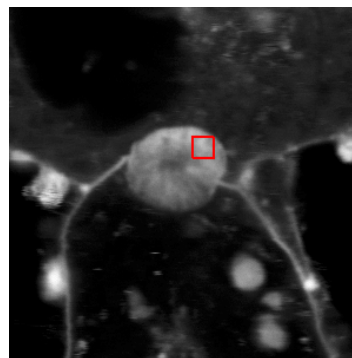
The data acquisition was done by the TimeHarp 200 TCSPC PC-board working in the special Time-Tagged Time-Resolved Mode, which stores all relevant information for every detected photon for further data analysis [4], i.e. the photon arrival time at the detector relative to the corresponding laser excitation pulse, the position of the sample and the number of the detection channel.

## Measurement results

First the sample was scanned at low resolution in order to define special regions of interest. These regions were then scanned again at higher resolution. The results of these two scans are shown in fig. 1a and 1b, displaying the measured fluorescence intensity in gray scale. These images are formed by integrating all collected photons in every pixel, thereby ignoring the temporal information.



*Figure 1a: Fluorescence intensity image of HepG2 cells labeled with NBD tagged phospholipids (80x80  $\mu\text{m}$ , 150 pixels/axis, 2 ms acquisition time/pixel).*



*Figure 1b: Enlargement of the red marked region in figure 1a. In the center, a canalicular vacuole is seen. The vacuole is enriched with fluorescent phospholipids. The small red rectangular is used to form and analyse a global histogram (see below). (21x21  $\mu\text{m}$ , 150 pixels/axis, 2 ms acquisition time/pixel).*

The next release of the MicroTime software (V 4.0) will also allow to generate a fluorescence lifetime image already during the measurement. However, though this lifetime image is already very useful for qualitative imaging purposes, it still needs to be analysed in detail for quantitative results.

## Calculation of the Lifetime Image

In general, the calculation of a fluorescence lifetime image is done by sorting all photons that correspond to one pixel into a histogram, which is then fitted to an exponential decay function to extract the lifetime information. This procedure is then repeated for every pixel in the image. As the fitting procedure relies on the quality of the start parameters for the fit, they are best extracted directly from the image. For this purpose all recorded photons inside the canalicular vacuole (red marked region in fig.1b) are used to form a global histogram, which was then analyzed (see fig. 2). In principle, the system software allows to fit the data to multi-exponential decay functions using tail-fitting as well as numerical deconvolution. However, in this case tail-fitting to a mono-exponential decay was found to be sufficient, giving small residuals and a  $\chi^2$  of 1.1.

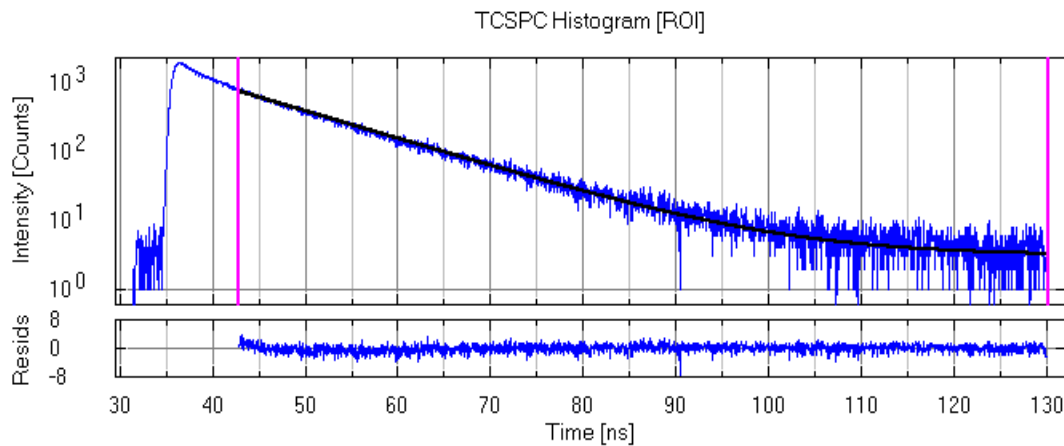


Figure 2: TCSPC histogram of photons detected inside the red region of Fig. 1b. The mono-exponential tail-fit (black line) for the fluorescence decay yields a lifetime of 10.7 ns and a  $\chi^2$  of 1.1.

Based on these results every pixel in the image was then analysed in the same way, but this time with a maximum likelihood estimator to account for parts with low signal-to-noise ratio. The final fluorescence lifetime image is shown in fig. 3 along with the lifetime distribution histogram and the corresponding color coding.

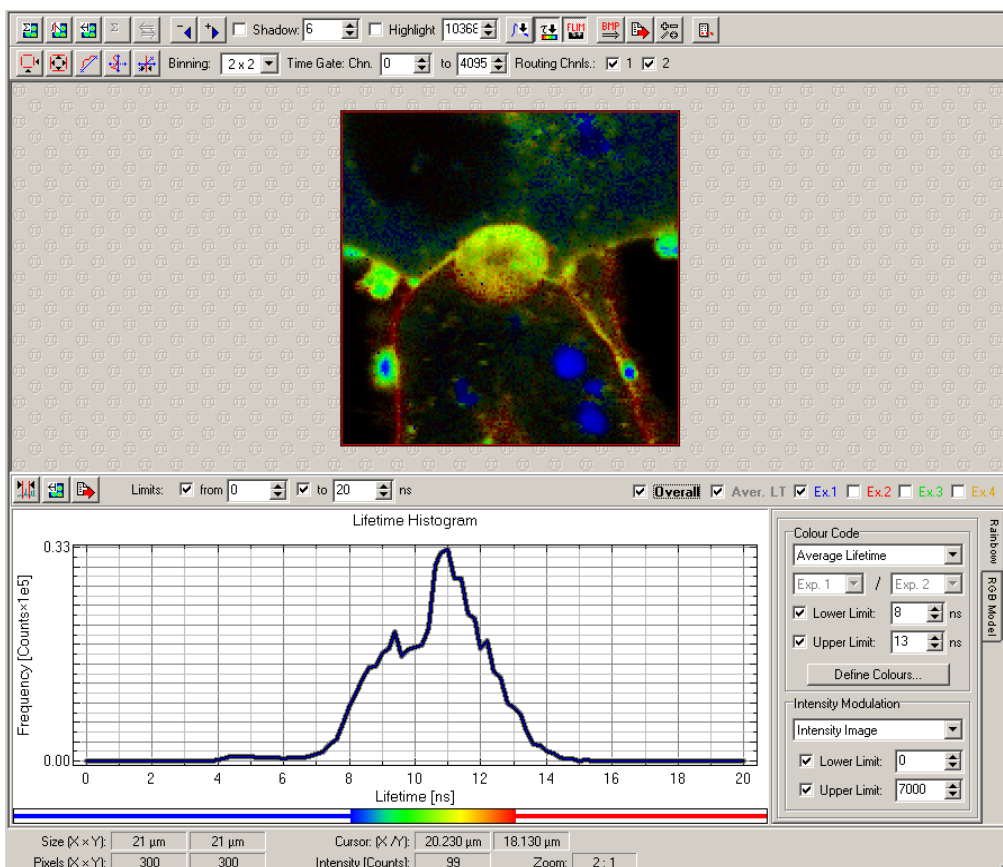


Figure 3: Fluorescence Lifetime Image of the image Fig. 1b (monoexp. tail-fit model with a maximum likelihood estimator applied to the photons in every image pixel separately). Long lifetimes (red) are found in the membrane of the cells while the cytoplasm exhibits lower lifetimes (green) indicating less ordered environment. The lowest lifetime values (blue) are found in vacuoles presumably due to destruction of the fluorescent lipid and cleavage of the NBD moiety. The canalicular space in the center of the image displays a relative long lifetime suggesting insertion of the lipid analogue into supramolecular structures.

The lipid bound NBD shows a broad range of lifetimes from approx. 8 ns up to 13 ns indicating different molecular environments of the fluorescent lipid inside the imaged hepatocyte sections. The longest lifetime of about 13 ns is found in the plasma membranes which are lipid bilayers. This relatively long lifetime can be explained by the fact, that these structures form a quite rigid environment with a low local water concentration. Therefore efficient quenching by water is prevented which could reduce the fluorescence lifetime.

A majority of pixels in the image has a lifetime of around 10 ns. In particular, such a lifetime can be observed for lipids in the cytosol (see fig. 3) and in the canalicular vacuole in the center of the image. This lifetime reduction, in contrast to the lifetimes of the plasma membranes, can be caused by a higher local water concentration and less rigid lipid structures enabling also enhanced collisional fluorescence quenching as it is typical for micelles. The lipid organization in the canalicular structure in the center of fig. 3 is therefore very likely of a bilayer type at the rim, resembling the canalicular membrane, whereas the canalicular lumen harbours micellar structures.

## Conclusion

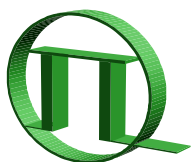
The presented results illustrate how the different molecular environments in various cell compartments can be investigated by fluorescence lifetime analysis. The results show a reduced fluorescence lifetime in the canalicular space between hepatocytes as compared to typical lipid bilayers like the plasma membrane. This indicates a less rigid and less hydrophobic lipid organization like in micelles.

Other possible applications for fluorescence lifetime imaging include:

- Lifetime analysis of autofluorescent cell domains
- Efficient discrimination of autofluorescence relative to fluorescent labels
- Differentiation of different fluorescent labels with equal spectral properties
- Measurement of intracellular pH of the aqueous medium in living cells
- Determination of local O<sub>2</sub> – concentration
- Measurement of proximity and concentration of quenching species
- Determination of calcium concentration inside living cells
- Time resolved Förster Resonance Energy Transfer (FRET) for:
  - Distance measurements on the nanometer scale
  - Quantitative measurements of environment-sensitive FRET probes

## Further reading

- [1] Wahl, M., Koberling, F., Patting, M., Rahn, H., Erdmann, R. , Curr. Pharm. Biotech., Vol. 5, p. 299-308 (2004)
- [2] Tannert, A., Wüstner, D., Bechstein, J., Müller, P., Devaux, P. and Herrmann, A., J. Biol. Chem., Vol. 278, p. 40631-40639 (2003)
- [3] Handbook of Fluorescent Probes (<http://www.probes.com/handbook/>)
- [4] Wahl, M., Erdmann, R., Lauritsen, K., Rahn, H.-J., Proc. SPIE, Vol. 3259, p. 173-178 (1998)



**PICOQUANT**  
Unternehmen für optoelektronische  
Forschung und Entwicklung

PicoQuant GmbH  
Unternehmen für optoelektronische Forschung und Entwicklung  
Rudower Chaussee 29 (IGZ), 12489 Berlin, Germany  
Telephone: +49 / (0)30 / 6392 6560  
Fax: +49 / (0)30 / 6392 6561  
e-mail: [photonics@pq.fta-berlin.de](mailto:photonics@pq.fta-berlin.de)  
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**Information:**  
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