Protocol

Designing a Generic Videography Experiment for Studying Mosquito Behavior

Florian T. Muijres,[1,3] Andrew K. Dickerson,[2] and Remco Pieters[1]

[1]*Department of Experimental Zoology, Wageningen University, 6708 PB Wageningen, the Netherlands;*
[2]*Department of Mechanical, Aerospace, and Biomedical Engineering, University of Tennessee, Tennessee 37996, USA*

In this protocol, we describe the basic design considerations and general method to set up a videography system to study mosquito behavior. A basic videography system to study mosquito behavior requires one or more cameras with an optical lens, camera lighting, a calibration setup, and a system to record the video data or otherwise control the camera. Here, we define two types of systems: (1) a real-time videography-based tracking system for determining the position of multiple moving (flying) mosquitoes, and (2) a high-fidelity videography system that can track the detailed movements of body, wings, and legs of a single mosquito at high spatial and temporal resolutions. These high-fidelity trackers are divided into single-camera systems for studying two-dimensional (2D) movements, and multicamera systems that can reconstruct three-dimensional (3D) movements of the mosquito.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Reagents

Mosquitoes

Equipment

Calibration objects, such as a simple ruler (see Step 6)

All videography systems require a calibration to convert the videography measurements in pixel coordinates into spatial units such as millimeters. To perform this calibration, a calibration object is needed. For single-camera systems, this calibration object can consist of a simple ruler with an easily distinguishable scale. The ruler should be placed in the same plane of focus as the mosquito, spanning most of the camera frame. For multicamera systems, more complex calibration objects are needed to determine how combined pixel coordinates in the set of cameras can be converted into 3D spatial coordinates (see Step 6).

Camera(s), either a one-, two-, or three-camera system

3D tracking requires two or more cameras (see Step 2).

Flight arena

[3]Correspondence: florian.muijres@wur.nl

From the Mosquitoes collection, edited by Laura B. Duvall and Benjamin J. Matthews.

Advanced Online Article. Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot107926

Lights for set illumination

Backlighting provides sharp silhouettes but hides on-body features (see Step 4).

Tracking software

For more information on choosing a tracking software, see Introduction: ***Using Videography to Study the Biomechanics and Behavior of Freely Moving Mosquitoes*** *(Dickerson et al. 2022).*

Video recording system (see Steps 7 and 8)

METHOD

1. Design and build the flight arena.

   The flight arena should permit optical access and allow mosquitoes to fly unencumbered but constrain the volume for easy filming. Tethered experiments require no enclosure.

2. Determine the required number of cameras.

   The most important design decision when developing a videography system to study freely moving mosquitoes is the number of cameras used. This decision is primarily driven by the aim of the study.

Tracking Basic Behavioral Measurements

If one is interested in basic behavioral measurements, a simple single-camera system, like that shown in Figure 1A, is often sufficient. Using such a system, one can track 2D positional data, wingbeat frequency, feet and proboscis placement on a substrate, and some basic behavioral metrics. However, a single camera cannot be used to accurately quantify movements out of the image plane, and only one side of the mosquito is visible. Thus, if one needs to quantify 3D movement and wants to reconstruct the position and orientation of the complete animal, a multicamera system is needed.

Tracking the 3D Position of the Mosquito

If one is only interested in tracking the 3D position of the mosquito, only two cameras are needed (see Fig. 1B), although using more cameras will generate higher precision positional estimates or encompass a larger volume-of-interest. In a multicamera system, it is imperative that at least two cameras capture each point within the volume of interest. Systems that are used to determine the 3D position of a mosquito need to run at frame rates of at least 50–100 fps, depending on the speed of movement. Thus, flying tends to require higher frame rates than walking or probing.

Tracking the 3D Movements of Body Parts

When one needs to quantify the 3D movements of body parts, such as head, thorax, abdomen, legs, wings, and proboscis, more sophisticated multicamera systems are needed. To prevent temporarily obscured body parts, a system with at least three cameras is required to be positioned around the mosquito. That way, all body parts are almost always visible from at least two cameras. Frame rate requirements are driven by the movement frequency or the number of pixels traversed by the body part of interest. This is particularly problematic when studying mosquito flight, as flying mosquitoes beat their wings at exceptionally high wingbeat frequencies (up to 600 Hz). To reconstruct the 3D wingbeat kinematics, one should film the wing movement at 30–40 images per wingbeat, resulting in a required frame rate up to 24,000 fps.

Regardless of the type of multicamera system used, there are a couple of critical design rules that should always be considered to accurately determine 3D movements, including camera synchronization (see Step 5) and viewing angles.

To minimize 3D reconstruction errors, the stereoscopic cameras should view the object from different angles. A difference in viewing angle of 90° is best. For viewing angle differences of 0° (cameras in the same position) and 180° (cameras opposite each other), 3D reconstruction is not possible. Therefore, it is best to aim for angles of 90° between the cameras, but angles of >45° and <135° are most often sufficient.

If one wants to reconstruct the 3D kinematics of different body parts, it is often best to use a three-camera system with one camera viewing the mosquito from the top, one from the side, and one from the front. This system allows viewing the mosquito from all sides, minimizing occlusions, and the viewing angle between all cameras is exactly 90° (see Fig. 1C).

3. Align camera and focus the lens and set the aperture for the desired depth of field (DOF). Place an alignment object in the center of the volume of view. Set the lens aperture to fully open. Focus the

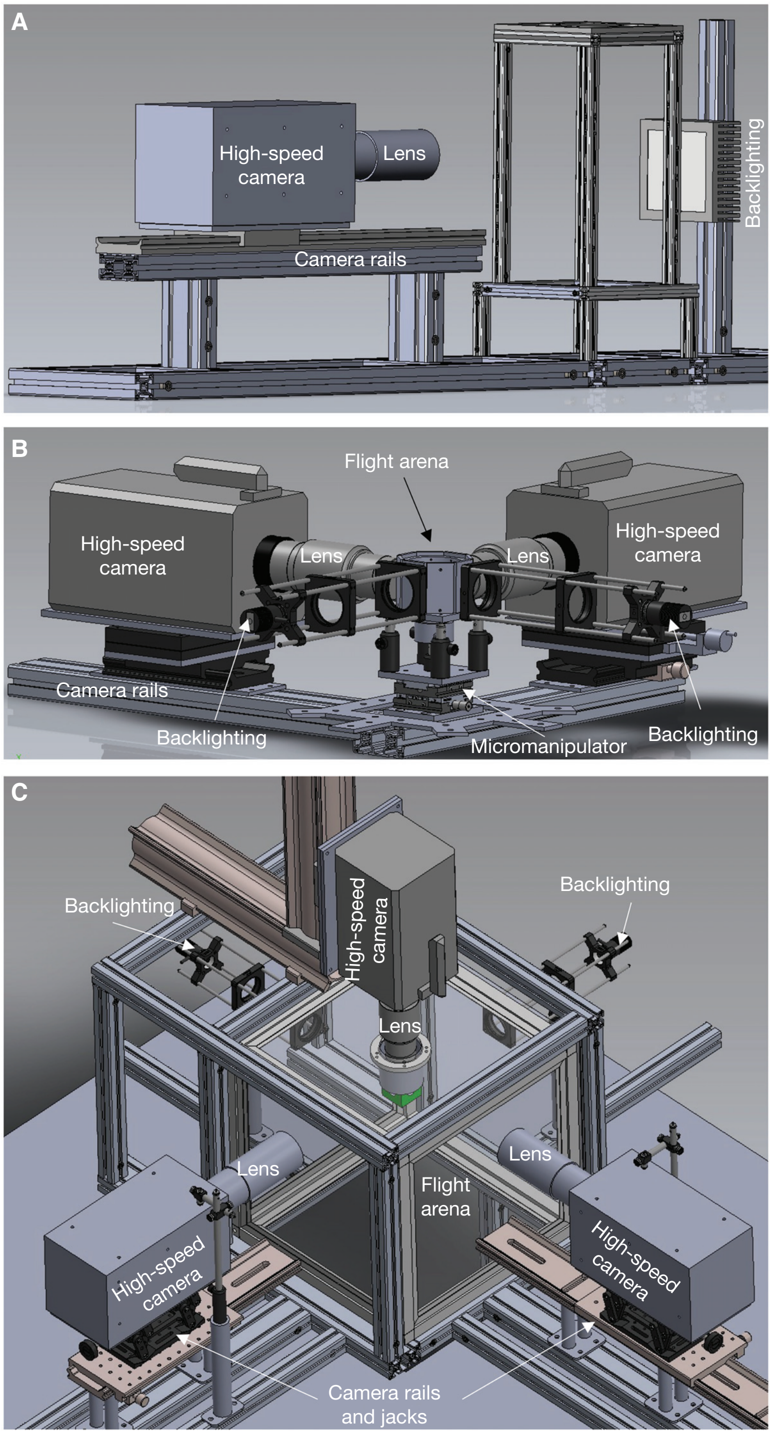

FIGURE 1. Example of videography setups with (*A*) one, (*B*) two, and (*C*) three cameras.

lens. Close the aperture to the desired DOF—a piece of printed text is an easy-to-use object to focus the lens. Frame rate is not important for Step 3.

Any videography system consists of either a single or multiple cameras that are positioned such that the viewing area of the camera(s) cover the volume of interest. In addition, the object of interest should be filmed at high enough spatial and temporal resolution. To achieve all three requirements (sufficient spatial and temporal resolution and coverage of the complete volume of interest), one should carefully design the *videography setup. This includes the choice of camera and lenses, the placement of the camera(s), and the camera and lens settings. The most important trade-offs include the following.*

Monochrome Versus Color Cameras

Full color recordings show the natural environment of the mosquito. But this color sampling comes at a cost: Color cameras use a color filter array (often a Bayer pattern) to capture the primary colors. For each pixel, only one color is measured directly. To get the full color representation of each pixel, it must be combined with the neighboring pixels. Therefore, monochrome cameras are more sensitive (three times more light per pixel), have more detail (∼20%), and can record video at higher frame rates than equivalent color cameras. Furthermore, monochrome cameras are in general sensitive to near-infrared light and can therefore be used in combination with infrared lights to simulate nighttime conditions for the mosquito. Thus, monochrome camera sensors have many advantages compared to color sensors, so when color information is not needed, a monochrome camera sensor is highly recommended.

Spatial Resolution and Volume of Interest

The pixel resolution of the video camera is limited, and thus for a given volume of interest, the maximum achievable spatial resolution of the filmed mosquito is also limited. Thus, when designing the camera setup, one should determine the minimum required field of view and spatial resolution, and thus choose a camera with a corresponding pixel resolution.

Spatial Resolution and Temporal Resolution

Most professional video cameras can record video at various combinations of frame rate (temporal resolution) and pixel (spatial) resolution, whereby the pixel resolution decreases with increasing frame rate. Thus, based on the research question, one should select the camera type that can best achieve the required combination of frame rate and pixel resolution.

Image Brightness and Motion Blur

When filming rapidly moving mosquitoes, one should be careful to prevent motion blur in the video. This is done by reducing the camera exposure time. However, reducing the exposure time reduces image brightness. Therefore, a balance needs to be found between image brightness and motion blur. It is again important to base such a balance on the research question: If one is interested in the rapid wingbeat movements of flying mosquitoes, then exposure time should be very short and images sharp; but if wing motion does not need to be digitized, blurred wing images are not problematic and exposure time can be increased to produce higher quality (brighter) images. The problem of image brightness and motion blur can also be solved by increasing the camera illumination (see next section) or increasing the sensor gain.

Image Brightness and DOF

When filming small animals such as mosquitoes, the DOF or focal depth of the camera system can be relatively small, especially when using macro lenses. In other words, one can only accurately digitize the movements of the mosquito within a very thin focal plane, and sometimes only portions of a complete mosquito are in focus. Thin focal planes are particularly problematic for multicamera systems, as the DOF should cover the complete volume of interest. One can increase the DOF by reducing the aperture of the lens, but doing so reduces the brightness of the image. Thus, similarly to motion blur, there is a trade-off between maximizing the DOF and image brightness, a problem that can also be reduced by increasing the camera image illumination.

4. Arrange the lights around the arena such that the cameras are viewing mosquitoes lit from either the front or back.

   Illumination of the camera setup is crucial for producing good quality video data. This is particularly relevant for high-speed stereoscopic videography because of the short exposure time and the small lens aperture required for maximizing the DOF.

Recording Nocturnal Mosquito Species

In contrast, high-intensity lights can disturb the behavior of mosquitoes, especially nocturnal species. To solve this problem, one can use near-infrared LED lights. Most insects cannot visually detect the near-infrared spectrum, minimizing the disturbance to mosquitoes and allowing one to recreate nighttime conditions while providing illumination for the cameras. There are a wide range of LED light sources available with wavelengths of 850 nm and 940 nm, generally designed for nighttime CCTV security applications. Also, most monochrome camera systems for machine vision application have good sensitivity in the 850- to 940-nm range. There are also cameras available that are specially designed to operate in the near-infrared range. Color cameras tend to have infrared filters in front of their sensor and should be avoided if using near-infrared

 Advanced Online Article. Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot107926

illumination. In general, monochrome cameras tend to be more sensitive to light, so if color vision is not needed, we suggest using monochrome cameras.

High-Contrast Lighting to Analyze 3D Body Kinematics

A high contrast between the mosquito and background is advantageous to detect the mosquito and analyze its 3D body kinematics. Very high contrast is easily achievable with backlighting by placing an array of LEDs in combination with a diffusing sheet behind the mosquito, thus shining the light straight into the camera. The mosquito will then be visible as a dark shadow in front of a bright background. This illumination setup has the added benefit that the light intensity is relatively high, resulting in sharp video images.

The downside of shadow images produced by backlighting is that detailed information about the body surface is often not visible. If this information is desired, one can use a conventional illumination setup with lights positioned next to the cameras. Thus, the cameras will record the light reflected from the mosquito, which will be seen as a light object with relatively high surface contrast in front of a dark background.

A less conventional camera illumination setup is to place a single LED in combination with a long focal collimator lens opposite the camera (Walker and Taylor 2021; Voesenek et al. 2019). This setup also results in backlit video images, but due to the collimated light beam, the DOF becomes relatively high. In this case, the downside is that the field of view becomes maximally equal to the size of the image sensor, or the maximum aperture of the lens.

5. When using more than one camera, synchronize the cameras using a (TTL) signal from a digital pulse generator or from a transmitting camera to the receiving camera(s).

   All cameras should be synchronized in time, which is best achieved using a synchronization signal. A synchronization signal can be produced by a digital pulse generator, or by a transmitting camera that sends the signal to all other receiving cameras. If synchronization is not possible because of hardware limitations, video data must later be aligned (e.g., using a filmed action such as an LED flash at a random time interval during the whole recording), but this method will inherently result in larger 3D reconstruction errors.

6. Calibrate the camera(s) using a calibration object.

   Camera calibration should be done regularly, especially after the camera or lens settings are changed or a camera is (slightly) moved or rotated.

   The calibration routine is often included in the analysis software, and each routine requires its dedicated calibration object. This can be a 3D object with many points with known 3D locations, or a checkerboard or stick that should be moved throughout the field of view of all cameras. The calibration object should be made very precisely and cover as much of the experimental volume as possible to minimize calibration error. Calibration routines using an object of known 3D location typically use Direct Linear Transformation (DLT) to convert pixel coordinates of multiple cameras into 3D spatial world coordinates; the resulting spatial coordinate orientation matches that of the calibration object. Calibration routines using a moving object typically first solve for the relative position and orientation of the cameras along with their lens properties; these may later be converted to DLT coefficients for calculating 3D spatial world coordinates. When the optics of the camera lenses are inferior, lens distortion corrections are needed. The most sophisticated calibration routines include such distortion corrections.

7. Perform your experiment and record videos of the moving mosquitoes inside the arena with your camera setup.

   Video recordings at low frame rates can be streamed to permanent storage in real time, but at high frame rates, the recording should be triggered manually or automatically.

8. Store video files on a data storage device.

   Most machine vision cameras do not have their own controller or data storage system. These cameras must therefore be connected to a video recording system, typically consisting of one or more personal computers. The video recording system sets the acquisition parameters: frame rate, exposure time, and sensor gain, and stores the video data. At frame rates on the order of 100 fps, video can often be streamed directly to the computer, where it will be stored or processed. For recordings at a higher frame rate, the camera footage must often first be stored on local camera RAM, after which it can be downloaded to the computer (typically via a gigabit ETHERNET or USB3 connection).

9. Extract kinematic data from videos using an appropriate tracking software.

   See Protocol: ***Real-Time Tracking of Multiple Moving Mosquitoes*** *(Straw et al. 2022) and Protocol:* ***Tracking the Body, Wing, and Leg Kinematics of Moving Mosquitoes*** *(Hedrick et al. 2022) for details.*

10. Analyze the kinematic data.

*See Protocol: **Quantifying and Analyzing Mosquito Movement from Video Tracking Results** (Muijres 2022) for details.*

DISCUSSION

We present three examples of generic videography systems in Figure 1, which represent nearly idealized systems. These setups incorporate rail sliders upon which cameras rest, micromanipulators to position flight arenas, and lights affixed to the rig. Such levels of build-out and fine adjustment allow for the precise placement and adjustment of the camera, lights, flight arena, and calibration object. The sophistication shown in Figure 1 is not required. Cameras can be mounted to tripods and lights affixed to arms. However, although more rudimentary setups have been the basis of successful studies, they are more prone to misalignment, calibration errors, and inconsistency over the course of experiments. The cameras in Figure 1 are positioned with mutually orthogonal viewing angles. This is ideal for reconstructing 3D position, but is not strictly required, and any pair of cameras, excluding the special case of cameras with viewing angles separated by 0° or 180°, can be used to collect 3D measurements.

REFERENCES

Dickerson AK, Muijres FT, Pieters R. 2022. Using videography to study the biomechanics and behavior of freely moving mosquitoes. *Cold Spring Harbor Protoc* doi:10.1101/pdb.top107676

Hedrick TL, Dickerson AK, Muijres FT, Pieters R. 2022. Tracking the body, wing, and leg kinematics of moving mosquitoes. *Cold Spring Harbor Protoc* doi:10.1101/pdb.prot107928

Muijres FT. 2022. Quantifying and analyzing mosquito movement from video tracking results. *Cold Spring Harbor Protoc* doi:10.1101/pdb.prot107929

Straw AD, Pieters R, Muijres FT. 2022. Real-time tracking of multiple moving mosquitoes. *Cold Spring Harbor Protoc* doi:10.1101/pdb.prot107927

Voesenek CJ, Pieters RP, Muijres FT, van Leeuwen JL. 2019. Reorientation and propulsion in fast-starting zebrafish larvae: an inverse dynamics analysis. *J Exp Biol* **222:** jeb203091. doi:10.1242/jeb.203091

Walker SM, Taylor GK. 2021. A semi-empirical model of the aerodynamics of manoeuvring insect flight. *J R Soc Interface* **18:** 20210103. doi:10.1098/rsif.2021.0103

Cold Spring Harbor Protocols

Designing a Generic Videography Experiment for Studying Mosquito Behavior

Florian T. Muijres, Andrew K. Dickerson and Remco Pieters

Cold Spring Harb Protoc; doi: 10.1101/pdb.prot107926; published online September 27, 2022