# Autofluorescent Biomolecules in Diptera

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Light-based phenomena in insects have long attracted researchers' attention. Surface color distribution patterns are commonly used for taxonomical purposes, while optically-active structures from Coleoptera cuticle or Lepidoptera wings have inspired technological applications, such as biosensors and energy accumulation devices. In Diptera, besides these optically-based phenomena, the ability of some biomolecules to fluoresce makes them to act as markers of bio-metabolic and structural features useful for vector control studies. Resilin or chitinous compounds, with their respective blue or green-to-red autofluorescence (AF), are commonly related to biomechanical and structural properties, helpful to clarify the mechanisms underlying substrate adhesion of ectoparasites' leg appendages, or the antennal abilities in tuning sound detection. Metarhodopsin, a red fluorescing photoproduct of rhodopsin, allows to investigate visual mechanisms, whereas the AF of NAD(P)H and flavins, commonly correlated to energy metabolism, favor the investigation of sperm vitality. Lipofuscins are AF biomarkers of aging, as well as pteridines, which can be involved in the synthesis of pigments and, similarly to kynurenines, are also exploited in metabolic investigations.

Keywords: endogenous fluorophores ; resilin ; chitin ; spectroscopy ; imaging ; Drosophila melanogaster ; mosquitoes ; high resolution morphology ; mechanical functions ; sensory perception

## 1. Introduction

Biological substrates can give rise to autofluorescence (AF) emission in the near-ultraviolet (UV), visible, near-infrared (IR) spectral interval when irradiated with proper excitation light [1]. The AF emission depends on the presence of biomolecules with chemical structures suitable to interact with light, and usually comprising covalent double bonds and aromatic moieties, commonly named endogenous fluorophores. The properties of the overall AF light signal, in turn, will strictly depend on the chemical nature, amount, distribution and microenvironment of the various endogenous fluorophores typically present in cells and tissues under investigation, in a close relationship with their morpho-functional conditions. Autofluorescence can thus act as a valuable intrinsic biomarker useful for the set-up of real time in situ analytical and diagnostic procedures to be applied in the most various fields, from biomedicine, industry, vegetable and animal food production and processing, to environmental surveillance [2].

In all cases, the efficiency of the measuring systems for a sensitive and specific detection of the target fluorophores will rely on the choice of the optical set-up ensuring the proper excitation and emission conditions for the light-based observations. When pure compounds are available, spectroscopic and time resolved analyses can provide information on the spectral shape and time decay kinetics of their AF, to assess the most suitable optical conditions for their detection in the biological substrates where they are naturally present. On these bases, increasingly sophisticated imaging procedures have been developed, improving both excitation efficiency and specificity of the AF detection. For example, the multiphoton excitation using the sequence of photons in the red or NIR spectral interval provides the energy equivalent to the violet or blue spectral interval, with the advantages of reaching deeper layers of the structure under study and decreasing the risk of photobleaching and UV radiation damage. The continuous development of sophisticate devices improves also the frequency and time resolution of the AF signals, advancing AF conventional imaging to multispectral, hyperspectral and lifetime imaging procedures for the detection and topological localization of specific fluorophores in living cells and tissues, up to the label-free mesoscopic applications in clinical diagnosis [3][4]. It is also worth to recall the development of procedures based on the multicomponent analysis transforming the fluorescence lifetimes in a phasor plot, which have been demonstrated to effectively solve different molecular species within single pixels. Such procedures permitted to verify the differences in the topological distribution of bound and free NAD(P)H and FAD in mammalian cell models with a different engagement in aerobic or anaerobic energy metabolism [5].

In insects, optical phenomena have deserved great attention since a long time. This is the case of Lepidoptera and early investigations on their wing fluorescing pigments, commonly referred as papilliochromes, and their components, identified with the support of combined optical analyses of compounds purified from natural extracts and autoradiographic imaging and chromatographic assays following administration of $^{14}C$ labelled dopamine and tryptophan [6]. Numerous additional

studies focused on the chemical identification of the pigments responsible for AF emission, as well as on effects like reflection and diffraction induced by the grating-like structures of the ribs located along the ridges of the scales, acting as photonic crystals [7][8][9].

The characterization of AF and of true color properties has prevalent implications as a support in taxonomical classification [10][11][12][13][14], while the studies on the mechanisms underlying the interaction of photonic structures with light and their responses to external factors have inspired the set-up of sensors for applications ranging from energy accumulation to the monitoring of environment and human health [15].

Similarly, in Coleoptera, the porous layers structurally organized in ordinated and periodic stacks localized in the cuticle of thorax and elytra have been regarded as photonic cells, and their ability to sense the refractive index of fluids by means of changes in light reflection or fluorescence has inspired the set-up of fluid biosensors [16][17][18]. In this respect, it is worth also to recall that reflectance spectra measured on adults of two beetle species (*i.e*., *Sitophilus zeamais* Motschulsky and *Cynaeus angustus* (LeConte)) with sclerotized exoskeleton allowed to monitor their response to treatment with killing agents, demonstrating the potential of the technique for further applications as development of a non-destructive and non-invasive approach to assess stress conditions in insects [19].

With specific reference to the biomolecules responsible for AF emission, besides the abovementioned fluorescing pigments of Lepidoptera, resilin and chitinous compounds have been greatly investigated with attention to their structural and functional implications. Resilin is the very resilient elastomeric, or rubber-like, protein detectable in various arthropod body structures, where it ensures important biomechanical properties such as elasticity, resilience and energy storage in the pterothorax cap tendons in the direct flying apparatus of Odonata [20][21][22][23]. The typical bluish AF emission of resilin favors the detection of structures with prevalently elastic properties, helping to distinguish them from material with different degrees of sclerotization. Chitin as a commercial compound purified from natural sources has been demonstrated both to be practically non-fluorescent [24] or to give rise to an emission in the 400–550 nm range [25]. The absence of AF from pure chitin is consistent with its polysaccaride nature. However, in a chitinous material, a variable presence of oxidised and undefined compounds can be responsible for the AF emission and the lengthening of the wavelength position. This suggestion is supported by an AF lifetime study performed on the cuticle of *Cimex lectularius* Linnaeus (Hemiptera: Cimicidae) [26] that reported the absence of long decay times relatable to chitin [25] On the other hand, the detection of decay times of 0.4 ns and 1.0–1.5 ns indicated the presence of resilin along with other fluorophores, such as likely melanin. On this basis, Reinhardt and colleagues proposed the possibility to distinguish areas rich or poor in resilin, respectively [26]. The progressive variation in the reciprocal content between chitinous components and resilin has been proposed to be directly reflected by the changes of AF emission from blue, yellow and green to reddish colors, detected by means of confocal microscopy at properly selected excitation/emission wavelengths [21]. Comparable AF measuring conditions have been also used by Eshghi and colleagues for the development of an algorithm based on a modulus color map able to assign each color of a confocal AF image to a specific type of material [27]. In this way, it is intended to improve and facilitate the investigation of the relationships between the biochemical components and functions of the body structure under investigation, as shown by the results obtained on different insect species. This is the case of *Coccinella septempunctata* Linnaeus (Coleoptera: Coccinellidae) and the tarsal seta, *Cassida rubiginosa* Muller (Coleoptera: Chrysomelidae) and the male flagellum, *Carausius morosus* (Sinéty) (Phasmatodea: Lonchodidae) and its hindleg tibia [27]. In addition, in the leaf beetle *Chrysomela populi* Linnaeus (Coleoptera: Chrysomelidae), the combined morphological and AF-based analysis allowed the identification of two subtypes of gustatory sensilla chaetica on the antennal apical flagellomeres. The structures were classified in two types, those fluorescing prevalently in blue, ascribed to the dominant presence of resilin ensuring flexibility, and those fluorescing prevalently in green, attributed to the stiffer chitin. The latter type, the subtype 2, has been proposed to sense both primary and secondary metabolites of the host plant by contact chemoreception, with promising insights for in situ investigations on the capacity of phytophagous insects to sense plant biochemical components [28].

Last, but not least, it is worth mentioning that AF rising from various tissues and organs of insects can often disturb the identification of target sites labelled with specific exogenous fluorescing dyes or the expression of fluorescent proteins introduced with genome manipulations. This issue is similar to what frequently reported in the case of fluorescence-based histochemical assays in biomedicine, with particular relevance when the targets consist in few and scattered positively labelled sites [2].

Technical procedures have been thus developed to treat the specimens in order to minimize the AF signal. In some cases, a proper decrease of AF can help the topological localization of the investigated target sites. This possibility has been elegantly exploited by Pende and colleagues, who developed a combined tissues clearing procedure to decrease the AF

with an ultramicroscopy approach. The resulting lowered signal of AF allowed to delineate the body of *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) larvae and adults, favoring the combined multi-view topological localization of the green fluorescent protein (GFP)-expressing neuronal network with single-cell resolution in *Drosophila* larvae [29].

In insects, AF was also reported as a real disturbance through confounding and impeding the reliable detection of the fluorescent signal from the reporter gene [30], or limiting the detection of 16S rRNA-targeted fluorescent in situ hybridization (FISH) applied to tissues for the analysis of symbiotic bacteria [31][32]. The disturbance can be due mainly to the presence of food particles in the gut, or to Malpighian tubules, chitinous exoskeleton and necrotic tissues, responsible for high AF signals that could be mistaken for the fluorescence of the exogenous markers, reducing or even hampering their specific detection [33][34]. In this context, efforts have been devoted to circumvent the problem. Different optical clearing methods and microscope imaging procedures have been compared, in order to improve the localization of *Plasmodium* parasites in the midgut of *Anopheles* vectors [35]. On the other hand, Koga and colleagues have developed a protocol based on the use of hydrogen peroxide able to strongly reduce AF in insect tissues and allow a reliable FISH detection of endosymbionts of aphids, lice and bat flies [36]. Anyway, several studies performed in insects on the development of transgenic individuals through germline transformation and consequent expression of fluorescent protein markers (e.g., green and red fluorescent proteins) have reported that AF signals did not impair the detection of the experimentally-induced fluorescence [27][28][29][30][31][32][33][34][35][36][37][38][39][40][41][42]. It is also worth to recall the particular case of the AF rising from the yolk granules and the vitelline membrane of dechorionated *D. melanogaster* embryos, which, together with the embryo thickness, size, and opacity, seemed to affect the application of optical fluorescence microscopy in the study of this developmental stage. However, the lower AF signal obtained from yolk and vitelline membrane in the green excitation condition (543 nm) than in the blue one (458 nm, 488 nm; two photons 820 nm), was exploited to exclude the AF hindering the detection of exogenous dyes or to provide an optical guide to detected specifically labelled sites [43]. In the same paper, additional strategies to avoid AF-related issues consist in the use of optical sectioning by confocal laser scanning microscope and by photobleaching or photoactivation procedures.

## 2. Autofluorescence in Diptera

An early attempt to investigate AF under UV light excitation was performed in the genus *Phlebotomus* (Diptera: Psychodidae). With the aim to circumvent the use of radioisotopes and related economical and management issues, as well as insect vitality loads, the observation of various AF patterns in different organs was successfully reported [44]. A next work on the AF of Diptera proposed confocal microscopy as a relatively simple and reliable means to assess and describe their structures, with the advantage to evidence details otherwise hard to distinguish by bright field imaging. Acquisition of AF images under the common excitation at 543 nm permitted the appreciation of the fine structures of the head, in particular mouthparts and antennae, and genitalia in three dipteran families, namely Campichoetidae, Camillidae, and Drosophilidae [45]. Remarkably, this work promoted the use of AF imaging applications as a support to facilitate structure identification and sharing of morphological, phylogenetic and taxonomical data among entomologists. Even more importantly, the study predicted also the helpful role of AF analysis for the interpretation of the functional role of soft structures as compared with the most sclerotized ones. This prediction has been gradually taken shape even exceeding the promised expectations, as illustrated by the following reports testifying the increasingly deserved attention to the fluorescing structures of Diptera, with consequent valuable implications for the study of mechanical and sensory functions, such as olfaction and hearing, metabolism in different tissues and developmental stages, including the response to different physiological states.

### 2.1. Mechanical Functions

Autofluorescence imaging analysis has greatly helped the characterization of the attachment system of the bee lousefly *Braula coeca* Nitzsch (Diptera: Braulidae), a kleptoparasite on the honeybee *Apis mellifera* Linnaeus (Hymenoptera: Apidae). The attachment system to the bee's hairy surface consists in tarsal appendages, with a pair of claws and two pulvilli. Images obtained by confocal microscopy at selected excitation/emission conditions showed AF patterns with differently colored areas. The blue regions indicated a dominating presence of resilin in the pulvilli, consistently with their soft mechanical properties ensuring adhesion to soft surfaces, while the yellowish, greenish and reddish regions were put in relation with increasing degrees of sclerotization, and thus of stiffness, in the tarsus and its structures, such as claws and grooming setae [46].

These findings are in a remarkable agreement with a previous report on the attachment system of the leg of *Crataerina pallida* (Latreille) (Diptera: Hippoboscidae), a non-flying ectoparasite of birds, in particular of the swift *Apus apus* (Linnaeus). In this research, AF allowed the researchers to detect a gradual change from the prevalence of the sclerotized chitinous-like components in the basal region of the claws, the pulvilli and the empodium, to an increasing presence in the

soft resilin at the apical tips of the setae. This AF characterization of the material based on laser scanning microscopy, combined with the morphological study by cryo-scanning electron microscopy and experiments on the attachment forces performed on both feathers and glass, silicone rubber and epoxy resin, as the respective native and artificial substrates, allowed to demonstrated how the attachment system is adapted to ensure the functional adhesion of the ectoparasite to the bird feathers [47]. In adults of *D. melanogaster*, resilin has been shown to contribute as a protein matrix to skeletal structures, organs and tissues that are involved in repeated movements, torsion or flexion [48]. In particular, resilin-enriched sites include spots at the leg joints, at the articulation of the wings (where resilin patches may act as muscle attachment sites) and on the abdominal flanks, in cibarium and labellum in the head, in the tracheal endings, at hair bases and in the spermathecal ducts, as observed in mosquitoes [49][50][51][52]. A recent study focusing on the analysis of resilin in the spotted-wing fruit fly *D. suzukii* (Matsumura) (Diptera: Drosophilidae) showed that the intensity and distribution of resilin signals are conserved with respect to *D. melanogaster* [53]. However, in the wing hinge and in the trochanter, the resilin signal is stronger in *D. suzukii* than in *D. melanogaster*, while there are no differences between *D. suzukii* and *D. hydei* Sturtevant (Diptera: Drosophilidae). These findings are indicative of potentially different biomechanical features responding to different lifestyles, and further investigations will be essential to clarify the functional adaptation of these structures.

### 2.2. Sensory Functions

The interpretation of AF-based data in the characterization of the material composing specific structures, besides being relevant to study mechanical functions as mentioned above, is increasingly receiving attention to describe sensory processes, which are target of intensive research across Diptera [54][55][56][57][58][59][60][61][62][63]. Sensory processes take place at the interface between insects and their environment. Therefore, the understanding of their molecular and physiological bases, together with the biomechanical features of organs and tissues, will allow gathering information essential for both basic and applied research. Sensory processes comprise, but are not limited to, chemoreception (including olfaction and taste), hearing and vision.

### 2.3. Metabolism

The AF-based metabolic studies in insects have greatly taken advantage from the detection of NAD(P)H and flavins. These coenzymes are indispensable for the oxidoreductive reactions. Their ability to fluoresce in the respective reduced and oxidized state has been since a long time exploited to investigate the functionality of the anaerobic and aerobic pathways of energy production, as well as for the reductive biosynthesis and defense against oxidizing species [64]. Besides the spectral shape properties, the shorter AF lifetime of free NAD(P)H, as compared with that of the bound state, has been proposed as a useful parameter to investigate the cell engagement in anaerobic and aerobic metabolism [65].

In general, the selective measurement of AF at different intervals during the decay of the emission signal following the excitation pulse is a valuable tool for the simultaneous detection of different fluorophores and lifetime imaging technique (FLIM) in cells, tissues and organs for analytical and diagnostic purposes [4]. In fact, many studies aimed at characterizing the morpho-functional properties of the most variegated biological substrates take advantage of the possibility to separate different endogenous fluorophores, exogenous dye markers and or fluorescent proteins expressed in genetically manipulated cells and animals depending on the lifetime of their emission signals. This possibility, associated with the advances in luminescence imaging, contributes to provide 3D spatial resolution [3].

### 2.4. High Resolution Morphology

Direct AF excitation, as well as the use of multi-photon AF excitation, second- and third-harmonic generation (SHG and THG, respectively) in the visible range and of near-infrared (NIR)-based approaches are particularly useful in achieving label-free imaging in samples with variable chemical composition. These techniques even allow deep tissue penetration (>500 µm), valuable for samples with nonlinear optical properties (see [66] for a review). Moreover, hyperspectral imaging (HSI), a technique able to acquire data simultaneously in hundreds of spectral bands with narrow bandwidths [67] has been recently applied also in entomological studies.

A label-free study performed on *Drosophila* larvae by using a 780 nm laser multiphoton excitation with the combined collection of both AF signal in the 435–700 nm ranges and SHG has provided a complementary structural information. Autofluorescence has evidenced internal organs such as the trachea and the digestive system, and SHG has revealed the larval muscles according to their repetitive structural organization, providing a promising supportive tool for combined physiological and developmental investigations [68].

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