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Autofluorescence lifetime variation in the cuticle of the bedbug *Cimex lectularius*

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ABSTRACT

The decay time of the fluorescence of excited molecules, called fluorescence lifetime, can provide information about the cuticle composition additionally to widely used spectral characteristics. We compared autofluorescence lifetimes of different cuticle regions in the copulatory organ of females of the bedbug, *Cimex lectularius*. After two-photon excitation at 720 nm, regions recently characterised as being rich in resilin showed a longer bimodal distribution of the mean autofluorescence lifetime τ_m (tau-m) at 0.4 ns and 1.0–1.5 ns, while resilin-poor sites exhibited a unimodal pattern with a peak around 0.8 ns. The mean lifetime, and particularly its second component, can be useful to distinguish resilin-rich from resilin-poor parts of the cuticle. The few existing literature data suggest that chitin is unlikely responsible for the main autofluorescent component observed in the resilin-poor areas in our study and that melanin requires further scrutiny. Autofluorescence lifetime measurements can help to characterise properties of the arthropod cuticle, especially when coupled with multiphoton excitation to allow for deeper tissue penetration.

1. Introduction

Measuring autofluorescence is an established way to characterise material properties of the arthropod cuticle, including properties of chitin and chitinous substances (Lin et al., 2008; Chien et al., 2011). Traditionally, the characterisation has been confined to spectral information, such as the emission spectra at varying excitation wavelengths. The quantification of an additional fluorescence parameter, the decay time of the fluorescence of excited molecules, known as fluorescence lifetime, can also hold information relevant for material properties. For example, fluorescence lifetime imaging microscopy (FLIM) has been successfully used to characterise different strata of the human skin *in vivo* (König and Riemann, 2003; König, 2008; Huck et al., 2016), the metabolic stage of different epithelia or tumours (Skala et al., 2007) and aspects of sperm metabolism (Reinhardt et al., 2015). Fluorescence lifetime characteristics may be advantageous if the spectra of fluorophores overlap (Pellet et al., 2006). Other advantages that may make the application of FLIM desirable in addition to, or even instead of, spectral analyses are i) faster measurements, ii) the requirement of lower photon yields, iii) the independence of the lifetime from the concentration of the fluorophore, from the absorption, thickness, photo bleaching and/or excitation intensity and iv) it is more robust than intensity-based methods.

FLIM can be carried out by one-photon or two-photon excitation. Two-photon excitation enables the use of near infrared light which experiences reduced scattering and absorption by the sample. Near infrared light has the ability to both penetrate deeper into the tissue compared to visible or UV light used in one-photon excitation, and to excite the material for long periods of time with low photodamage. Two-photon excited autofluorescence (TPEF), therefore, enables the capture of longer time series. These advantages make two-photon autofluorescence lifetime measurements a potentially suitable tool to characterise biological materials of, or through, greater thickness or of higher sensibility to photodamage. However, few previous applications exist in this area (but see König and Riemann, 2003; König, 2008; Huck et al., 2016), especially non-medical ones (e.g., Rabasovic et al., 2015). Yet, the clinical use of multiphoton autofluorescence lifetime on human

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skin (König, 2008) and previous applications on arthropod cuticle (e.g., Rabasovic et al., 2015) suggest this method can be useful to characterise the material composition of the arthropod cuticle.

During investigations of the properties of a unique copulatory organ of females of the bedbug *Cimex lectularius*, a cuticle area with substantial autofluorescence was discovered resembling that of the elastomeric protein resilin (see Andersen, 1964; Andersen and Weis-Fogh, 1964). This cuticle area was suggested to contain large proportions of resilin (Michels et al., 2015) and we follow this suggestion here, although the biochemical and genetic confirmation of the presence of resilin is still lacking. Recently it was shown that pro-resilin genes are much more diverse in the bedbug genome (Benoit et al., 2016) than in the genome of the related species *Rhodnius prolixus* (Mesquita et al., 2015). The patch of material with large resilin proportions is situated on the right, ventral side of the female abdomen (Michels et al., 2015; Fig. 1A). The structure forms part of an evolutionarily novel organ, called the spermalege (Carayon, 1953; Usinger, 1966), which protects females from male-imposed mating damage (Morrow and Arnqvist, 2003; Reinhardt et al., 2003; Benoit et al., 2011) by reducing tissue rupture and haemolymph loss (Michels et al., 2015).

In the current study, we ask whether fluorescence lifetime characteristics of resilin-rich and resilin-poor cuticle areas in the arthropod cuticle can be identified. We had the specific expectation that at the site where autofluorescence under one-photon excitation revealed the presence of resilin, we would also find distinct lifetime properties. Dityrosine, one of the two autofluorescing amino acids that form the cross-links in resilin (Andersen, 1964; Andersen and Weis-Fogh, 1964) can be used to predict fluorescence lifetime characteristics. Dityrosine exhibits a dual-fluorescence behaviour with complex decay characteristics consisting of a sub-nanosecond rise phase and a subsequent double exponential decay with decay times in the nanosecond range (Kungl et al., 1992). In aqueous solution, with 285 nm excitation at pH = 6.94, a mean decay time of 2.86 ns can be expected, as calculated from the table in Kungl et al. (1992). The most abundant molecules in the arthropod cuticle are chitin, melanin and proteins (Sugumaran, 2002; Morgan, 2010; Kim, 2011; Merzendorfer, 2013; Moussian, 2013). The spectrum and lifetime of the dityrosine autofluorescence can, therefore, be obscured by those of other autofluorescences exhibited by these substances. Accounting for this, we here conclude that TPEF is a useful tool to study differences in the chemical composition of the arthropod cuticle.

2. Material and methods

2.1. Study animals

Female bedbugs of the species *C. lectularius* were taken from a large culture maintained for several years at the University of Sheffield (U.K.) as described previously (*e.g.*, Stutt and Siva-Jothy, 2001; Reinhardt et al., 2003, 2009, 2011; Otti et al., 2013). Bedbugs were killed by pressing the head with a forceps, just before dissection. The ventral side of the abdomen was separated from the dorsal abdomen side by cutting along the body and tearing both sides apart with a pair of fine scissors. The ventral cuticle of the abdomen was placed in a drop of phosphate-buffered saline (PBS) (pH = 7.4) on a microscopic slide, covered with a cover slip and examined under the microscope to which the two-photon excitation source was attached. To examine the copulatory organ, we chose one resilin-rich and two resilin-poor sites as defined by analyses of intense autofluorescence resembling the typical autofluorescence of resilin (see Figure 2 in Michels et al., 2015). Three regions of interest (ROI) were determined for further analysis: ROI 1 – the resilin-rich site in the spermalege, ROI 2 – a resilin-poor site directly adjacent to ROI 1 and ROI 3 – an area situated at the location of the spermalege but situated one segment away in cranial direction (Fig. 1).

2.2. Two-photon autofluorescence imaging

Measurements were performed using the multiphoton tomograph DermaInspect (JenLab GmbH, Jena, Germany), described in detail elsewhere (König, 2008; Breunig et al., 2010; König and Riemann, 2003; Breunig and König, 2011; Reinhardt et al., 2015). Briefly, the system consists of a tuneable femtosecond (fs) laser (MaiTai XF1 with a DeepSee unit, Newport/Spectra Physics, Newport, USA), a scan-detector module and high-numerical-aperture focussing optics. The scan-detector module contains a pair of galvo-scanning mirrors, a beam expander and a dichroic mirror to separate excitation and signal light. The fs laser provides sub 100 fs pulses at a repetition rate of 80 MHz in the tuning range of 710–920 nm with an output power of 0.5–1.1 W depending on the centre wavelength. Fluorescence is exclusively excited within the focal volume by two-photon absorption. Signal light that reaches the focussing optics is synchronously pixel-wise detected. Images are recorded by scanning the laser focus pixel-wise over the whole sample area or parts of it, so-called regions of interests (ROI). For every pixel, the number of arriving photons is counted within a pre-determined time interval, representing the fluorescence intensity. The fluorescence intensity, displayed in grey-scales, is visualised as the brightness of the pixel. The arrival time of the signal photons is measured by time-correlated single photon counting (TCSPC) (Becker et al., 2004) and is used to determine the fluorescence decay times for each pixel. Decay times are represented as pixel averages across selected regions of interest in false-colour classes (FLIM images).

The multiphoton tomography allows optical sections with a maximum field-of-view of 250 μm × 250 μm perpendicular to the optical axis at an adjustable depth between 0 and about 200 μm (König, 2008). The temporal resolution used was approximately 200 ps. The fluorescence decays were fitted using commercial fitting software (SPCImage Becker & Hickl GmbH, Berlin, Germany; Fig. 2 for an example output). During the measurements, the scanning time for an image of 256 × 256 pixels was set to 13 s for FLIM imaging. The mean laser power incident on the sample was adjusted to 12 mW. The signals were detected with photomultiplier tubes (Hamamatsu H 7724 for intensity and PMH-100, Becker & Hickl GmbH, for TCSPC imaging). A broadly transparent blue-green colour-glass filter (BG39) was used to protect the detectors from residual laser light in all measurements. Two-photon FLIM images were taken at several wavelengths but only those of 720 nm are presented. All measurements were carried out at room temperature.

2.3. Fluorescence lifetime measurements

The TCSPC data of the autofluorescence were pixelwise fitted with a two-exponential decay using the software packages SPCImage (Becker & Hickl, Berlin). A detailed example output showing the fitting curves, the FLIM images and the model specifications is given in Fig. 2. We used two-exponential decay models because they provided a better fit to the data than mono-exponential fits (720 nm: $chi^2 = 1.28$ compared to 2.26). A three-exponential fit did not perform better the two-exponential fit. The lifetimes within both of the two fitted decay curves can themselves show different maxima in the frequency distribution, which may be used to suggest the presence of additional components.

The distribution of lifetimes in a region of interest is represented in a histogram, whereby FLIM images are generated by false-colour coding of the decay times resulting from the fit (Fig. 2).). The number of pixels the decay curves were based on was the same in each ROI (cf. Fig. 1) was similar (ROI1: 4355, ROI2: 4260, ROI3:

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