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Cars as a Method for the Detection of Toxic Pollutants in MIBA: The Case of Phthalates on Danio renio's Larva

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Abstract

Living organisms (eggs and larvae of Danio rerio) have been incubated in an innovative microincubator MIBA with various concentrations of Di-N-Butyl Phthalate (DBP) derivatives for different times. Images were acquired with a macromultiphoton or CARS microscope and probe DBP (control) and its derivative N3-aDBP were detectable thanks to the signal emitted by the alkyne and a signal possible thanks to the auto-fluorescence of the aromatic ring. Using fluorescence imaging and CARS microscopy methods, we investigated the value of Raman-CARS that could vibrate unnatural groups (N3 or C≡C) previously grafted onto the DBP molecule in zebrafish. Contrary to other products, N3-aDBP probe has been detected continually by macro-fluorescence in larva but CARS signal was only detected in FLIM mode (Fluorescence Lifetime Imaging Microscopy). To conclude, CARS microscopy can be used to image toxic pollutants even by integrating photo-activatable probes. In our case, photo-physical reactions following the irradiation of the probe lead to new photoproducts that are not identified and difficult to characterize in fluorescence and specific CARS signal.

Introduction

Phthalates have an impact on the environment, as they are one of the most persistent and frequently detected pollutants in environmental analyses and are often described as endocrine disruptive chemicals that can affect reproduction and development (Endocrine-disrupting chemicals, EDCs). A new method for the efficient and rapid detection in aquatic organisms of phthalate derivatives, specifically Dibutyl Phthalate (DBP) is sought with transparent eggs and larvae of *Danio* rerio (zebrafish) [1,2] in multiway microincubator MIBA as a popular model organism to evaluate environmental toxic effects. In this work, we plan to detect specifically various concentrations of Di-N-Butyl Phthalate (DBP) derivatives for different times using fluorescence imaging and CARS microscopy methods [3].

Materials and methods

Microscope and macroscope setup

The Leica SP8-CARS microscope. (Leica Microsystems) is an optimized setup for the detection of CARS signal, and combined with a PicoEmerald OPO IR laser (80Mhz, a 1064 nm line and a beam accordable between 780 nm and 943 nm) and a FLIM workstation (PicoQuant. Gmbh, TCSPC) which will allow to perform CARS and its time-correlated signal. The SP5-CFS Macromultiphoton (MacroMP) is a prototype designed by Dr. Dominique Dumas and Sébastien Hupont [4,5]. The multiphoton mode was useful for a complete view of eggs or larvae with its low magnification objectives (10, 5, 2 or 0.5 fold) and epi-fluorescence mode (dichroic cubes I3: blue/green, N21: green/red, A4: UV/blue) for best optical compromise with selected irradiation (multiphoton laser or lamp light). To optimize CARS signal with range of pump wavelength, a cube CARS 2000 was used from 780 nm to 880 nm and a Cube CARS 1200 from 880 nm to 943 nm. Two ranges of Raman values are expected for this molecule (from 2,100 cm^{-1} to 2,260 cm^{-1} and from 3,270 cm^{-1} to 3,330 cm^{-1}).

Modified phthalate probes

The first probe to have been created (Bioactive Molecules laboratory of the UMR 7199 CNRS in Strasbourg) is called N3-aDBP, due to the grafting of two alkynes, thus two carbon-carbon (-C ≡ C) triple bonds, and an aryl azide (N_3). This N_3 is conjugated with the aromatic of the starting molecule, DBP. This probe has auto-fluorescence from the aromatic ring, alkyne fluorescence, and fluorescence from N_3 which is a photo-activatable component and highly sensitive to oxidation.

Sample preparation

A protocol was set up by placing eggs in a LabTek plate embedded in MIBA and incubated in the presence of N3-aDBP (250 nM) for a minimum of 2 hours. MIBA is an biomedical device for incubating under variable conditions with a thermostation, and two transparent sides for observing macroscopy and microscopy in real time (Biositech.com, Frouard, France). For longer incubations, from 2 to 10 days, the concentration of N3-aDBP was decreased to 25 nM for contact with zebrafish eggs and embryos in MIBA. Both modes could be used, the fluorescence mode to reveal N3-aDBP by activating it, and the multiphoton mode to detect CARS and FLIM signals in the sample eggs. Observations were made daily against a control in the absence of N3-aDBP with aDBP as positive control [6].

Results

After verifying that the natural DBP probe did not emit a CARS signal, tests were performed on the two modified probes. Auto-fluorescence signal was found on the other two compounds N3-aDBP and aDBP, with a CARS detect ion that is conjugated over the same emission bandwidth (530-680 nm). Under these conditions, fluorescence signal has been quenched as a function of the irradiation time using the laser source. Strong CARS signals have previously been found on the aDBP solution in the range of 3,080 to 3,300 cm^{-1}. The acquisitions of these larvae in multiphoton mode (at 815 nm) on the macroscope show more intense signals for N3-aDBP.

Discussion

These probes were brought into contact with fertilized eggs or young Danio renio larvae in order to carry out the CARS detection using multiphotonic infrared lasers, allowing a non-invasive detection with high penetration into organisms. The experiments were able to follow a characteristic signal (wave number value for N3-aDBP by CARS) but none of the multiphoton experiments were able to discriminate the fluorescence of N3-aDBP from controls (DBP and aDBP).
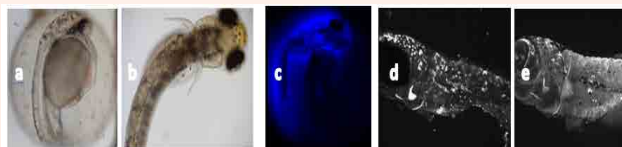
Figures 1: x10 objective in MIBA.
(a)   Danio renio egg
(b)   Larva
(c)   Observed in white light, by macromultiphoton N3-aDBP fluorescence
(d)   CARS for control
(e)   Incubated with N3-aDBP larvae

Conclusion

Some additional studies are needed to conclude on modified phthalate probes when the derivative product has an unknown mode of bioavailability [7] and bioproducts could be formed under irradiation by photo-physical reactions with some unidentified components that are difficult to identify in fluorescence.

References

1.   Ankley GT, Johnson RD (2004) Small fish models for identifying and assessing the effects of endocrine-disrupting chemicals. ILAR Journal 45(4): 469-483.

2.   Bambino K, Chu J (2017) Zebrafish in Toxicology and environmental health. Current Topics in Developmental Biology 124: 331-367.

3.   Cheng JX, Jia YK, Zheng G, Xie XS (2002) Laser-Scanning coherent anti-stokes raman scattering microscopy and applications to cell biology. Biophysical Journal 83(1): 502-509.

4.   Dang Z (2016) Interpretation of fish biomarker data for identification, classification, risk assessment and testing of endocrine disrupting chemicals. Environment International 92-93: 422-441.

5.   D. Dumas et al, World patent WO2012160312 A3, 2012. UL/CNRS.

6.   Segner H (2009) Zebrafish (Danio rerio) as a model organism for investigating endocrine disruption. Comp Biochem Physiol C Toxicol Pharmacol 149(2): 187-195.

7.   Souder JP, Gorelick DA (2018) Assaying uptake of endocrine disruptor compounds in zebrafish embryos and larvae. Comp Biochem Physiol C Toxicol Pharmacol 208: 105-113.