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15

Skin Biopsies as Tools to Measure Fish Coloration and Colour Change

P. Andreas Svensson[1] and Helen Nilsson Sköld[2]
[1]*Deakin University, School of Life and Environmental Sciences*
[2]*University of Gothenburg, Marine Ecology-Kristineberg*
[1]*Australia*
[2]*Sweden*

1. Introduction

1.1 Preliminaries

A biopsy is a link between the whole organism and its cells. Many recent advances in the field of fish coloration have been gained through experimentation on skin biopsies. In particular, this strategy has allowed the in-depth knowledge from pigment cell physiology to be applied to wider topics, such as behavioral ecology. Moreover, melanophores from fish and frogs have long served as models for a general understanding of intracellular transport and organelle positioning. For this purpose, fish skin biopsies have frequently been used for isolation of pigment cells but also for isolation of fibroblasts. In fish pigmentation research, skin biopsies have also been used to understand what causes changes in the skin's overall appearance, especially in the context of short or long-term modulation of tissue coloration. Examples of this are how many fishes rapidly change their colour during courtship or aggressive displays. Here, we review our methodology for the use of skin biopsies in research on fish coloration – from pigment cell physiology to animal behavior. We also highlight some of the most significant findings from this research, and present unsolved issues and suggestions for future directions. In this chapter, we will provide a description of the experimental procedures we have used to manipulate and analyze fish skin biopsies in the context of fish coloration - from chromatophores to animal behavior. Since our research has mainly focused on marine fishes, the protocols (e.g. the choice of buffers) were chosen to suit such animals. However, with minor modifications, these methods will likely work also on most fresh water species. Many of the experimental procedures are fast to assess and easy to perform, and we therefore encourage the experimentation on fish biopsies for use in education. The protocols described in this chapter are also suitable for such exercises.

1.2 Overview

Body colour patterns are important for animals because they can function in inter- and intra species communication and provide camouflage, thermoregulation and protection against solar radiation. In many taxa, colour patterns are caused by large star-shaped pigment containing cells, chromatophores, which are located in the skin. The chromatophores are grouped into subclasses based on the colour of their pigment containing organelles: xanthophores (yellow), erythrophores (red/orange), iridophores (reflective/iridescent), leucophores (white), melanophores (black/brown) and the more rare cyanophores (blue).

As opposed to birds and mammals, where feathers and fur merely absorb pigment from pigment producing cells, fish, amphibians, reptiles and certain invertebrates display the pigment that reside inside chromatophores in the skin itself (Figure1).
Fish chromatophores are found in dermis, epidermis and on the scales that cover the skin. Interestingly, chromatophores and especially melanophores are also located in the peritoneum, the epithelium that covers the abdominal cavity at the inside of the fish. The belly of the fish is typically whitish, both at the outside and the inside of the fish, as a result of large densities of leucophores in these areas. The dorsal areas are on the other hand typically dark as a result of high densities of melanophores. As a result of sexual selection, males and females may display different body colours and patterns. Often juveniles have distinctive colours that differ from the adults of the same species or gender. In adults, particularly in males, the colour patterns can change over the year to be cryptic in winter and more conspicuous during the breeding season. Such a relatively slow and long term change in coloration, is caused by changes in the number of chromatophores in the skin, and called morphological colour change. Skin patterns and colours can also be modified within minutes by reflective changes in iridophores as well as through the aggregation or dispersion of the pigment-containing organelles inside the chromatophores. Both these more rapid changes are referred to as physiological colour change. Vision, direct light, hormones and neurotransmittors can all be used in the regulation of physiological colour change. Dispersal of the pigment inside the chromatophores will increase body pigmentation while pigment aggregation causes reduced pigmentation. A reduced coloration due to chromatophore pigment aggregation will typically also result in increased skin transparency.



Fig. 1. An adult male cuckoo wrasse, *Labrus bimaculatus*, displaying its characteristic red and blue skin pattern in the wild. This fish does not have blue pigment cells but the blue colour of the skin is instead an effect of chromatic interactions between melanophores and iridophores. Females of this species lack the blue colours (not shown). The photograph was kindly provided by Dr Mattias Sköld.

On a cellular level, melanophores, with their distinctive dark melanin-containing organelles, melanosomes, are relatively well studies compared to the other chromatophore types. The cellular regulation of melanophores is therefore rather well understood. To achieve maximum lightness of the skin, the melanosomes are tightly aggregated to a spherical pigment mass in the center of each melanophore. A common observation is that fish often appear pale during sleep at night. The melanophore pigment aggregating effect of melatonin, which is released at night, causes this body paling. Both aggregation of pigment and the maintenance of the pigment within the spherical central pigment mass (CPM) depend on the molecular motor dynein and the presence of low levels of intracellular cAMP. Dyneins carry the melanosomes while they move along the microtubule tracks towards the center of the melanophore. On the other hand, to achieve maximum darkness of the skin, the pigment needs to be evenly dispersed throughout the cells. Such dispersion depends on a kinesin-like motor that moves along microtubules in the opposite direction of the dynein motor, and on high levels of cAMP. Dynein is inactivated during dispersion to allow rapid dispersal from the cell centre. The pigment do however not all move all the way to the periphery of the cells, but they stop at different position to become evenly dispersed throughout the cells. This even distribution is achived by movements of both dynein and the kinesin, together with actin and the actin-dependent motor myosin V, also located on the melanin pigment organelles. How the degree of dispersion is coordinated and maintained at the intracellular level is not yet fully understood.

Chromatophore-based pigment patterns are used by animals in a variety of behavioral contexts. In fishes, the regulation of physiological colour change has been mostly studied in the context of crypsis (i.e. camouflage). Recently, studies have been conducted on the often more complex nuptial colour patterns that are used for courtship and aggression. For a more comprehensive overview of fish coloration and patterns, including regulation and mechanisms behind the different types of colour changes, we refer to recent and classic reviews as well as some particularly relevant original publications (Kasukawa et al., 1987; Fujii and Oshima, 1994; Fujii 2000; Amundsen and Forsgren 2001; Nilsson Sköld et al., 2002; Siebeck 2004; Nilsson Sköld et al., 2008; Aspengren et al., 2008; 2009; Mills and Patterson 2009; Leclercq et al., 2010).

To fully understand short-term regulation of fish coloration one needs to address a wide range of phenomena: from the very fine details of the intracellular motile machineries to the complex chromatic interaction between tissue types and the signalling behaviours of the animals. We believe the use of skin biopsies work as a link between these traditionally disparate scientific disciplines. Skin biopsies can therefore become a bridge between physiology at cellular and organismal levels to animal behaviour and ecology.

2. Methodology for assessing fish coloration and colour change

2.1 Assays for analysis of rapid colour change

2.1.1 Skin biopsies

Skin biopsies can be excised from various parts of the fish body depending of purpose and area of interest. We have recently used biopsies from the abdomen (Nilsson Sköld et al., 2008; 2010) because these contain both epidermal (external) and peritoneal (internal) chromatophores (Figure 2). Melanophores, erythrophores, xanthophores, leucophores and iridophores are the most common chromatophore types found in the skin of fish.

Although blue cyanophores have been described, most blue and green skin colors are the result of chromatic interactions between melanophores, xanthophores and the reflective iridophores.

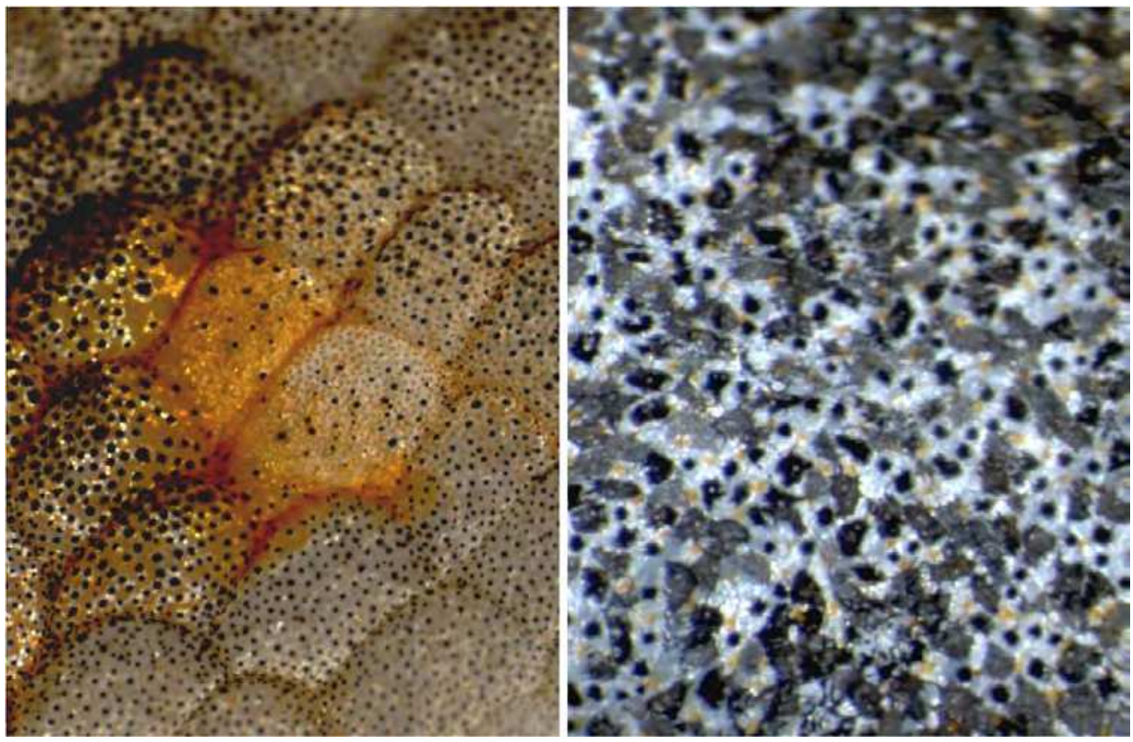


Fig. 2. Example of how the skin (left) and peritoneum (right) of a fish, *Pleuronectes platessa*, appears on close up photographs of an abdominal biopsy. In both photographs, examples of chromatophores with either aggregated or dispersed pigment can be observed.

In order to better understand the roles of different chromatophores on skin coloration or to test possible effects of certain hormones, the rapid color change reaction can be manipulated and monitored using skin biopsies excised from the fish of interest. A list of extracellular hormones and effectors reported to regulate skin coloration in various fish species is listed in Table 1. In principle, the biopsies are immersed in such hormones and the colour effect monitored. In our studies, we used abdominal biopsies which were excised so that they included the ventral and some of the lateral skin. Cutting the biopsy down the middle allows it to be used in both treatment and control. The biopsies were placed in saltwater or in Atlantic cod Ringer´s saline at pH7.5 (150 mM NaCl, 5.2 mM KCl, 1.8 mM $MgSO_4$, 7.0 mM NaHCO, 1.9 mM NaH_2PO_4 and 1 mg/ml glucose). In saltwater, melanophores, erythrophores and xanthophores become fully dispersed. In the Ringer's saline, the chromatphores are more or less dispersed. In order to trigger the pigment aggregation response, the biopsies can then be transferred into Nunc plastic wells (VWR International AB Stockholm) containing either Atlantic cod Ringer´s saline as a control, or freshly diluted 10 μM melatonin or norepinephrine (Sigma Aldrich, St. Louis, MO, USA), to induce pigment aggregation (Nilsson Sköld et al., 2008). Norephinephrine appears to be a general pigment aggregating agents across species, but melatonin varies in effectiveness among species. We

had often problems with the consistency of the effect of epinephrine or melatonin in natural saltwater, but defined solutions such as Atlantic cod Ringer's saline were more reliable. The biopsies should be incubated in dark during the treatments since chromatophores in many (but not all) fish species are light sensitive. The light intensity also needs to be standardized during microscopy. It is advisable to do paired treatments where each fish produces both the control and the treatment biopsy. Both the epidermal and peritoneal side of the abdominal biopsies can be tested at the same time, if of interest. Cutting and pinching with forceps can cause cell damage, so the cell morphology and response to treatments are not representative in those parts of the biopsy. Soft insect forceps are advisable to use when handling the biopsies or scales.

The rate of colour change can be categorized subjectively under an inverted brightfield microscope using the Melanophore Index, MI (Hogben and Slome 1931), where fully aggregated melanosomes give MI = 1 and fully dispersed melanosomes give MI = 5. Occasionally, a hyperdispersed state can occur where the pigment is located only at the extremities of the cells, and this is given MI = 6 (Nilsson 2000). See figure 3 for illustration of the melanophore index. The MI shall be monitored just prior to applying the treatments, and at regular intervals thereafter; for example at 5, 10, 15, 20, 25, 30, 60, 120 and 180 minutes. Sometimes it is sufficient to use longer time intervals. In some species, the reaction occurs within minutes but in others it is slower. However, in most species the response is complete within one hour. Pilot observations testing a suitable timing of observations and hormone concentrations, is advisable when investigating a new species for the first time. The MI index works best for melanophores and erythrophores. For skin xanthophores, the aggregated state is easily identifiable but the different levels of dispersal are less distinct. MI is also less applicable when analysing whitish chromatophores. An alternative to MI scores is to quantify the coloration of the biopsy (see 2.2). However, a hyperdispered state will not then be distinguishable from MI 2-3. Although we have not ourselves investigated the rapid colour change of iridophores and its effects on the skin coloration, detailed information of the technical procedures for such investigations is given in an excellent study on the spectacular blue damselfish (Kasukawa et al., 1987).

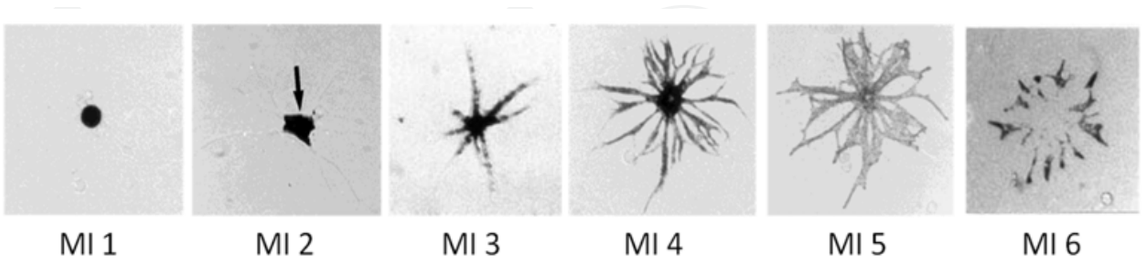


Fig. 3. Description of the Melanophore Index (MI) used to manually score the degree of pigment dispersal in fish melanophores. Arrow points at MI 2 which is similar to the shape of a deformed central pigment mass. MI 6 is normally a reversible state after a fast dispersal or an artefact seen after inhibition of dynein (in this case) or after modulation of the actin network. Damaged cells in the periphery of biopsies can also be hyperdispered. The depicted cells are primary cultures of cod skin melanophores.

Aggregation :	Colour response	Dispersion :	Colour response
Melatonin	pale	Adrenalin	dark
Noradrenalin	pale	Nitic Oxide	dark
Endothelins	pale	MSH	dark/red/yellow
MCH	pale	Prolactin	(dark)red/yellow
Light	pale	ACTH	dark/red/yellow
		UV	dark
		Light	dark
		*T/11kT	dark/red/yellow
		*E2	dark/red/yellow

Summarized from Fuji and Oshima, 1994; Fujii, 2000; Nilsson Sköld et al., 2008

Table 1. Extracellular signals reported to mediate fast or slow colour change in skin of fish. The colour response refers to the appearance of the skin. *The steroid hormones regulate morphological but not physiological colour changes directly. The other signals and hormones listed mediate both physiological and morphological colour change.

Skin explants in cultures have successfully been used for treatments with non-cell permeable agents to investigate intracellular mechanisms behind maintenance of the circular central pigment mass (CPM) and melanosome movements (Nilsson and Wallin 1997). The culture procedure makes the melanophores more exposed to the agents compared to the original biopsy, and the colour change reaction therefore occurs much faster. We here describe our own procedures for testing the aggregation inhibitory effects of the phosphatase inhibitor and putative dynein inhibitor vanadate. The same experimental principles can be used to assess effects of other agents, cell permeable or non-cell permeable. Skin pieces of about 1 mm^2 were prepared from Atlantic cod dorsal fins and allowed to adhere to the bottom of sterile plastic culture dishes in one drop of medium for about 1 hr before addition of a cell culture medium (medium 199 with Hank's salts supplemented with 10% fetal calf serum, 1% antibiotic/antimycotic solution, and 1% L-glutamine, all from Life Technologies, Renfrewshire, Scotland) and further incubation at 12°C. To facilitate permeabilization of cell membranes to the otherwise non-cell permeable agent vanadate, 0.02% of the mild detergent saponin was included in the lysis buffer during subsequent experiments, as described below. The experiments were performed the day after at 12°C in medium 199, or in lysis buffer at pH 7.0 made according to Grundström et al. (1985). Pigment aggregation was induced by addition of norepinephrine to the medium. Explants were tested before the treatments for the aggregation capacity, and explants that did not respond by pigment aggregation within 3 minutes were discarded. If the treatment induced aggregation, pigment granules were allowed to redisperse in fresh medium until the next day. Explants were then incubated in different concentrations of vanadate for 10 minutes in lysis buffer with saponin. The time of treatment was standardized to 10 min, as longer time in 0.02% saponin was found to inhibit the norepinephrine-induced pigment aggregation. Controls were treated with lysis buffer with or without saponin. Norepinephrine was added and the rate of aggregation monitored every 30 seconds for 6 minutes.

To investigate if the cytoskeleton is involved in maintenance of the spherical shape of the CPM (i.e. maximal aggregation), we tested the effects of microtubule and actin inhibitors in the shape of the CPM. For these experiments, the melanophores on the biopsy cultures were induced to aggregate pigment completely by addition of norepinephrine. After this, microtubule or actin disrupting agents were added for 2 hours of incubation in the presence

of norepinephrine. Controls were incubated in norepinephrine only. The skin explants were then fixed in ice-cold methanol for 6 minutes and rinsed in phosphate buffered saline for subsequent scoring of the amount of deformed versus spherical CMP´s on each explant. The results showed that microtubules, but not actin (not shown), maintain the spherical shape of the CPM (Nilsson and Wallin, 1997). Also here, the non-cell membrane permeable agent vanadate was added at different concentrations, now together with norepinephrine in the saponin containing lysis buffer. Controls were treated with lysis buffer with or without saponin. The treated explants were fixed after 10 minutes of treatment and the degree of aggregation (MI) evaluated as described above.
To investigate possible effects of vanadate on dispersion, melanophores were first induced to aggregate pigment for 4 minutes and then incubated for 10 minutes in vanadate together with epinephrine in lysis buffer with saponin, or in lysis buffer only with or without saponin together with 1 µM NA as controls. Measurements of dispersion were performed after removing the NA by rinsing the explants with lysis buffer including only vanadate, or lysis buffer without vanadate for the controls. The MI was scored every following minute until controls were fully dispersed (after approximately 20 minutes). We then statistically compared both time for onset of dispersion, which was faster in presence of vanadate, and the rate of dispersion, which did not differ from controls. Other ways to initiate dispersion than rinsing off norepinephrine, is to add agents that elevate intracellular cAMP levels such as the cell-membrane permeable forskolin or the melanocyte stimulating hormone (MSH) to the buffer. This speeds up dispersion to be completed within 5-10 minutes.

2.1.2 Scales

Scales have been widely used to investigate regulation of rapid colour change using both melanophores and erythrophores. Many scales can be collected from each fish, which allows multiple treatments and controls to be applied while also controlling for inter-individual differences. This is very useful when analyzing dose response effects. In our studies (Nilsson 2000; Andersson et al., 2003) scales from the area of interest were collected with fine forceps and placed in either Atlantic cod Ringer's saline or in phosphate buffered saline (136.9 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4 at pH 7.3). Fish scales can also be collected into culture medium for weeks of storage, but this has reported to create some response artifacts (Mårtensson and Andersson, 1997). In either condition, melanosomes and erythrosomes become fully dispersed within the chromatophores. Aggregation, shape of the CPM and dispersion can be tested using different hormones and inhibitors, as for the biopsies described above, and distribution of pigment scored under microscopy using the MI index. The reactions are slightly faster on scales than when using biopsies. As with the biopsies, care shall be taken to standardize the light intensity and the time of light exposure. Not all chromatophores within a scale will start translocation of pigment organelles simultaneously. The cells around the edges will often react first, so for reliable results, score either only the cells around the edges or use an average MI of the entire scale.

2.1.3 Melanophore primary cultures

For a direct assessment on the role of different molecular motors, second messengers, receptors or extracellular signals on the chromatophores, these cells need to be isolated. For this purpose, we developed and refined a procedure to obtain primary cultures of cod (*Gadus morhua*) skin melanophores (Townsley et al. 1963; Nilsson et al., 1996; Nilsson and Wallin, 2007). We here describe the final protocol and some of the experiments we did.

A slice of a dorsal fin is removed and placed in a sterile glass dish with cell culture medium 199 with Hank's salts and the supplements described above for the biopsies. Tissue pieces of about 1 mm^2 are cut and placed in a drop of the medium on sterile glass or plastic coverslips in a sterile culture dish. The explants are kept at 12°C for about an hour without drying in order to enhance the adhesion before further addition of medium. The cultures are incubated in darkness at 12°C and medium changed twice a week. A monolayer of skin cells including melanophores are under these conditions formed around the fin explants. In order to obtain more isolated and free melanophores, the medium is changed to serum-free CO_2-independent medium supplied with 1% antibiotic/antimycotic solution and 1% L-glutamine (Life Technologies), 5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml sodium selenite (Boehringer Mannheim, Mannheim, Germany) and 1 mg/ml glucose. After a few days in this medium, most cells except melanophores will detach from the coverslips. Isolated melanophores could be kept for weeks under these conditions if the medium was changed twice weekly. In both mediums, pigment was evenly dispersed in the melanophores. Pigment aggregation is then induced with micromolar concentrations of noradrenaline and pigment dispersal by removal of the noradrenaline by adding fresh culture medium. For a faster dispersal, the cAMP elevating agent forskolin could be added. Melanophores isolated by this procedure were used for immunocytochemistry, microinjections of anti-dynein antibodies, melanosome trackings and various other manipulations. Altogether, melanophore primary cultures have experimentally been a very powerful system for the present theory of the intracellular transport mechanisms in these cells – from the cytoskeleton to second messengers and receptors (Nilsson et al., 1996; Nilsson and Wallin 1997, 1998; Aspengren et al., 2003; Nilsson Sköld et al., 2003).

2.1.4 A fixed-time in vitro melanosome motility assay

As a complemental method to microinjections and other cell based methods for investigating intracellular aspects of melanophore colour change, we developed fixed-time in vitro melanosome motility assay that mimics the cellular situation. The preparation and application of melanophore lysate, including the MI scoring and photography, can all be done within one working day. The method is originally described in Nilsson et al. (2001).
Melanophores from dorsal scales of *Fundulus heteroclitus* were used to prepare melanophore lysates. These lysates were then used for the fixed-time in vitro melanosome motility assay. The scales were liberated from several individuals according to a method described in detail by Haimo (1998). In brief, the scales were rinsed three times in Marine Ringer's containing 50 mM ethylenediaminetetraacetic acid (EDTA), transferred into 15-ml falcon tubes with 5 ml of Marine Ringer's containing 5 mg/ml collagenase type 2 and 10 mg/ml bovine serum albumin and mixed slowly with a rotary shaker for 40 minutes at room temperature. Scales were allowed to settle, the supernatant collected and the melanophores pelleted by centrifugation at 500*g* for 5 minutes at room temperature. The supernatant was collected and added back to the scales. The melanophore pellet was carefully resuspended in cold freshwater Ringer's (Haimo, 1998) containing protease inhibitors (leupeptin, pepstatin A, aprotinin, and chymostatin each at 10 mg/ml and 1 mM phenylmethylsulphonyl fluoride). Solutions were prepared once a week and oxygenated 15 minutes before use. At this stage, the isolated melanophores contained dispersed pigment. In some treatments, cells were treated with isobutylmethylxanthine or forskolin (agents known to increase intracellular cAMP levels and thus facilitate dispersion) for 15 minutes before lysis. Aggregation was induced by incubation of the cells with norepinephrine for 30 minutes. Aggregation was not

always successful after liberation. Only preparations where 80% of cells aggregated their pigment within 30 min were used for the subsequent fixed-time in vitro motility assay. In the next step, the liberated melanophores were pelleted and resuspended in a small volyme of ice cold IMB50 buffer containing protease inhibitors, a buffer previously developed for an in vitro motility assay with frog melanosomes (Rogers et al., 1998). In our case either norepinephrin to induce aggregation or the combination of IBMX and forskolin to induce dispersion, were added to the buffer. Melanophores were then lysed in the buffer by 5–10 passes through a 22s gauge syringe. Cell fragments and remaining dermis were pelleted by centrifugation at 500*g*, for 5 minutes. The supernatant containing the melanosome solution was handled carefully to avoid bubbles and 100 µl pipetted using a tip with a cut end. Everything was kept cold during the procedure.

KCl activated oocytes of the clam, *Spisula solidissima* were used to produce lysates with microtubule asters for the motility assay (Palazzo et al., 1988) and kept as aliquots at -80 °C. Such oocyte lysates contain centrosomes that spontaneously nucleate microtubules arrays when warmed. For visualization of the asters, 0.1 mg/ml of rhodamine-labeled bovine tubulin (Cytoskeleton, Inc., Denver, CO) was added to 20 µl of ice-cold activated lysate. The mixture was then warmed to room temperature for 15 min to allow aster formation. The mixture was then diluted 1:5 into IMB50 buffer containing 10 mM of the microtubule-stabilizing agent taxol (Sigma Chem Co.) and the antifade agent oxyrace (Oxyrace, Inc., Mansfield, OH) at 1:100, and placed on ice. The mixture was then diluted 1:2 into the freshly prepared melanophore lysate and this mixture was then divided into aliquots for each subsequent treatment, for example addition of an inhibitory anti dynein antibody. For the in vitro pigment translocation to start, ATP was added to 2 mM. The total assay volume for each treatment was 10 µl. Each reaction mixture were then placed on a glass cover slip, incubated as a droplet at room temperature for 30 min in a humid chamber, and then overlaid with a glass slide. No pre-coating of the glass was necessary. The distribution of pigment on the asters in the different samples was immediately scored and recorded using a microscope with a 63x magnification. With this procedure, more than 50 asters could be scored on each slide. Melanophore lysates prepared from dispersed melanophores induced dispersal of pigment in vitro, and melanophore lysates prepared from aggregated melanophores induced aggregation of pigment in vitro. Addition of an inhibitory anti dynein antibody blocked the in vitro aggregation and pigment ended up in the periphery of the aster (Nilsson et al., 2001), similar to what happened after microinjection of the antibody into cultured cod melanophores (Nilsson and Wallin, 1997; Nilsson Sköld et al., 2002).

2.2 Measuring biopsy coloration and transparency

In this section, we will describe how to measure coloration and colour change with digital photography and computer-aided image analysis. The methods described have been used by us in the following papers: Svensson et al. (2005), Sköld et al. (2008), Svensson et al. (2009a,b) and Nilsson-Sköld et al. (2010), but also by others (Heflin et al., 2009). They are applicable to both biopsies and whole (living) fish.

2.2.1 Digital photography and colour

To quantify the degree of pigmentation in fish tissues, we developed methods to analyse the transparency and colour intensity of biopsies as well as whole animals. Many different approaches are possible for this task, all with their own limitations. Spectrometry and microspectrometry have long been the colorimetric methods of choice for many biologists

because of their objectivity and wide spectral range. However, carefully standardized digital photography has been put forward as a strong alternative (Stevens et al., 2007). After much experimenting, our choice fell on digital photography coupled with image analyses in the CIE Lab colour space (Svensson et al., 2005; Sköld et al., 2008; Nilsson Sköld et al., 2010). Importantly, photography is much faster than spectrometry, which is advantageous when dealing with tissues prone to rapid colour change. To obtain a photograph takes only a few seconds, but to take multiple readings with a spectrometer can be very time consuming. Photographs can also easily be taken without having to remove the biopsy from a reagent solution. This can be important in repeated measures and time-series designs. Furthermore, digital photography allows measurements of pre-defined areas by using anatomical landmarks on the biopsy/animal. Another benefit is that images can be analysed and re-analysed at a later date, allowing the researcher to decouple, in space and time, colour quantifiaction from the experimental work. Photographs also offer superior spatial resolution, which is crucial for analysis of complex colour patterns (Stevens et al., 2007). By using all the pixels inside the selected area, rather than a few point samples, one can obtain very precise estimates of colour parameters. It is important to point out that digital cameras have potential issues with non-linearity and spectral bias, which may affect the measurements. In many situations this may not be a great concern, for example when the degree of colour difference is relatively small. However, non-linearity and spectral bias can be formally addressed through calibration procedures, making the colorimetric data equal in quality to spectrometry, as described by Stevens et al. (2007). A potential disadvantage of photography is a limitation to the wavelengths of human colour vision. Importantly, certain fish colour patterns are only observable in the UV. However, cameras that can detect UV and IR light are now commercially available (e.g. the Fujifilm FinePix S3 Pro UV IR and a range of specialist cameras). For examples and methodologies for assessing the little studied UV-aspects of fish coloration, we refer to the excellent work by Siebeck (2004).

When measuring coloration it is important to realize that colour is not a physical property of an object but a property of the visual system of the observer (Grether et al, 2004). Quantifying colour in a way that is relevant to the animal in question may therefore be non-trivial. It is important to choose a method that is suitable for the question and study species at hand. Consequently, the methods described here may not be appropriate in all circumstances; they should rather be seen as a starting point for method development. The painstaking process of finding the best way to quantify coloration will ultimately have to be done anew for any new study system (Dawson, 2006). The aim should be to arrive at a method that maximizes relevance, accuracy and precision of the measurements. In other words, the obtained data should have both high within-specimen repeatability and high between-specimen variation.

2.2.2 Image analysis

After digital photographs have been taken, the information contained in them has to be converted to data. The simplest method is to let a panel of people score the photographs. However, digital image analysis provides a finer discrimination than human observers (Villafuerte & Negro, 1998). The next question is what to measure. There are several different colour parameters in various colour spaces available. For example, some early studies quantified red-orange colour intensity using a red index, Ri = R / (R+G+B), based on the RGB (Red, Green, Blue) colour space (Frischknecht, 1993; Barber et al., 2000; Pelabon et al., 2003). Other studies have used cylindrical representations of the RGB colour space, such as HSL,

HSB and HSV (Hue, Saturation and Lightness/Brightness/Value) (Hatlen et al., 1998; Nordeide, 2002). The RGB-colour space is device dependent and countless versions exist, even within the same software (e.g. Adobe RGB, apple RGB and sRGB IEC61966-2.1). In contrast, CIE L*a*b* is a standardized, perceptually uniform and device independent colour space (Chen *et al*, 2004), designed in 1976 by the International Lighting Commission (Commission International de l'Eclairage, CIE). CIE Lab measurements based on spectrometry have become standard in the field of animal coloration (e.g. Skrede & Storebakken, 1986; Smith et al., 1992; Craig & Foote, 2001), but few studies have applied CIE Lab to quantify animal coloration from digital photographs. Adobe Photoshop™ has a colour space called "Lab colour mode" which is based on CIE Lab. As it name suggests, it consists of three parameters: L*, a* and b*. In Photoshop, the asterisks have been dropped, but for clarity, we have chosen to retain them. The L* channel stands for lightness (or luminosity) and describes the relative lightness from total black to total white. In an 8 bit/channel image, L* will range from 0 (black) to 255 (white). The a* channel is the balance between green and magenta (Margulis, 2006). Thus, a* ranges from 0 (pure green) to 255 (pure magenta), while a* = 128 (or, more specifically 127.5) describes a neutral grey. The b* channel describes the balance between blue and yellow, where 0 is pure blue, and 255 is pure yellow, while b* = 128 (127.5) describes a neutral grey. Typically, however, Lab values are expressed differently. L* is often described as ranging from 0 to 100, while a* and b* range from -100 to 100, with zero corresponding to neutral grey. It is easy to convert 8-bit a* and b* values to this scale by the following formula: $a^* = (a^*_{8bit} - 127.5) / 1.275$. This conversion is important if one aims to use a* and b* values to calculate chroma or hue (Little, 1975):

$$\text{chroma} = \sqrt{a^{*2} + b^{*2}} \quad (1)$$

$$\text{hue} = \text{atan}(b^* / a^*) \quad (2)$$

A major advantage of Lab colour is that it separates colour from contrast (lightness), as opposed to RGB where all three channels affect both these parameters. Thus, a* and b* affects only colour while L* affects only contrast. In theory, the a* and b* channels are therefore completely insensitive to variations in light intensity. However, even when analysis takes place in Lab, the image capture does not, because all digital cameras use a RGB sensor to capture the image. Differences in the light intensity of the image can therefore affect both contrast and colour information. This is a major reason why the photographic conditions must be highly standardized if one is to accurately quantify biopsy coloration. A second source of concern is that the algorithms used to convert RGB to Lab may differ between software versions. For example, an a* value obtained from Photoshop 4.0 will not exactly match a value from Photoshop 5.0. In more recent software versions, however, ICE colour management can be used to control the RGB-Lab conversion.

When choosing how to quantify colour, it is important to select colour parameters that maximize relevance, accuracy and precision for the investigation at hand. For example, we found untransformed values of the a* channel to be superior in describing the orange belly ornament in female *G. flavescens*, as verified by a strong relationship between a* and actual pigment concentration (measured by HPLC, Svensson et al., 2005; Svensson et al., 2006). For blue and yellow hues, the b* channel may be more relevant. In other circumstances, parameters like chroma and hue (which take both a* and b* into account) may prove a better option. When quantifying the transparency of biopsies and/or the effect of melanophores, the L* channel is typically the most relevant parameter (see below).

2.2.3 Standardization and setup

As already mentioned, it is important that the entire photographic procedure is standardized. For example, one should minimize variation in the handling time of the biopsies when taking the photographs. It is also vital that all camera settings are kept constant. This includes the light conditions, the lens zoom setting, flash settings and, on the camera itself: shutter speed, aperture, ISO, white balance and image file storage settings. The exposure should be set so that it maximizes the dynamic range of the image file. This means that the lightest area (typically the background) should be close to 100% exposure, without being overexposed. Likewise, the darkest area (typically the zero-light reference) should be close to 0% exposure. In other words, the tonal histogram of the biopsy should not be cut off at either end. Ideally, images should be saved without any in-camera processing (i.e. saving in "camera raw" format), and subjected to a standardized conversion to the target format (e.g. tiff or jpeg) before analysis. The image analysis should, if possible, be done blindly so that the person analysing images is unaware from which treatment they originate.

We have used two camera setups, either a Canon D30 dSLR camera with Canon 50 mm f/2.5 EF Compact Macro lens (Svensson et al., 2005; Sköld et al., 2008) or a Nikon D80 dSLR camera with a Nikon 105 mm AF Micro Nikkor lens (Nilsson Sköld et al., 2010). If dealing with small biopsies it is important to choose a camera/lens with good macro capabilities. Alternatively, cameras can be mounted on a dissecting microscope.

2.2.4 Method to quantify biopsy transparency

To measure the transparency of skin biopsies (figure 4), we used back-lit digital photographs and quantified the lightness channel, L*, (Svensson et al., 2005). This method is also good at detecting pigmentation changes caused by regulation of melanophores. In relatively small-bodies fish species, the same method can be used to quantify the transparency of live animals (figure 5).

Biopsies were placed on a light table, ideally still submerged in their respective treatment reagent. If necessary, the biopsies were flattened with a glass slide. When quantifying transparency of whole (live) animals, the fish were quickly transferred and placed either ventrally of laterally on the light table. The picture was taken with the camera affixed directly above the sample, with an opaque object placed next to it for calibration purposes (a zero-light reference). It is important that photography takes place in a dark room, or that the whole setup is covered by dark fabric, so that the light table is the only light source. Care should be taken that all biopsies are placed on the same area of the light table. One should also verify that the light levels do not change over time (e.g. from ageing fluorescent lights).

The image analysis was carried out in Adobe Photoshop, and photographs converted from RGB colour to Lab colour space. We further standardized the tonal range in the image by setting the centre of the opaque area to L* = 0 and the background to L* = 255. In Photoshop this is done with the pipettes in the curve or levels tool. The area of interest was selected with the polygon lasso tool with zero feathering. After the selection was made, the mean value of L* in the selected area was noted (in Photoshop this is given in the 'histogram/all channels view' dialogue). Lightness was converted to a measurement of transparency by calculating the percentage of L* in the selected area relative to the L* of the background (transparency = $100 \times L^*_{biopsy} / 255$).



Fig. 4. Examples of photograph used to quantify abdominal skin coloration and transparency in *G. flavescens*. After excising the biopsy, it was placed next to a black piece of plastic (zero-light reference). It was placed on a light table and photographed with the light shining through the skin. The dashed line indicates the two areas selected for analysis (Sköld et al., 2008).

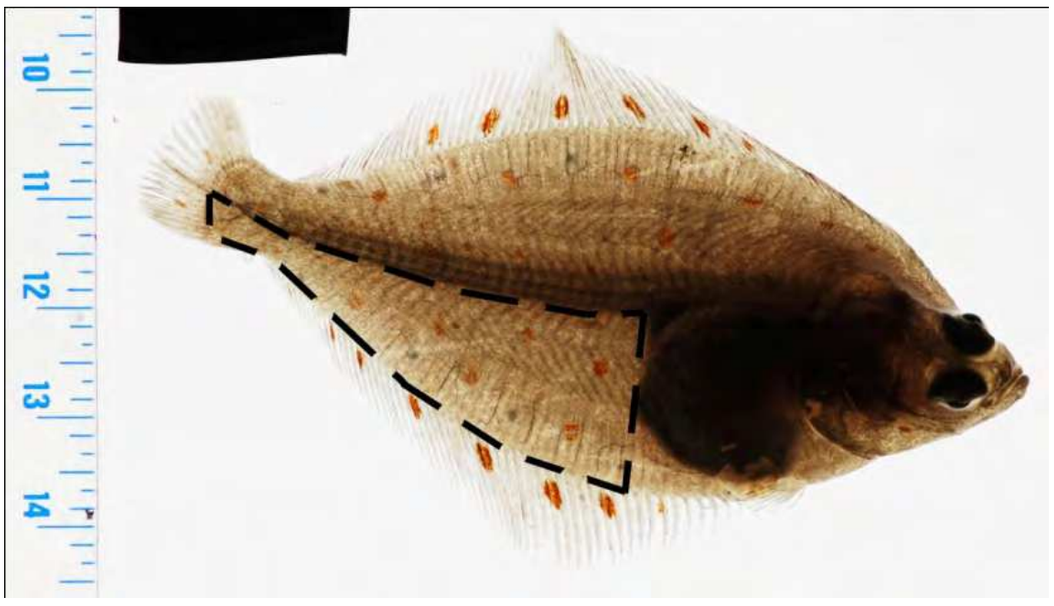


Fig. 5. Example photograph of whole-body transparency in juvenile plaice *P. platessa*. The live fish was placed on a light table and photographed with the light shining through the skin and body. The dashed line indicates a pre-defined area selected for analysis (Nilsson Sköld et al., 2010).

2.2.5 Method to quantify biopsy coloration

The coloration of a biopsy may be measured with the light passing through the biopsy. In this case the same method as described above (2.2.4) was used (figure 4). Alternatively, the biopsy may be lit from above (figure 6). For illumination our choice fell on photographic flashes, which are consistent, broad-spectrum light sources. We used two flash heads, fired at obtuse angles to avoid glare. Having the biopsy submerged and using diffusing flash filters may reduce glare further. It is important that the settings and position of the flash is kept constant, and that it is allowed to recharge fully between photographs. Apart from a matte black object as a zero-light reference one can use cut-outs of colour standards (e.g. Kodak™ Q13 colour separation guide) to assist in calibration. After converting the RGB image file to Lab and selecting the area of interest, the mean a* and b* values were noted. These values were used to calculate chroma and hue as described above.

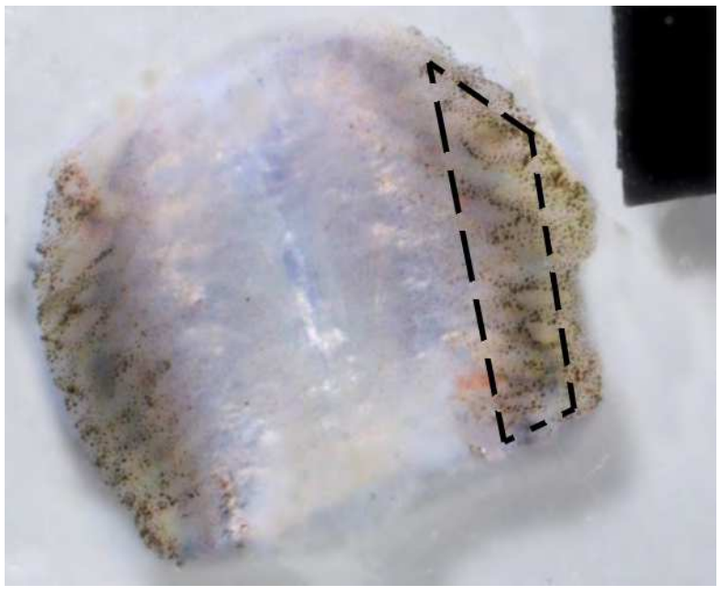


Fig. 6. Examples of photograph used to quantify abdominal skin coloration in *G. flavescens* after exposure to norepinephrine. In this case, the biopsy was lit from above with dual flash heads. The dashed line indicates the anatomically predefined area analysed.

3. Some notes on statistics

A major benefit of using scales or excised biopsies is the ability to apply treatments that would be impractical or unethical to administer to live animals. A major limitation is the inevitable, and non-randomizable, effect of time passing from the moment of euthanasia/excision. Time effects can be addressed with appropriate controls, for example by comparing "time only" with "time + reagent". A statistically powerful method is to collect several scales or to divide biopsies and to then expose the sub-samples from the same individual to different treatments. Such within-subject designs, also called block designs, can be analysed by using the identity of the animal as a random factor in a mixed model. In an experiment with only one factor (treatment) at only two levels, this will be equivalent to a paired t-test.

MI-scores and data describing the shapes of the CPM's are subjective by nature. It is therefore advisable that the same person do all such scoring within a study. Ideally, the person that scores shall also be blind with respect to treatments.
Percentages, and many colour parameters, such as L*, a* and b*, will always be bounded by a maximum and a minimum value. This may cause data to be non-normally distributed, especially if many values are close to the upper, or lower, limit. An arcsine square root transformation may improve normality in these cases (Crawley, 2007). The following formula is used will return a transformed value in degrees (i.e. ranging from 0 to 90):

$$transformed\ value = \frac{180}{\pi} \times \operatorname{asin}\left(\sqrt{p}\right) \quad (3)$$

where p is the parameter converted into a proportion (i.e. ranging from 0 to 1).
For the determination of differences in amounts of spherical shapes of CMPs as well as for differences in of the rate of colour change reaction, we used factorial analysis of variance with treatment as fixed factor, individual fish as a random factor and percentage deformed CPMs or slope of MIs/minute during the linear phase of colour change reaction as response variables. MI score at a specific time point can be used to measure of the rate of a response, but as the data are discrete, non-parametric tests are typically more suitable.

4. Conclusions

We have described several different ways in which skin biopsies can be useful for studies of animal coloration and the mechanisms for colour change. Manipulations and observations of skin biopsies have given us an increased understanding of regulation of rapid colour change in general, and have been successfully applied to the study of colour signals in animals. Similarly, by using abdominal skin biopsies (containing both epidermis and the peritoneum) we have acquired novel insights into the functions of internal pigment cells in transparent fishes. At a cellular level, fish biopsies has been successfully used to isolate melanophores for immunocytechemistry, to trace the movements of individual melanosomes, for microinjections and other more direct manipulations. This cell-based approach has been instrumental in the construction of the current physiological model for rapid colour change in fishes. This model includes the function of the cytoskeleton and the coordinated actions of various molecular motors, and has increased the understanding intracellular organelle transport and positioning in all cell types.
However, many things remain to be discovered, and current methods have important limitations. In fish, the type of kinesin used in pigment dispersal during rapid colour change is still unidentified. We are yet to understand how the pigment organelles at an intracellular level are maintained in an evenly dispersed state. Although the important roles of cAMP and its downstream protein kinase A in pigment movement are clear, the possible roles of other second messengers, including the exact functions of calcium, are far from understood. From image analysis of skin biopsies, changes in a* and b* channels are thought to reflect changes in erythrophores and xanthophores, respectively, but this needs to be verified though measurements of isolated pigment cells. Many fishes perform rapid and complicated colour modifications during specific behaviours (such as courtship displays). Our photographic/microscopic techniques are not well suited for quantification of such ephemeral signals. Likewise, the quantification of other types of coloration, such as iridescent and UV colours will require a refined methodology. The use of skin biopsies as a

tool to address questions from cell physiology to animal behaviour does hold a lot of promise. We strongly believe that future studies will lead to many exciting discoveries.

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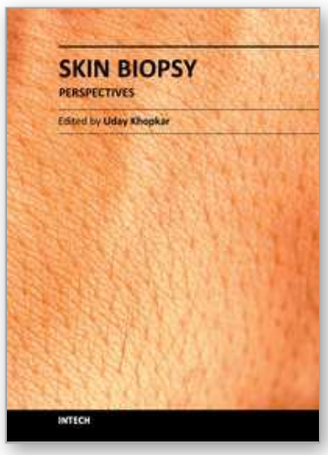
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Skin Biopsy - Perspectives

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Skin Biopsy - Perspectives is a comprehensive compilation of articles that relate to the technique and applications of skin biopsy in diagnosing skin diseases. While there have been numerous treatises to date on the interpretation or description of skin biopsy findings in various skin diseases, books dedicated entirely to perfecting the technique of skin biopsy have been few and far between. This book is an attempt to bridge this gap. Though the emphasis of this book is on use of this technique in skin diseases in humans, a few articles on skin biopsy in animals have been included to acquaint the reader to the interrelationship of various scientific disciplines. All aspects of the procedure of skin biopsy have been adequately dealt with so as to improve biopsy outcomes for patients, which is the ultimate goal of this work.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
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Unit 405, Office Block, Hotel Equatorial Shanghai
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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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