Fly Imaging

(Nicolas Gompel, Eva Ayla Schröder, June 2016)

This protocol is for imaging of live, anesthetized adult drosophilids, using extended depth of field (or focus stacking).

Material

Anesthesia

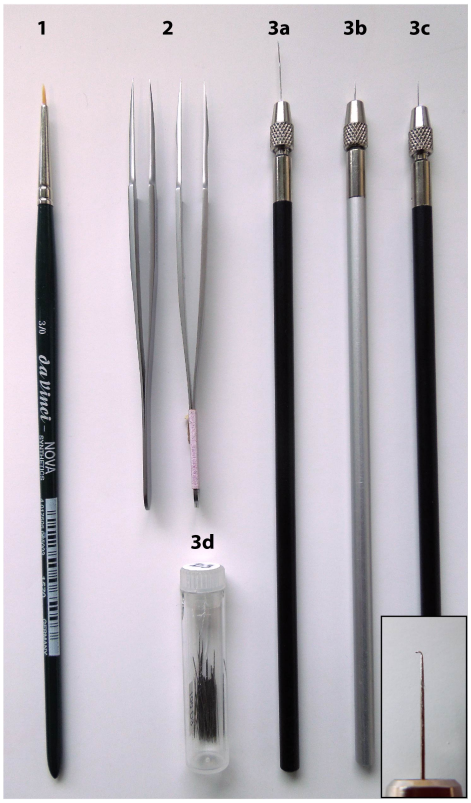
Flies are anesthetized with vapors of triethylamine (the main component of FlyNap, Carolina Biologicals). Place cotton pads in a clean squirt bottle (Fig. 1). Under a fume hood, dispense 1 ml of triethylamine on the cotton. This makes a source of triethylamine vapors lasting days to weeks and can directly be puffed into a vial containing living flies (Fig. 7). Flies exposed to the vapors become idle after minutes, and the anesthesia may last for hours. The advantage of this anesthetic is that it leaves the flies relaxed, allowing to mount them in natural postures. Different fly stocks or fly species have different sensitivities to this chemical.

Figure 1: squirt bottle with triethylamine vapors

Mounting

Specimen mounting is monitored under a dissecting stereoscope.

To manipulate the flies, to position them on a slide, spread their appendages, clean off dust particles and deposit glue droplets, we use different kinds of tools (Fig. 2):



-a fine brush (type 1 or 0) (No. 1 in Fig. 2)
-1 or 2 pairs of forceps (*e.g.,* 5-Dumoxel-H-Dumont, No. 2 in Fig. 2; www.finescience.com)
-2 mounted needles. We use insect pins #3 or headless stainless steel pins (www.watdon.co.uk E3 pins 0.31 mm x 25 mm) and needle holders from www.roboz.com or www.finescience.com (No. 3 in Fig. 2). A needle with a bended tip as shown in the inset in Fig. 2 facilitates the fly manipulation and appendage spreading.

Flies are glued onto a microscope slide using transparent, water-soluble glue. We use "l'Adhésive" from Cléopâtre® (www.colles-cleopatre.com). Tissue-Tek® O.C.T. embedding medium, used for cryosection, is a reasonable alternative. The viscosity of the glue can be adjusted empirically by letting it dry or adding drops of water to the tube.

Figure 2: toolset for fly mounting; **1** brush type 1 or 0; **2** 2 pairs of forceps; **3a, b** needle holders stainless steel needle; **3c** needle holder with bended needle [see also inset]; **3d** insect pins

Upon imaging, to position the slide with the mounted fly at a desired angle, we use clay (ideally matching the color of the background used for the photographs; Fig. 9).

Camera and microscope

Images are taken on a single-beam path microscope, with parallax-free movement along the z-axis (*e.g.*, Leica Z-Series Macroscopes). The microscope is equipped with a digital color camera (ccd or cmos), as shown in Fig. 3.

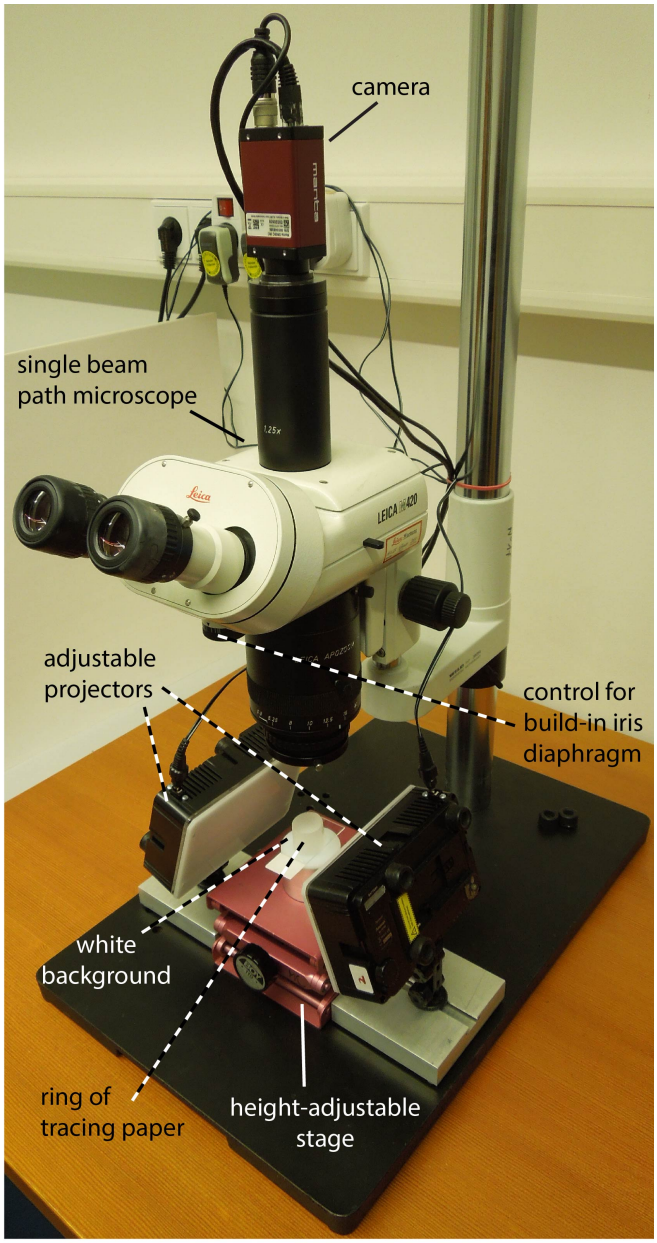


Figure 3: single-beam path microscope with camera

Although a motorized stage for automatized z-movement facilitates the acquisition of an image volume of the specimen, it is dispensable to image objects of the size of a fly. Stepwise focusing can also be done manually, with small increments.

If the microscope has an iris diaphragm, this diaphragm should be largely or completely open to minimize the depth of field of each image of the stack.

Specimens should be imaged at the highest possible magnification, such that they occupy most of the field of view.

Mounted specimens are placed on a height-adjustable stage with a white or black smooth surface (Fig. 3, Fig. 6). The adjustment of stage height facilitates the control of lighting (see below).

Once the light conditions (see below), the zoom magnification and other microscope parameters are set, adjust the camera settings using a mock specimen (*e.g.*, a dead fly). These settings include exposure time, white balance, gamma and if applicable the definition of a region of interest (ROI).

Adjusting camera settings can take time, and it is convenient to save all parameters into a setting file that can be recalled for another imaging session. Several camera control software have this “save settings” function.

Lighting

Homogeneous, smooth lighting is key to the final image quality. The nature of the light source, its position relative to the object, and the addition of light diffusion filters determine the quality of object illumination.

Light source

1) Dome lighting: commercial and homemade solutions

-Ready-made and costly solutions such as integration spheres with orange peel paint efficiently suppress shadows and give an excellent rendering. (*e.g.*, Leica high diffuse dome illumination).

-Significantly cheaper alternatives, sometimes involving a bit of tinkering, give equally good results and may offer more flexibility on light control. The following reference describes a simple solution for dome lighting:

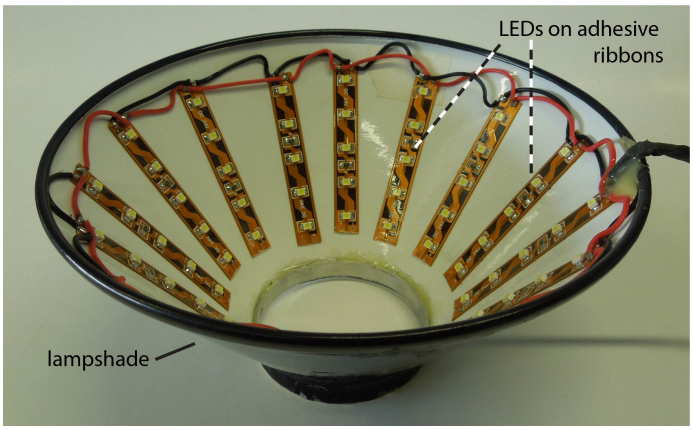


Figure 4: homemade lighting dome

Kerr, P. H., Fisher, E. M., & Buffington, M. L. (2008). Dome lighting for insect imaging under a microscope. *American Entomologis*t, 198–200.

Fig. 4 shows a homemade alternative, we have designed using adhesive ribbons of LEDs, positioned inside a recycled lampshade. This simple dome contains about 100 LEDs and results in a reproducible and homogeneous light source.

2) LED plates with adjustable intensity

Retailers of photography material sell arrays of LEDs with adjustable intensity. These can be used as projectors and give more flexibility for adjusting light orientation than dome illumination. Fig. 3 shows a typical setup using such mini-projectors. Their orientation and distance to the object have considerable impact on the image rendering. The angle and height of the lateral projectors can indeed affect shadows or uniformity of the background lighting, which may help to visualize certain features.

Light diffusion

When using any of the light sources described above, using filters can further increase light diffusion. These filters need to be placed between the light source and the object, without obstructing the optic path to the camera.

Figure 5: an insect imaged without (left) or with (right) diffusion filter. These 2 conditions result in highlighting different morphological features, such as here the punctuation or the pubescence.

We typically use two different filter types: either a cylinder of tracing paper or an open Styrofoam cup. Fig. 6 shows how these filters look like, and how they are positioned on the stage.

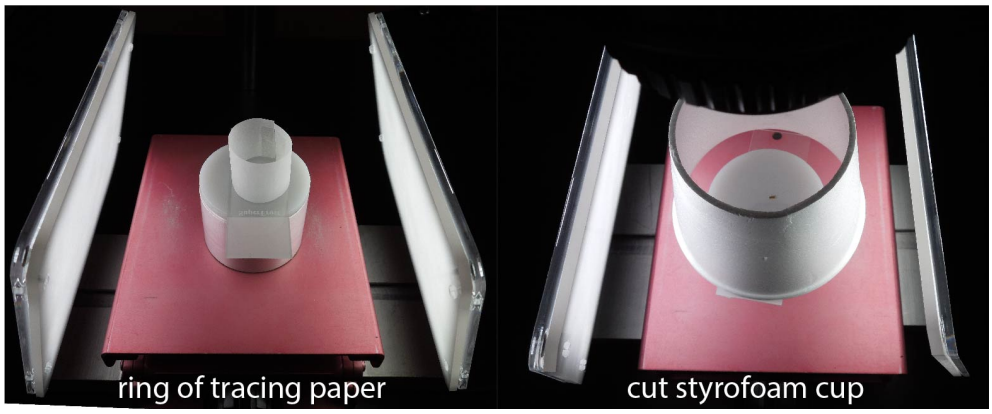


Figure 6: different filters for light diffusion: a ring of tracing paper, a cut Styrofoam cup.

Methods

Fly collection and anesthesia

Flies of interest are selected under CO_2 anesthesia and placed into a new vial.

The following steps should only be started when the whole camera and lighting setup is ready for imaging.

Vapors of triethylamine are used for prolonged fly anesthesia. The vapors are directly puffed into the fly's vial (Fig. 7).

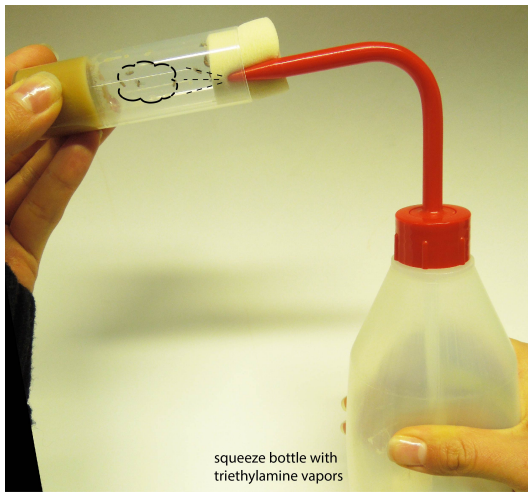


Figure 7: application of triethylamine on flies.

Mounting

The mounting proper consists in gluing a fly to a microscope slide and arranging its appendages as desired.

-Place a microscope slide under a stereomicroscope and transfer a single anesthetized fly to the center of the slide. The followings steps are illustrated in Fig. 8:
-Spread and roughly position the fly's appendages, flip the fly on its belly, so that it assumes a natural posture.
-Deposit a droplet of glue onto the slide, near the fly.
-Pull the fly with forceps over the droplet, quickly but gently, before the droplet dries. Push the fly's thorax down softly to fix it to the slide.
-Arrange the fly's legs as desired. A needle with a small hook (inset in Fig. 2) can prove helpful to move the legs. Should a leg be stuck in too much glue, a humid fine brush can be used to delicately dissolve the glue and free the leg.
-The fly's wings can optionally be spread open and glued to the slide, one after the other.
-If the mounted fly starts to move again, or shows spontaneous appendage movements, the slide can be placed for a few more minutes into a 50 ml Falcon tube also filled with vapors of triethylamine.

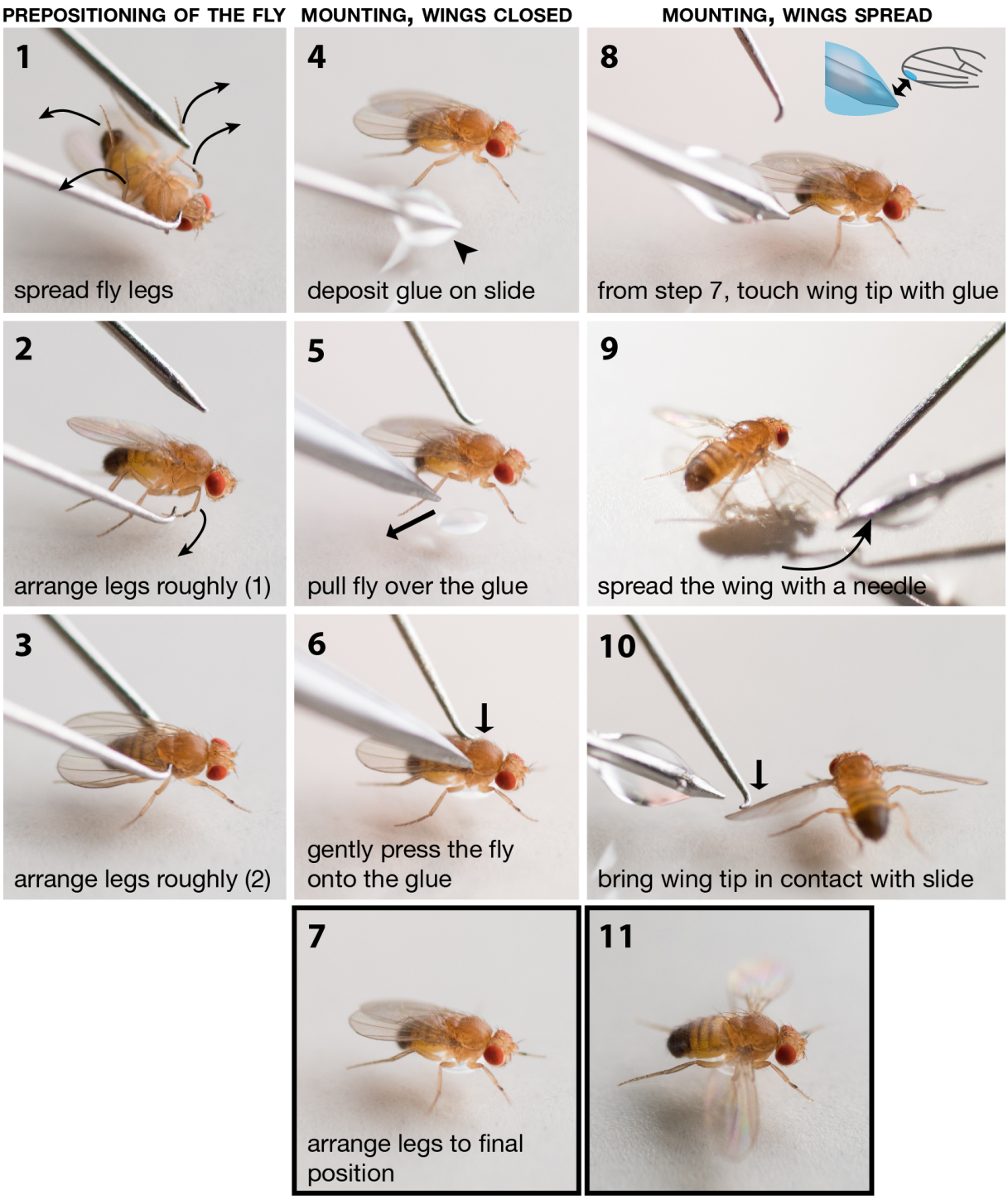


Figure 8: fly mounting procedure. Step 7 and step 11 show possible positions for imaging. (Photos: ©Carolin Bleese).

Imaging

As soon as the fly is mounted and still, the slide is transferred onto the stage, either flat or maintained at an upright position with clay (Fig. 9).

Figure 9: left: upright slide, held in place with clay for lateral imaging; right: final image, (the reflection of the fly on the glass slide can later be photoshoped out).

The final position of the slide (and the fly) is monitored on the screen under live acquisition. The cylinder of tracing paper or the Styrofoam cup is then placed around the fly, as shown in Fig. 3 and 6 (or around the entire slide maintained with clay).

-Under live acquisition, set the upper focal plane slightly above the fly. This will be image 1 of a stack. All images should be saved as independent tiff files (*e.g.*, stack_001, stack_002, …, stack_n).
-Acquire this first image and move down the z-axis manually by a few tens of microns.
-Acquire the second image and repeat the procedure until the fly is completely out of focus.
-Altogether, a stack of 10-15 images should suffice to capture an entire fly. Any image should share some elements in focus with the one that follows (Fig. 10)

Figure 10: Stack of images obtained by focusing through the fly from dorsal (left) to ventral (right).

Image processing

To obtain an extended depth-of-field image, the stack of 10-15 pictures is projected into a single image retaining every sharp bit (*e.g.*, Fig. 11). Several freeware and shareware programs can be used to this end. Here are two examples that we like:

-Stack Focuser, a plugin for ImageJ: rsb.info.nih.gov

-Helicon Focus, a more versatile solution for a reasonable price: www.heliconsoft.com

Figure 11: projection of a stack of 10-15 images.