Optical Coherence Tomography: Age estimation of *Calliphora vicina* pupae *in vivo*?

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Abstract

Necrophagous blowfly pupae are valuable contributors to the estimation of post-mortem interval, should an accurate age estimate be obtained. At present, this is reliant on a combination of rearing and destructive methods conducted on preserved samples, including morphological observation and gene expression analyses. This study demonstrates the use of optical coherence tomography (OCT) as a tool for *in vivo* morphological observation and pupal age estimation. Using a Michelson OCT microscope, alive and preserved four and ten-day old *Calliphora vicina* pupae were scanned in different orientations. Two and three-dimensional images were created. Morphological characteristics such as the brain, mouthparts and legs were identifiable in both living and preserved samples, with distinct differences noted between the two ages. Absorption of light by the puparium results in a vertical resolution of 1-2mm, preventing observation of deeper tissues. The use of contrast agents or a longer wavelength laser would improve the images obtainable. At present, the data suggests OCT provides a primary view of external and internal morphology, which can be used to distinguish younger and older pupae for further analysis of age and PMI estimation.

Key words

Calliphora vicina, forensic entomology, pupae, age estimation, optical coherence tomography, microscopy

Highlights

- We examine OCT microscopy as a tool for *in vivo* pupal age estimation
- Alive and preserved *Calliphora vicina* pupae were observed in 2D and 3D
- Brain, mouthparts and legs were identifiable in four- and ten-day old pupae
- The current maximum of 1-2 mm depth penetration limits observations
- We suggest OCT provides a complimentary method of pupal age estimation

Introduction

Pupae, a sedentary and long-lasting stage of the blowfly lifecycle, are often reported at crime scenes in association with a cadaver [1–3]. They are valuable contributors to post-mortem interval (PMI) estimation, provided an accurate age estimate can be obtained. This is generally conducted by rearing pupae under controlled conditions [4], which takes up to two weeks at room temperature and the accuracy of estimates decrease with later lifecycle stages [5]. Consequently and additionally, rapid age estimation from pupae can also be conducted from preserved samples, giving a more reliable estimate of PMI.

Age can be estimated from correctly preserved pupae using analysis of external morphology, histology and developmental gene expression [6–11], with an accuracy of +/- 12 hours (at 22°C) [10]. The dissection and/or homogenisation required for these methods can destroy the pupa. Whilst this causes minimal problems when large numbers of pupae are collected from the scene, when only a small sample is available, the preferred multidisciplinary approach to age estimation may not be possible. For example, it is likely that initial external morphological examination of a single pupa may inhibit subsequent gene expression analysis on the same organism, therefore it is advantageous if age estimates can be made without damaging the pupa.

Negating the need for histological sectioning and resulting tissue loss [6], methods such as X-ray imaging, micro computed tomography (micro CT), synchrotron X-ray microtomography (SR-μCT) and magnetic resonance microscopy (MRM) enable production of 2D and 3D images of insects [7,12–15]. Whilst SR-μCT resolution is ~1 μm [16], the equipment size and costs are prohibitively high for routine forensic use. In contrast, the resolution of micro CT and MRM is low [7], and this can only at present be improved using freeze-drying [17], staining with phosphotungstic acid (PTA) [13] or iodine [7] or injection with contrast agents [18]. Undesirably, these modifications would interfere with other age estimation methods and may kill and detrimentally alter the pupae.

The optimal non-destructive technique for age estimation is the analysis of living pupae, as this permits multiple further analyses including rearing. *Sarcophaga peregrina* pupal head eversion has been observed *in vivo* using MRM [19] however movement generally causes blurring [20]. The highest quality images are those of static organs such as the brain [21–24] but as mentioned previously resolution is still low and this technique is still considered complementary to histology on larger insects [20].

Optical coherence tomography (OCT) is a more recently developed microscopy technique than MRM and micro CT. The OCT microscope is considerably smaller (bench-top sized) and cheaper than other high-resolution microscopy equipment, and it is relatively simple to operate. It displays a resolution of <5 μm, superior to those achievable using MRI and some micro CT equipment. Slice thicknesses of 2-

20 µm are achievable, with imaging depths of up to 3 mm depending on tissue type [25,26], making the method comparable to histological preparations. This study assesses the suitability of OCT for *in vivo* morphological examination of *Calliphora vicina* pupae, with the aim of reducing reliance on destructive observation methods for age and PMI estimation.

Materials and Methods

Colony establishment

Adult *Calliphora vicina* were collected in Portsmouth, UK (50° 47' 51.08" N, 1° 5' 46.87" W) using liver-baited flytraps made from 2L plastic bottles. Colonies, housed in BugDorms (http://bugdorm.megaview.com.tw), were established from these individuals and maintained at 22°C, 40-60% relative humidity and under a 16L:8D photoperiod. Flies were fed water and sugar, and provided liver for oviposition.

Collection of pupae

Under the same environmental conditions for adults, eggs were collected on ox liver placed in a small plastic container (17cm x 11cm x 5cm) in the BugDorm. The container was then removed after a few hundred eggs were laid and placed in a larger plastic container (28cm x 28cm x 11cm), on a bed of sand for pupation. Upon hatching, the larvae were reared on liver *ad libitum*. The aim of this study was to assess the technique for observation of gross morphological change, therefore precise pupal age was not required and so in-colony temperatures were not recorded. Pupae remained undisturbed in the larger container where they were reared to 30-40% and >90% developed and five were sampled (Table 1) at four days (4d) and ten days (10d) post-puparium formation respectively. They were collected alive and also fixed by piercing and hot-water-killing [8]. The emergence of live pupae, after scanning, was briefly monitored.

OCT microscopy

Puparia remained intact for living pupae and were removed from fixed pupae prior to visualisation. The EX 1301 swept source OCT microscope (Michelson Diagnostics) used in this study employs multiple, simultaneously stacked lasers to scan the sample, up to a speed of 30 frames per second. Pupae were placed on the motorised stage in different orientations and the microscope was set to scan at 8-12 µm (Table 1). The two-dimensional cross-section images obtained were produced from the attenuated and backscattered near-infrared light.

Scans were saved as stacks of TIFF files, and viewed in Voxx (http://www.nephrology.iupui.edu/imaging/voxx), Image J (http://rsbweb.nih.gov/ij) and Vaa3D (http://www.vaa3d.org) as two-

dimensional stacks and in rendered 3D models. 3D models were virtually sectioned on all three axes (x, y and z) to reveal internal anatomy.

Results

The purpose of this study was to explore OCT microscopy as a potential tool for age estimation of pupae *in vivo*, for PMI estimation. For comparison and assessment of OCT capabilities, pupae were also examined *in vitro*, after preservation.

Dorsal longitudinal sections of 4d live pupae allowed visualisation of some internal structures through the puparium such as the abdomen, wings, posterior trachea as well as the developing brain (Fig. 1a, b). The transverse section shows everted respiratory horns (Fig. 1c). Saggital sections also show the brain to a depth of ~1mm (Fig. 1d). Longitudinal ventral and transverse sections of live 10d pupae similarly revealed external features such as the labellum, labrum, brain and leg musculature (Fig. 1f, g, h). 3D rendering of live pupae (Fig. 1e) resulted in a view of the external puparium surface. The pupae emerged successfully within the same time frame as the cohort from which they were sampled.

Whole 3D rendered images of preserved 4d pupae showed antennae, wing and leg detail on the ventral surface (Fig. 2a). Leg musculature and tarsi were viewed both in ventral transverse (Fig. 2b) and longitudinal (Fig. 2c, d) sections respectively. These longitudinal cut away sections also showed outlines of wing tissue, eyes and antennae, and mouthparts in considerable detail (Fig. 2c, d). 3D surface rendered images and cut away sections of 10d pupae showed compound eyes, orbital bristles and optic neurons (Fig. 2e, g). Sagittal and dorsal stacks showed eyes, antennae, abdominal macrochaetae, legs and wing folding detail (Fig 2f, h).

Discussion and Conclusion

OCT is commonly used for *in vivo* examination of retinas, blood vessels and skin carcinomas (summarised in [25]; see also [27]) but also in developmental biology to examine mammals (reviewed by [28]) and *Xenopus* spp. [29–31]. This pilot study using OCT microscopy for imaging of blowfly pupae aimed to test whether enough morphology information could be obtained without dissection or histology for age estimation. Non-destructive *in vivo* methods are clearly advantageous and would permit further age estimation analyses, such as gene expression [9,11], to be performed.

The data shows that whilst many important morphological developments, e.g. mouthparts, brain and bristles [32] were visible *in vivo* using OCT, many of the features lacked in detail due to the main limitation of the OCT system used here; the lack of vertical resolution. The tissues, especially the puparium, absorb the light strongly, limiting penetration to 1-2mm and resolution is lost. Preservation and puparium removal allowed further details in wing folding and bristle formation to be observed; this permits more reliable differentiation between the two ages of pupae, demonstrates the

significance of the back scattering effect and finally highlights the technique's potential could back scattering be reduced. This problem was also evidenced in *Drosophila melanogaster* larvae [33,34]; their transparent organs lie beneath an opaque cuticle which causes much of the light back scattering before penetration of the sample can occur. In contrast, daily development of lepidopteran wings [35], which lie just beneath the surface of the puparium, has been observed.

There have been no previously reported cases of damage or death of any tissue analysed by OCT; similarly and importantly in this study, pupae were observed to emerge as normal. Clearly, extensive quantitative analysis of any possible effects on pupal age and morphology effects caused by OCT scanning must be conducted prior to acceptance as a suitable *in vivo* technique for PMI estimation.

The tissue contrast required for the operation of OCT was enhanced by the sclerotisation of 10d pupae. This increased the light back scattering and improved surface and internal resolution. It is considered that OCT would therefore be most suited to age estimation of older pupae. Recent advances to the equipment are that of enhanced tissue differentiation based on elasticity/stiffness (Michelson Diagnostics, pers. comm.) and this could facilitate identification of external features and internal tissue types, such as bristles and trachea respectively. Unfortunately, the use of OCT for age estimation of preserved pupae offers no advantage over standard external morphological examination, which provides more accurate age estimates. Age estimation of live pupae using OCT would, however, be advantageous to PMI estimation; estimates may be accurate to within a few days (based on gross morphological changes), providing an initial assessment prior to rearing of the pupa to eclosion [4], or performing targeted age estimation using other methods such as gene expression [9].

Current costs of an OCT microscope are within the realms of a research-standard stereomicroscope - one-quarter that of a micro CT system - making this a more likely and realistic addition to a forensic laboratory. Prior to investment however, for OCT to be adopted for morphological analysis, the vertical resolution or penetration depth must be improved. This could be achieved by using a laser with a longer wavelength than 1130nm, such that the tissue absorbs less light. A more recently developed microscope (OCS1300SS; www.thorlabs.de) utilises a 1300nm laser, which provides a slightly better penetration depth of 3mm, however depths of 4-5mm would be desirable for pupae.

The injection of contrast agents such as microspheres into living organisms has been shown to improve image quality, by increasing the light-scattering properties of the tissues [36,37]. This method would require the pupa to remain alive for a currently unknown period of time sufficient for this to occur, reducing the accuracy of age and PMI estimation. Microspheres may also then inhibit additional age estimation methods, such as gene expression analysis or rearing to eclosion and therefore negate the benefit of *in vivo* observation.

In conclusion, this work has demonstrated that rudimentary morphological analysis of pupae *in vivo* is possible using an OCT microscope. Differences in 4d and 10d pupal morphology was distinct enough such that ages were identifiable, thus this technique shows potential as a tool for approximate age and PMI estimation. Although the results are promising, for OCT microscopy to be considered a more useful tool for age estimation using external morphology and/or histological examination, technological improvements must be made.

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Table 1. Description of pupae observed using OCT microscopy. Living and fixed 4 and 10-day old pupae were observed using the OCT microscope with their dorsal, ventral or lateral surfaces upwards. The lateral scanning resolution was either 8 or 12 μm as indicated.

Pupa #	Age (days)	Condition	Orientation	Resolution
1	4	Alive	Dorsal	12 μm
2	4	Fixed	Ventral	8 μm
3	10	Fixed	Dorsal	12 μm
4	10	Alive	Ventral	12 μm
5	10	Fixed	Lateral	12 μm

Figure legends

Figure 1. OCT images of pupae *in vivo* 4-day (Table 1: 1) and 10-day old pupae (Table 1: 4) remained alive whilst their dorsal and ventral surfaces were scanned respectively. Images a-d show the 4-day pupa; images e-h show the 10-day pupa. Longitudinal (a, b, g and h), transverse (c and f) and sagittal sections (d) were taken. A 3D rendered image of the puparium is also shown (e). Ab = abdomen, Br = brain, La = labellum, Lb = labrum Le = legs, Pu = puparium, Re = respiratory horn, Tr = trachea.

Figure 2. OCT images of pupae *in vitro* 4-day (Table 1: 2) and 10-day old pupae (Table 1: 3, 5) were preserved and scanned in multiple orientations after puparium removal. Images a-d show the 4-day pupa; images e-h show the 10-day pupa. Whole and cut-away 3D rendered images (a, e and g) were modelled. Transverse (b), ventral longitudinal (c and d), dorsal longitudinal (h) and sagittal sections (f) were viewed as single tiff images and taken as slices from the 3D models. An = antennae, Ey = Compound eye, La = Labellum, Lb = labrum, Le = Legs, Mc = macrochaetae, Ob = orbital bristles, On = optic neurons, Ta = Tarsi, Wg = wing.

Brown, K. & Harvey, M., 2014. Optical Coherence Tomography: Age estimation of Calliphora vicina pupae in vivo? *Forensic Science Inte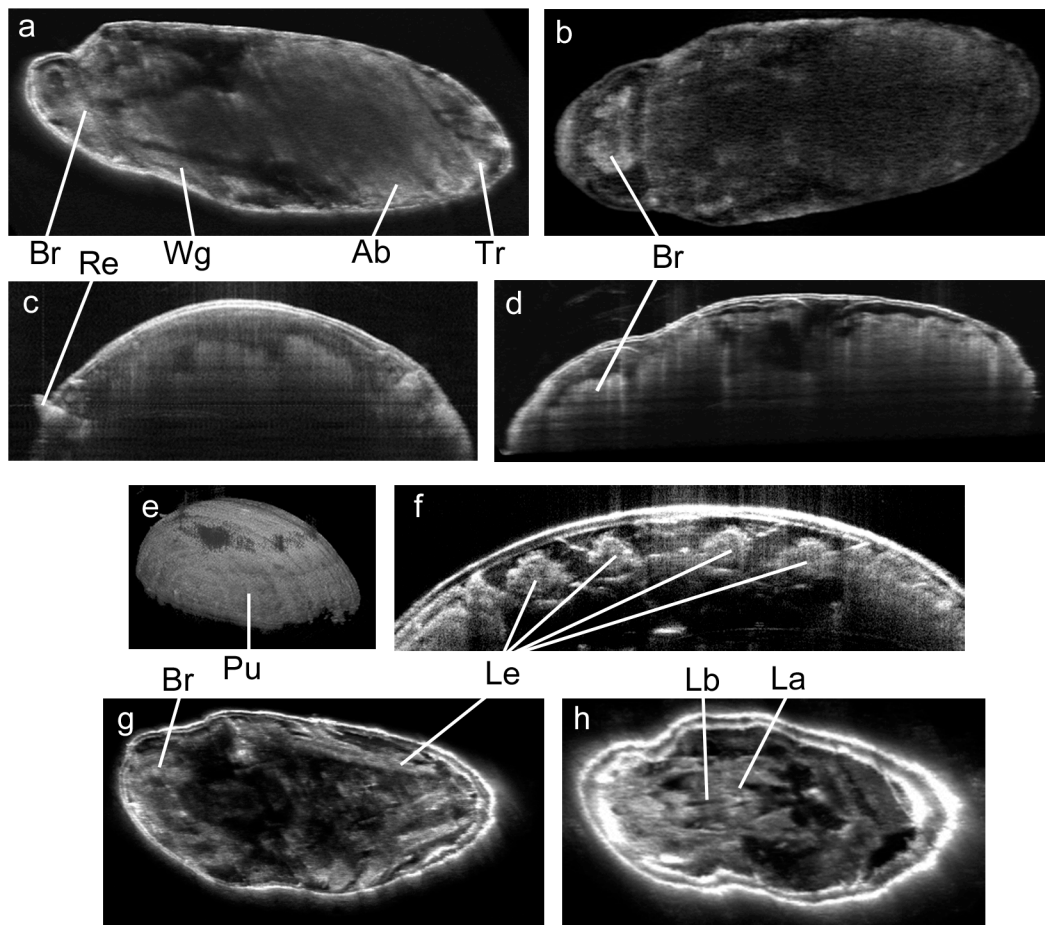rnational*, 242(September 2014), pp.157–161. Available at: http://www.fsijournal.org/article/S037907381400276X/fulltext [Accessed July 17, 2014].

Figure 1

a   Br   Re   Wg   Ab   Tr

b   Br

c

d

e   Br   Pu

f   Le

g   Le   Lb   La

h

Figure 2