Chapter 2
Methods used to study butterfly wings and wing scales

The methods and systems used during the present investigation are described. An innovative method to catch single scales with a microelectrode-like glass micropipette is presented. The equipment and techniques used to study the optical properties of butterfly wings as well as that of single wing scales are described. The back- and forward-scattering of intact and denuded wings were measured with an integrating sphere. For single scale analysis, a microspectrophotometer was used to study the transmitted as well as reflected light. Furthermore, a special optical set-up for studying the angular distribution of reflected and transmitted light by single scales is described. The anatomical structure of butterfly wing scales was studied with scanning and transmission electron microscopy.

2.1 Catching scales

Measuring the optical properties of single butterfly scales requires the possibility to accurately position the sample in the optical set-ups. We therefore developed a method to catch single scales, which includes the following steps:

1. Isolation of scales from the wing substrate. As described in Chapter 1, butterfly wing scales are sustained from their base to the remaining cell in the wing substrate. To separate the scales, the wing is gently pressed upon a glass microscope slide.

2. Production of micropipettes. Using a microelectrode puller, a glass tube is heated in its centre and pulled apart, which yields two usable micropipettes. The puller variables (heat, velocity, pull strength) have been set so that a long tip with a diameter of approximately 5 µm results. One micropipette is put in a micromanipulator and its tip smeared with glue (not fast drying).
3. **Positioning of micropipette and glass slide under the stereoscope.** The glass slide with scales is placed obliquely under the stereoscope (Fig. 1a) and the pipette is moved slowly, approaching the scales (Fig. 1b).

4. **Selection of scale suitable to catch.** Most of the times it is useful to have the scale with its ridges either perpendicular or parallel to the pipette. A scale that approximates one of the two ideal situations is selected (Fig. 1c). The scale is approached until it sticks to the pipette with glue, so that it can be removed from the slide.

5. **Use of a second pipette to manipulate the scale.** Even though the best scale has been selected, it often needs adjustment. This is done with the aid of a second (clean) pipette, which is kept vertical and stable.

The final result is a single scale (or two, as in Fig. 1d) conveniently sustained by a very thin holder that can be translated and rotated. Different types of holders were used (e.g. polished needles and eyelashes), but a microelectrode-like glass pipette showed the best results, because a very thin tip, which is still flexible and stable, is easy to produce. Furthermore, the electrostatic charge created by touching the tip lightly with the fingers is very helpful during the preparation of samples for scanning electron microscopy (Section 2.3).

![Fig. 1. Sequence of the procedure to catch single scales.](image)
Methods used to study butterfly wings and wing scales

The scales in Fig. 1d are conveniently placed in such a way that rotation of the pipette results in rotation around the longer and the shorter axis of the scale, respectively. Those directions are convenient in optical measurements, because the ridges, which run along the longer axis, frequently cause diffraction of light. This can result in a linear pattern of the reflected light (e.g. *Morpho aega*) or in a transmitted diffraction pattern (e.g. *Dione juno*). In order to study these optical effects, an accurate adjustment method is necessary.

2.2 Spectrophotometry

Spectrophotometric measurements are performed on a given sample in order to know how it interacts with light. To study the spectral properties of single scales, a microspectrophotometer was used. For larger samples (a few millimetres of diameter) a fibre optic connected to a probe was used. Most of the samples do not behave as good diffusers and thus light is partially specularly reflected. To collect all the light reflected (back-scattered) or transmitted (forward-scattered) an integrating sphere can be used. To know precisely the angular distribution of the scattered light, an optical setup which consists of aligned lenses and a goniometer was used. In the following, this equipment is briefly described.

2.2.1 The microspectrophotometer

A microspectrophotometer (MSP) is fundamentally a microscope combined with a spectrophotometer. Its advantage is that microscopical areas can be accurately measured.
Butterfly wing scales - pigmentation and structural properties

Fig. 3. Integrating sphere. Diagram of a reflectance and b transmittance modes. c Photograph of the integrating sphere, set in transmittance mode. The incident light is focused on the sample with a microlens. After multiple scattering on the inner surface of the sphere, the reflected (transmitted) light is collected with a fibre optic and conducted to the spectrometer. The arrow in (c) shows the socket where the fibre, F, is screwed on for measurements in reflectance mode.

selected. Furthermore, transmittance as well as reflectance by epi-illumination can be studied. The latter means that incident light as well as reflected light pass through the objective of the microscope. Fig. 2 shows a diagram of the MSP. Light emitted from a broad band Xenon lamp is collected by a lens and filtered if necessary. A half-mirror deviates the beam, which then passes through the objective to be focused on the sample. After the area of interest has been selected by a diaphragm, the reflected light is collected by the objective and focused on a fibre optic. Transmitted light can also be studied by redirecting the light with mirrors and focusing it on the sample (dashed lines in Fig. 2). The path is then the same as for the reflected light.

2.2.2 The integrating sphere
Independently of their characteristics, scattered light can be effectively collected by using this equipment. It consists of a sphere, the inner surface of which is coated with a highly diffusing reflective material (e.g. spectralon). Three small holes exist for placement of the sample and two fibre optics; one for the incident light and one for the collector (Fig. 3a). Transmitted light can also be measured by changing the incident light fibre to the position indicated in Fig. 3b. The integrating sphere is particularly useful for large samples. Single scales, however, require a very long measuring time.

2.2.3 The angular-distribution setup
The angular distribution of the reflected (and transmitted) light reveals the diffuser and specular characteristics of butterfly wing scales. Experiments can be performed for single scales as well as for scales in situ. By using this setup, reflectance and transmittance spectra in a plane over a 360° angle can be measured.
Methods used to study butterfly wings and wing scales

Fig. 4 Diagram of the optical system used for measuring the angular distribution of the scale reflectance. Light from a light source is focused on a pinhole, which is imaged on the scale. A white screen with a small hole is placed in between the imaging lens and the scale. The light reflected by the scale causes a light pattern on the screen, which is photographed. The light reflected by the scale is collected by a lightguide, which relays the captured light to a spectrometer. The lightguide is mounted on a stage rotating in the horizontal plane (top view drawing).

Fig. 4 shows a diagram of the angular-distribution setup. Light from a light source is focused on a very small pinhole (typically 30 µm) and imaged on the scale. In order to know the spatial distribution of the light reflected by the scale, a white screen with a small hole (to let the incident beam pass through) is placed between the imaging lens and the scale (Fig. 4a). For transmitted light, a semitransparent paper is placed behind the scale. Light is reflected (transmitted) by the scale on the screen and its pattern can be photographed. To measure the reflectance spectra as a function of angle, the reflected light is collected with a fibre optic, which is mounted on a rotating stage and then is relayed to a spectrometer.

2.3 Scanning electron microscopy

For scanning electron microscopy (SEM) of butterfly wing scales, small pieces of wing were cut and placed on a conductive carbon-made sticker, on a SEM holder. Before putting the samples inside the microscope’s chamber, they were sputtered with palladium, to minimize the charging and consequently improve the quality of the images. The layer of palladium had a thickness of a few nanometer that did
Butterfly wing scales - pigmentation and structural properties

not perturb the much larger sized butterfly structures, which range from hundred nanometer to a few micrometer. The voltage used in the microscope for biological tissues (2-12 kV) is much lower than that for metallic and solid samples (30-60 kV). Large voltages will cause contraction of the structures and deformation. It is preferable to use a low voltage of 3 kV and a small size spot, although higher voltages usually give better resolution.

The way of cutting and positioning the samples is also important when exploring the inner structure of the scales by SEM. Making an image of the cross section of a scale will require cutting the scale, perpendicular to the ridges, by using a razor blade or eye surgical scissors. By putting the sample almost vertically on the holder and using the tilting stage of the microscope, a good image can be achieved, as long as there is good support for the scale. When the scale is still attached to its socket on the wing, the electron beam momentum usually moves it during the slow scanning period necessary for making an image. A good solution is to glue single, isolated scales to the sticker on the holder. The method described above for attaching single scales to the glass pipette can be used for that; but

Fig. 5. a Wing pieces embedded in Epon ready for the microtome. b Microtome cutting a thin layer of the sample by using a glass knife. c Sample holders for TEM (up) and SEM (down). d Screen at the bottom of the TE microscope, showing the light transmitted through the microtomed sample of a *Morpho peleides* cover scale.

instead of glue, electrostatic forces will help to keep the scale attached to the pipette while positioning on the SEM holder. A ridge in the carbon sticker, made previously with a razor blade, can be useful to introduce an enhanced tilt, so that better images of cross sections can be obtained.

2.4 Transmission electron microscopy

For transmission electron microscopy (TEM) of butterfly wing scales, the samples were processed and embedded in Epon, using standard protocols. After embedding, sections ~ 80 nm thick are cut with an ultramicrotome (Fig. 4b) and put on copper grids previously covered with paraffin. The contrast of the samples for the microscope is made with uranyl acetate in methanol during 2 min and lead in water during 1 min. The preparation of the samples takes about a week but the procedure to make the photos in the microscope is rather simple.