

## Assessing Senescence in *Drosophila* Using Video Tracking

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### Abstract

Senescence is associated with changes in gene expression, including the upregulation of stress response- and innate immune response-related genes. In addition, aging animals exhibit characteristic changes in movement behaviors including decreased gait speed and a deterioration in sleep/wake rhythms. Here, we describe methods for tracking *Drosophila melanogaster* movements in 3D with simultaneous quantification of fluorescent transgenic reporters. This approach allows for the assessment of correlations between behavior, aging, and gene expression as well as for the quantification of biomarkers of aging.

**Key words:** Aging, Biomarkers, *Drosophila melanogaster*, GFP, In vivo imaging, 3D video tracking

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### 1. Introduction

Aging of living organisms (senescence) has traditionally been assayed by lifespan, and altered lifespan is still generally considered the “gold standard” for verifying interventions in aging (1). However, assay of lifespan is problematic in that it takes a long time to determine, and once determined there is limited additional information that can be obtained from the animal. For these reasons there has been extensive interest in identifying predictive biomarkers of aging, i.e., parameters that can be assayed in a non-destructive way in young animals that will be indicative of aging rate, future performance and lifespan (2–4). Aging in both *Drosophila* and mammals is characterized by changes in gene expression including upregulation of stress response and innate immune response genes (5–8). Expression of transgenic reporter constructs derived from such genes correlates with *Drosophila* lifespan (9, 10) and is predictive of remaining lifespan when assayed in young animals including *Drosophila* (7, 11) and *C. elegans* (12). Movement behaviors are also promising as aging biomarkers: aging in *Drosophila* and humans is characterized by

reduced gait speed (13) and disrupted sleep/wake cycles (14, 15), and gait speed is predictive of remaining lifespan in elderly humans (16). We have previously reported methods that allow for 3D video tracking of *Drosophila melanogaster* movement behaviors (17), while simultaneously quantifying gene expression in aging flies using fluorescent reporter proteins (18, 19). Video tracking in 3D has also been applied to studies of aging in additional fly species (20). We have recently developed a system called FluoreScore that utilizes two synchronized cameras and recorded videos and provides a simplified operating system and a user-friendly interface (21). FluoreScore allows for quantification of fluorescent transgenic reporter expression in groups of free moving flies and provides 3D movement patterns with simultaneous fluorescence quantification for single flies. Here we provide a detailed protocol for FluoreScore, including instructions on assembling the hardware and running the software.

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## 2. Materials

### 2.1. Hardware

The physical setup consists of the following parts:

- Desktop computer with FW800 PCI-E cards to connect the cameras.
- 2× video cameras (Grasshopper type GRAS-20S4C, Point Grey Research, Scottsdale, AZ).
- 2×8mm Megapixel fixed focal lens (Edmund Optics Inc., Barrington, NJ).
- 2× Adaptor with external SM30 threads and internal SM1 threads (SM1A15, Thorlabs Inc, Newton NJ).
- 2× Lens tube, Ø1", 0.3" long (SM1L03, Thorlabs Inc, Newton, NJ).
- 2× blue LED lights (Blue 3 Watt LED MR16 bulb, Super Bright LEDs, Inc., St. Louis, MO).
- 2× MR16/MR11 Socket—GX5.3/GZ4 (MR16S, Super Bright LEDs, Inc., St. Louis, MO).
- 2× 12V DC Single Color LED Dimmer (LDK-8A, Super Bright LEDs, Inc., St. Louis, MO).
- 2×0700C24F 17W Xitanium LED Driver, 700mA, plus Power Cord (XI-LED120A, LED Supply, Randolph, VT).
- 2×Xitanium Driver Connector (1365323-1, LED Supply, Randolph, VT).
- MDF-GFP filter set (Thorlabs Inc, Newton, NJ).
  - 2× MF469-35 filters (pass range: ~450–490 nm).
  - 2× MF525-39 filters (pass range: ~500–550 nm).

- Standard glass *Drosophila* culture vial as observation chamber.
- Aluminum Breadboard, 24"×24"×1/2" (MB2424, Thorlabs Inc., Newton, NJ).
- 2× Mounting Post, Length = 14" (P14, Thorlabs Inc., Newton, NJ).
- 2× 1.5" Mounting Post Bracket (C1505, Thorlabs Inc., Newton, NJ).
- 2× 3" One-Arm Package, Two Knuckles, 3" Shaft (NT53-885, Edmund Optics Inc., Barrington, NJ).
- 2× Mounting Post Base Ø2.40"×0.50" High (PB2, Thorlabs Inc., Newton, NJ).
- 2× U-shaped Base Support Stand, Cast Iron (12985-078, VWR, Brisbane, CA).
- 2× Talon Rod, Aluminum 457 mm (60079-381, VWR, Brisbane, CA).
- 2× Talon 3-Prong Dual Adjust Extension Clamp (21570-302, VWR, Brisbane, CA).
- Cardboard box.

Cameras are connected to the desktop computer via FW800 PCI-E cards. A low-end desktop computer is used, including Intel 64-bit Quad-Core Xeon Processors (2.13 GHz/Core) with 6GB of RAM and running Windows 7 enterprise (64 bit). The two LED lights have the MF469-35 filters fitted in front of them using electrician's tape, and are pointed toward the observation chamber. These filters pass light in the range of approximately 450–490 nm, which overlaps the eGFP absorption peak at approximately 488 nm. Each camera has an Edmund Optics 8 mm Megapixel fixed focal lens, with an MF525-39 filter fixed in front using lens tube and adaptor. The emission peak for eGFP is approximately 509 nm and is within the pass-range of the filter (500–550 nm). The observation chamber is a standard glass *Drosophila* culture vial. The bottom of the vial and the vial stopper are covered with nonreflective black cotton cloth. For longer tracking periods where water and nutrition are required we have found that grape agar media (Genesee Scientific, San Diego, CA) produces the least background. Typical fly media was found to produce significant background, which can be reduced by adding dark food coloring. Plastic (polystyrene) *Drosophila* culture vials can also be used but reflect more light, and although these reflections can be removed in processing, glass vials are preferable (see Note 1). After making cameras and lights rigid and stable on the breadboard, using mounting posts for the cameras and ring stands and clamps for the lights, the setup (lights, cameras, observation chamber) is isolated from outside light by covering with a cardboard box. The chamber and other parts are accessible from one side of the box that can be opened and closed to make a light-tight seal.

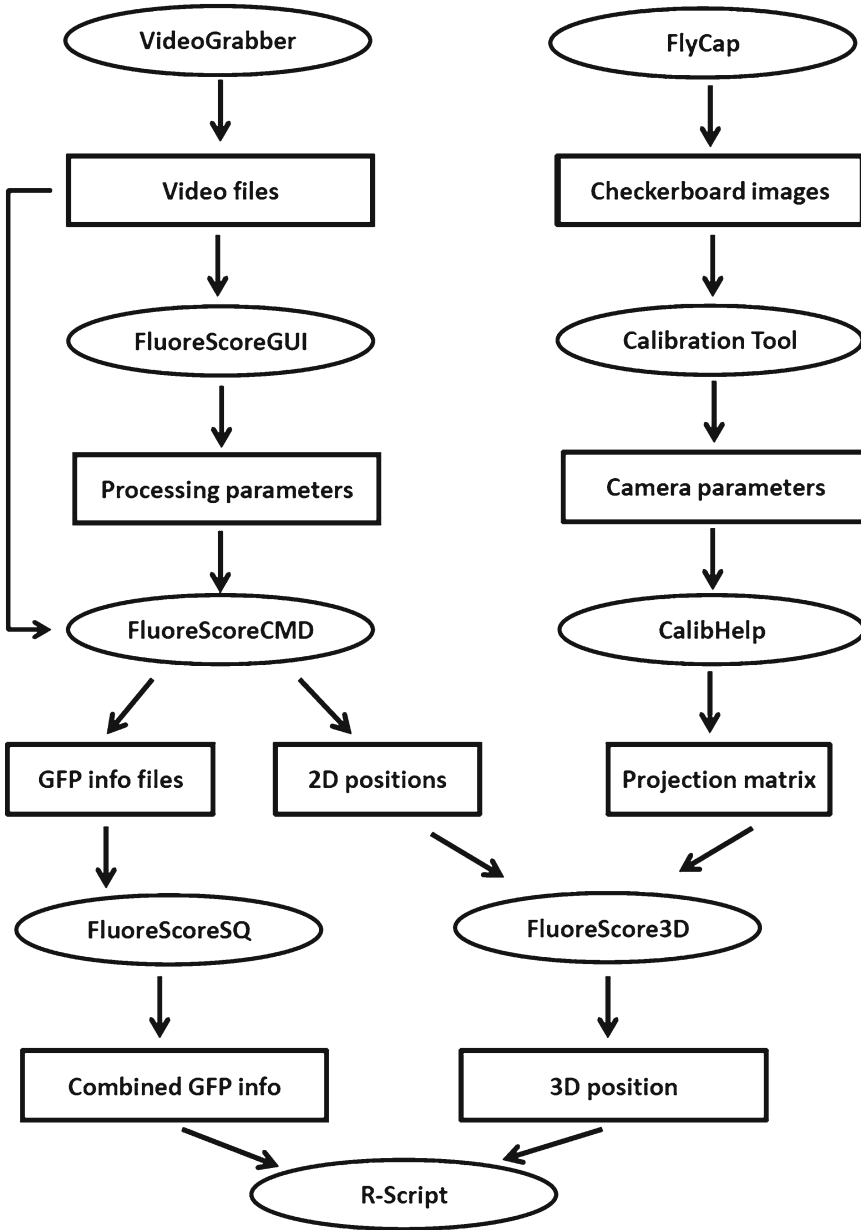


Fig. 1. FluoreScore suite modules and data flow. Each module is shown in an *ellipse*. Input and outputs are shown in a *rectangle*. Arrows show data flow between different components.

**2.2. Software**

The FluoreScore software suite has multiple modules. Each module is implemented independently to facilitate ease of use and future developments. Figure 1 shows components and data-flow of the FluoreScore suite. Custom software called VideoGrabber is

used to capture videos from the cameras (<http://code.google.com/p/video-grabber/>). VideoGrabber exploits FlycapSDK software (provided by Point Grey Research with the cameras) to capture synchronized videos from multiple cameras. Cameras are calibrated using software called Camera Calibration Tools, which is freely available at <http://www.cs.ucl.ac.uk/staff/Dan.Stoyanov/calib/>.

In addition, we provide five additional custom software modules, written in Visual Studio C++ (2005):

- CalibHelp (version 1.0): converts intrinsic and extrinsic parameters of cameras generated by Camera Calibration Tools to a projection matrix.
- FluoreScoreGUI (version 1.0): includes a graphical user interface (GUI) and is used to determine processing parameters such as the detection threshold. It also can be used to process videos, for example for extracting GFP information as well as 2D position of flies.
- FluoreScoreCMD (version 1.0): for experiments involving longer tracking periods the user needs to process multiple videos. A command line version of FluoreScore (FluoreScoreCMD) is provided that allows for batch processing of videos.
- FluoreScoreSQ (version 1.0): removes noise and irrelevant measurements from the data. In particular, it removes the data from objects with size smaller than a user-chosen threshold. In addition it combines the data from the two cameras.
- FluoreScore3D (version 1.0): generates 3D tracking using the 2D positions generated by FluoreScoreCMD.

More details about each module are provided in the next heading. FluoreScore suite is freely available at <http://fluorescore.cmb.usc.edu>.

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### 3. Methods

Here, we describe the methods for conducting experiments, assuming that the hardware setup is ready. More specifically, cameras are connected to a workstation and have a full view of the chamber (see Note 2) and lighting conditions are appropriate (see Note 3). The major steps for running experiments after having the hardware setup and flies ready are as below:

1. Recording videos.
2. Calibrating cameras for 3D reconstruction.
3. Parameter findings.

4. (Batch) processing of videos and obtaining GFP and 3D trajectories.
5. Data analysis.

Details are described for each step and sub-step.

### 3.1. Recording Videos

A custom program called VideoGrabber is used to record compressed videos (see Note 4).

Steps for recording videos are:

1. Create a new folder, copy “VideoGrabber.exe” to the folder and run it by double clicking on it.
2. Indicate the number of frames that you want to record. If the videos are recorded at 30 fps, this means for each second you will record 30 frames. For example to record a 5 min video, you will need to input 7,200 (= 5 min  $\times$  60 s/min  $\times$  30 fps).
3. After indicating other options including display On/Off (choose Off) and recording option (choose openCV AVI) as well as the number of cameras (input 2) the display will show the camera configuration GUI where you can define the resolution, frame rate, and other options for videos. A typical setting is resolution 800  $\times$  600, frame rate 30 fps, and YUV 422 color space for recording.
4. After pressing ok, it will ask about the codec; press Enter to choose the default codec, which is FFDS and stands for ffdshow codec (see Note 5) and it will begin to grab frames from the cameras and save them in a file. It will show a “done!” message after it grabs the number of frames that you have indicated at step 2.

### 3.2. Calibrating the Cameras

To reconstruct 3D points the projection matrix must be obtained for each camera. The camera projection matrix  $P$  describes the mapping between the real 3D points  $(X, Y, Z)$  to the 2D points  $(x, y)$  in the image (22). Freely available software called Camera Calibration Tools is used to find the intrinsic and extrinsic parameters of the cameras. A small custom program called “CalibHelp” converts these parameters to a projection matrix for the cameras.

#### 3.2.1. Camera Calibration Tool

Camera Calibration Tools (<http://www.cs.ucl.ac.uk/staff/Dan.Stoyanov/calib/>) has been developed by Daniel Stoyanov. This software calibrates cameras using multiple images of a checkerboard in different positions. The checkerboard should be an  $N \times N$  grid with squares of the same size. Figure 2a shows a 13  $\times$  13 checkerboard that we used for calibration, and the 10  $\times$  10 square in the middle of the checkerboard was used as follows: Each corner is marked by a number to indicate the  $x$ -axis and  $y$ -axis and origin. Since we want to keep the axes and the origin the same in all 2D

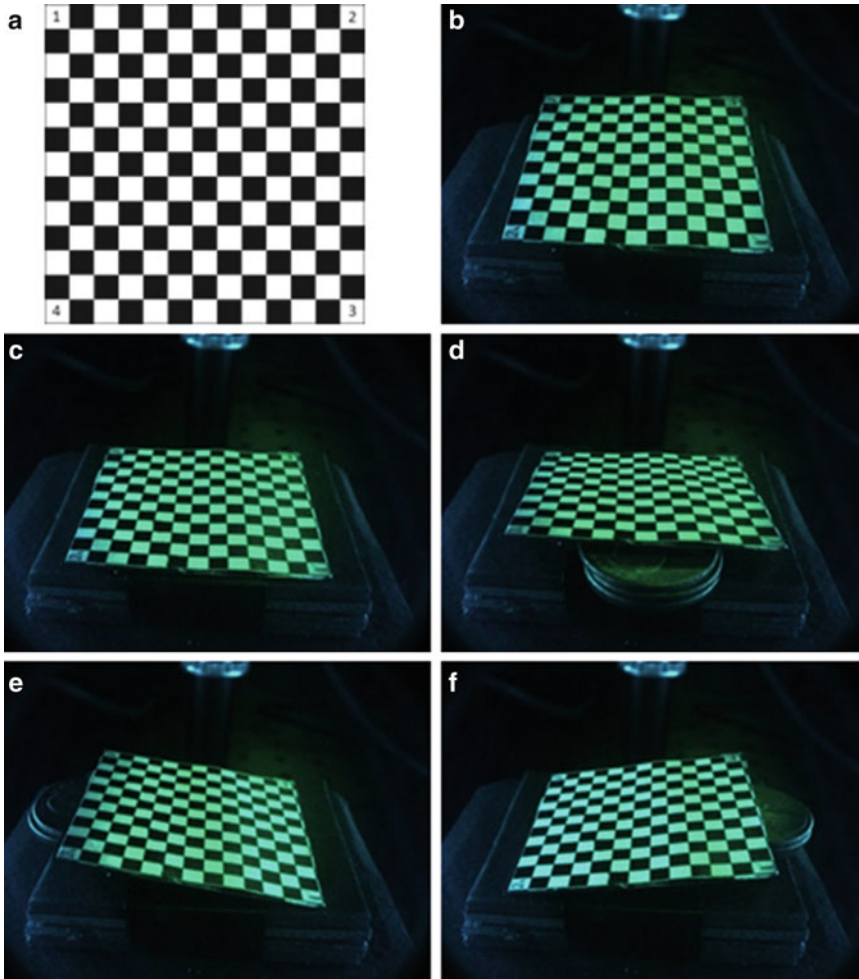


Fig. 2. Camera Calibration. (a) Sample checkerboard for calibration. (b–f) Checkerboard in five different orientations. Except for position one, at each position one side of the checkerboard is elevated to cover different 3D points in space and provide more sample points for Camera Calibration Tools.

images, we indicate them with a number to make sure the origin (O) is the same for all 2D images.

The steps for calibration are:

1. Create an  $N \times N$  checkerboard (at least  $5 \times 5$ ).
2. Take five different images for each camera and in each image incline the checkerboard at different angles (Fig. 2b–e).
3. Add images to the Camera Calibration Tool, define the size of the checkerboard (here  $10 \times 10$ ) and choose the corners for each image. Make sure the order of choosing the corners stays

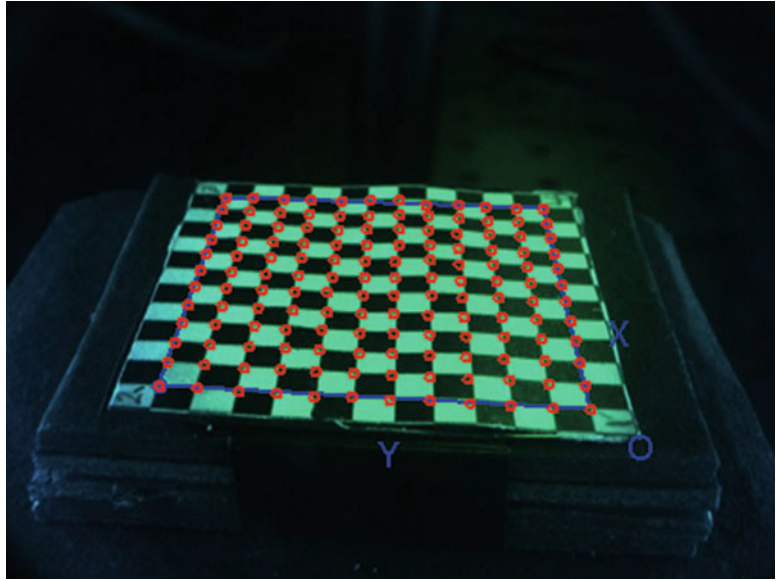


Fig. 3. Camera Calibration Tools. Camera Calibration Tools selects the vertex of each square in the checkerboard.

the same (i.e., corner1  $\rightarrow$  corner 2  $\rightarrow$  corner 3  $\rightarrow$  corner 4). After selecting corners, Calibration Tool extracts intersection points of inner squares (Fig. 3).

4. Press the calibration button for each camera and save the project. All intrinsic and extrinsic parameters will be saved in an output file.

For more details please refer to the manual of Camera Calibration Tool that is available online.

### 3.2.2. CalibHelp

This program converts intrinsic and extrinsic parameters into a projection matrix. To use CalibHelp, copy “CalibHelp.exe” to the same folder where the output of Camera Calibration Tool is located and run it. It will create a file named “projMat.txt” in the same folder. This file contains the projection matrix of the cameras, which will be used by the FluoreScore3D module to generate 3D points from 2D points (see Note 6).

### 3.3. Parameter Finding

1. There are three parameters that should be determined before processing using FluoreScoreGUI. The most important one is the threshold for detecting flies (21). The optimal value for this threshold will vary based on the lighting conditions and specific experimental setup. Once the user starts to (pre)process a video, they can see what will be detected with different thresholds. In the processed window flies should be marked, and background pixels should be minimized or eliminated. Figure 4 shows examples of detected fly silhouettes with different threshold values (see Note 7).



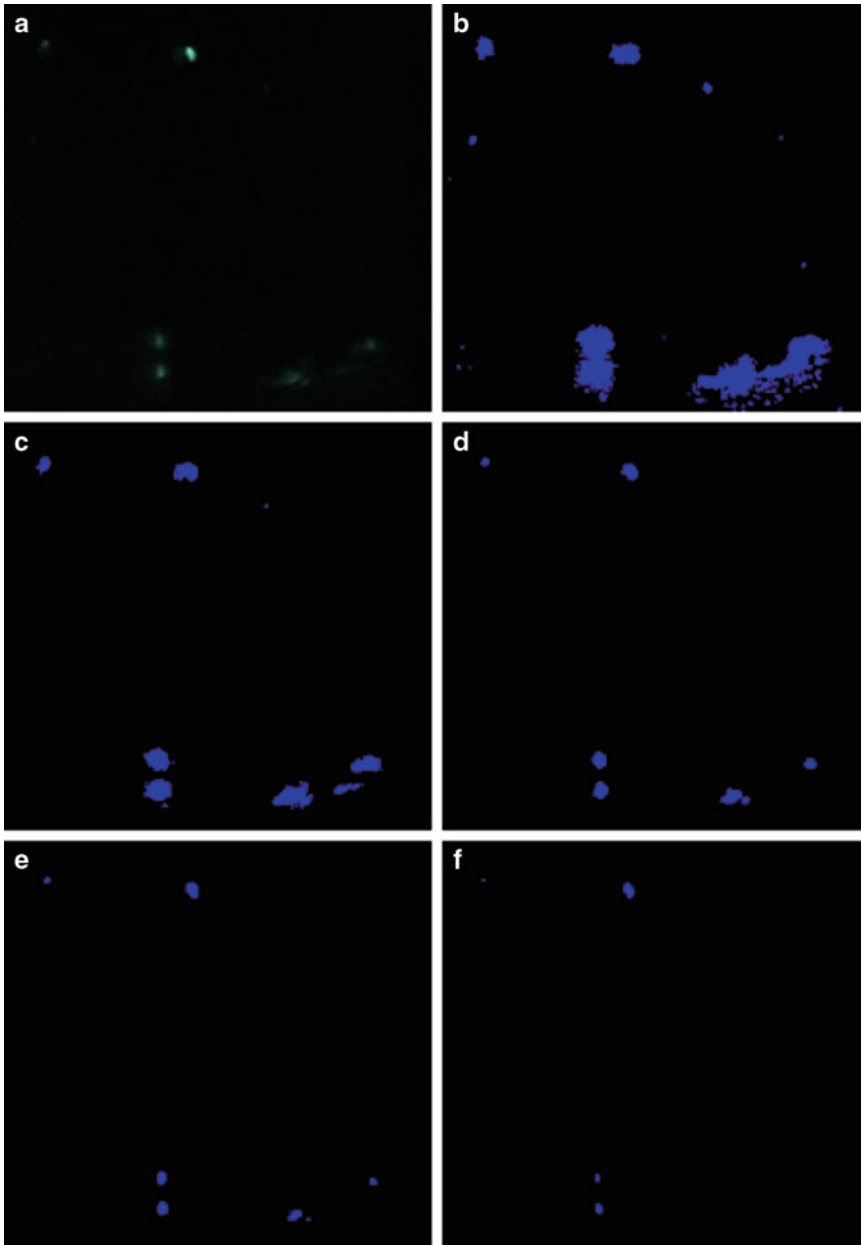


Fig. 4. Selecting threshold for silhouette detection. (a) Original image from the camera. (b–f) Detected components using different thresholds: threshold = 5 (b), threshold = 10 (c), threshold = 20 (d), threshold = 30 (e), or threshold = 50 (f). Different thresholds lead to different detected components. FluoScoreGUI enables the user to see the detected components and choose appropriate thresholds.

2. Because of the LED lights, sometimes there is glare in videos on the wall of the vial or on the base of the chamber. To exclude such glare from the analysis, the user can define a processing mask for each view by free-hand drawing; the mask marks regions that will not be included in the quantification. FluoreScoreGUI saves the mask in a .jpg file, which is used as an input parameter for FluoreScoreCMD so that it can be applied to multiple videos recorded under the same conditions (see Subheading 3.4.1).
3. To make the processing faster as well as more accurate the user can also define a region of Interest (ROI) in a view, which is a rectangle that just covers the chamber in the view. By defining an ROI, FluoreScore does not process the part of the images that are not useful, thereby increasing processing speed. An ROI rectangle is defined by two points (top-left and bottom-right). These points are saved in a text file and used as input parameters for FluoreScoreCMD.

### **3.4. Processing Videos**

After finding parameters, the user can start processing the videos. Processing videos has several steps. The first step is to generate from the videos the raw data files that contain GFP values and 2D positions of the fly, using FluoreScoreGUI or FluoreScoreCMD. The next step is to process the data and combine the information from both views using FluoreScoreSQ. These files along with the projection matrix file (see Subheading 3.2) will be used in FluoreScore3D to generate 3D position of the fly.

#### **3.4.1. FluoreScoreCMD**

FluoreScoreCMD should be called from the command-line to start processing videos. It will generate two CSV files for each view. One file contains 2D positions of the flies in that view. The other file contains intensity information on detected flies. More specifically, for each frame, intensity values (between 0 and 256) are calculated for red, blue, and green channels for pixels that represent flies. For each channel the pixels are separated into four bins based on the pixel intensity values. For example, for the green channel, pixels are separated into bins G1, G2, G3 and G4 of increasing intensity. For each fly, the histogram of pixel intensity values is calculated along with the sum of intensities for each bin of the histogram. Below, the input parameters and command line format for FluoreScoreCMD are shown.

**Command:**


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**FluoreScoreCMD.exe** <a1> <a2> ... <a16>

- a1: inputAviFile for View 1
- a2: threshold value for View1, should be between 0 and 127
- a3: inputAviFile for View 2
- a4: threshold value for View2, should be between 0 and 127
- a5: showing videos while processing (y or n).
- a6: Number of frames to process, put -1 to the whole video
- a7: Mask file name for view 1 (jpg file)
- a8: X-coordination of top-left for view 1, min = 0, max = 800
- a9: Y-coordination of top-left for view 1, min = 0, max = 600
- a10: X-coordination of down-right for view 1, min = 0, max = 800
- a11: Y-coordination of down-right for view 1, min = 0, max = 600
- a12: Mask file name for view 1 (jpg file)
- a13: X-coordination of top-left for view 1, min = 0, max = 800
- a14: Y-coordination of top-left for view 1, min = 0, max = 600
- a15: X-coordination of down-right for view 1, min = 0, max = 800
- a16: Y-coordination of down-right for view 1, min = 0, max = 600

**Example:**

```
FluoreScoreCMD.exe Case_View0.avi 15 Case_View1.
avi 15 y -1 mask1.jpg 200 200 400 400 mask2.jpg 100
100 500 500
```

**3.4.2. FluoreScoreSQ**

1. After processing videos from each view, FluoreScoreSQ is used for de-noising and for combining information from both views. FluoreScoreSQ combines the intensity values from two views and generates a CSV file including the intensity values for each frame. Moreover, if there are some detected components whose size is smaller than a chosen threshold, they will be removed (see Notes 8 and 9). Below, the use of FluoreScoreSQ is exemplified.

**Command:**


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**FluoreScoreSQ.exe** <a1> <a2> <a3> <a4> <a5>

**a1:** input intensity CSV file for view 1 (contains intensity info and is generated by FluoreScore)

**a2:** input intensity CSV file for view 2 (contains intensity info and is generated by FluoreScore)

**a3:** output CSV file for combined results of view 1 and view 2

**a4:** fly number (should be 1 for 3D tracking)

**a5:** threshold of the size of fly in pixels (with current setup it would be around 20–50)

**Example:**

```
FluoreScoreSQ.exe 0_0.csv 0_1.csv 0.csv 0_pos1.csv 0_pos2.csv 1 20
```

*3.4.3. FluoreScore3D*

2. FluoreScore3D uses the 2D position files for each view as well as the projection matrix file, and generates 3D position for each frame where 2D positions were available in both views (see Notes 10 and 11). FluoreScore3D is used as described below:

**Command:**


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**FluoreScore3D.exe** <a1> <a2> <a3> <a4>

**a1:** input CSV file for view 1 (contains 2D positions of fly, is generated by FluoreScore)

**a2:** input CSV file for view 2 (contains 2D positions of fly, is generated by FluoreScore)

**a3:** projmat.txt (generated in Subheading 3.2.1)

**a4:** output CSV file containing 3D position of the fly

**Example:**

```
FluoreScore3D.exe experiment_pos1.csv experiment_pos2.csv projmat.txt experiment_3dpos.csv
```

*3.4.4. Batch Processing*

Experiments involving longer tracking periods will generate multiple videos. For example, if an experiment is for 48 h, VideoGrabber (with default settings) will generate 48 1-hour video segments. Processing these videos one by one is laborious. Because we have provided command line versions of the software modules, it is straightforward to have a BAT file that runs all the modules on

multiple videos. A file called “runFSInBatch.bat” is provided that processes all segments of an experiment at once (see Note 12). Below, the use of FluoreScoreBatch.bat is shown:

**Command:**

**FluoreScoreBatch.bat** <a1> <a2>

a1: first hour to be processed

a2: last hour to be processed

**Example:**

**FluoreScoreBatch.bat** 1 48

*3.4.5. Data Analysis*

After running the previous modules, GFP data as well as the 3D positions of the fly have been calculated. R is then used to perform basic statistical analysis, including mean and standard deviation, calculation of motion parameters including velocity and frequency of directional heading changes, and data visualization.

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## 4. Notes

1. When preparing the chamber make sure the following steps are done; (a) clean the chamber with water followed by ethanol, using cotton swabs. (b) Clean the chamber plug using scotch tape to remove any lint and prevent background reflection. (c) Put flies into the chamber and push the plug into the vial until 2.0 cm from the bottom. (d) Place observation vial in defined location in front of the cameras. For optimal results the cameras should be inclined slightly downwards towards the observation chamber. A stand for the observation chamber can be fixed in place in front of the cameras; we have used stacked plastic eppendorf tube racks, taped together, with a circle drawn on top to indicate the location for placement of the observation chamber. We have also found it useful to tape pieces of thick black paper to the top of the stand to create a slot into which the observation chamber fits. The ring stands holding the LED lights can be moved closer or further away from the observation chamber to optimize lighting conditions and to make lighting equal for the two cameras; alternatively lighting can be adjusted using the LED Dimmers.
2. Once the cameras are calibrated they should not be moved or rotated; make sure they are sturdy and fixed in place before starting the calibration process.

3. To get the best signal possible make sure there is no light changing during an experiment. To do so make sure that the observation chamber and cameras are completely isolated from outside light.
4. The data storage capacity of the operating system employed here requires that files be smaller than 2 GB; therefore VideoGrabber will record videos in separate files of a length of 108,000 frames (equal to 1 h at 30 fps).
5. Recording uncompressed videos requires a lot of space and storing long experiments is practically impossible. VideoGrabber can record compressed videos. However the appropriate codec should be installed. More specifically, to be able to read/write compressed videos, codecs are needed. There are many codecs available for this task. We chose “ffds-show” due to its compatibility with openCV library and high compression rate. It is freely available at (<http://sourceforge.net/projects/ffds-show/>).
6. HelpCalib writes the projection matrices of cameras in “proj-mat.txt” with the same order that they have been added in Camera Calibration Tool. It is arbitrary to choose which camera be the “first” view. However, the order should be same for the FluoreScore3D to assign projection matrices to cameras correctly (see Note 10).
7. To choose an appropriate threshold for fly detection adjust the threshold such that the fly is detected in the majority of frames, and background (scattered) pixels are eliminated. Once you find the optimal threshold make sure you keep it the same across any additional samples that you wish to compare so that data are analyzed consistently.
8. Fly Size choice means the minimum size of the fluorescent region of the fly, as that is what is being detected. If this parameter is set too large, you will never detect flies. At the same time you want to set it large enough that you filter noise (which is mostly small connected components consisting of a few pixels). Larger settings will tend to eliminate frames where your fly is farther away and/or oriented such that only a part of the fluorescent region is detected. This is not ideal for a movement assay as you want to detect your fly in as many frames as possible. Typical values for expression specifically in eye tissue is 15–20, and for expression throughout the fly ~50.
9. Fly Number usually is equal to the number of flies in the observation chamber. However, it is possible to have multiple flies in the observation chamber and to set Fly Number to a smaller value if you want to recognize only a subset of the flies (for example the brightest or biggest, depending upon how the detection threshold and fly size have been set).

10. The order of inputs is important. Make sure the first file (view 1 csv file) belongs to the camera that is chosen to be the “first” view in the calibration step.
11. There are some frames in which a fly is not visible in either view. In these frames the 3D position of the fly cannot be obtained because two views of 2D points are needed to be able to perform 3D reconstruction.
12. To be able to use the batch file make sure you do not change the name of the video files that are generated by VideoGrabber.

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