LSRtrack and *LSRanalyze*

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Background

- LSR (larval swim response) software measures the movements of zebrafish larvae from video recordings in the wells of 96, 48, 24, 12 or 6-well plates.
- The software can be run on any machine (Windows, Linux, Mac) running Matlab (http://www.mathworks.com/products/matlab/)
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- For commercial use, please contact the authors.
- No technical support is provided with this software.

Setting Up the System for Windows and Mac

1. Ensure correct codecs are installed for the videos you wish to analyze.
2. Install Matlab including the image processing, signal processing, and statistical toolboxes.
3. Create a directory for the LSR code and extract the files from LSR-windows.zip into it.

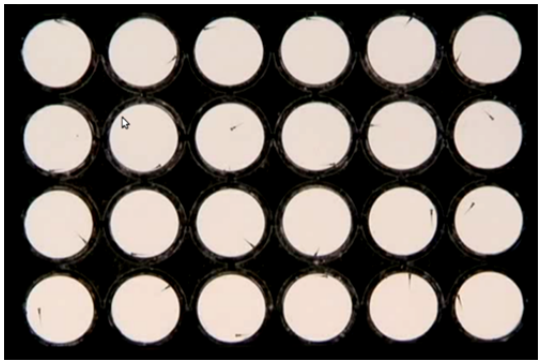
Setting Up the System for Linux (Debian based distributions)

On some Linux installations, the above steps will work without further action. If not, the following additional steps can be performed:

1. Extract the files from LSR-unix.zip into the LSR directory, overwriting all files.
2. Use the precompiled mex files or download, compile, and install mPlayerMex for Matlab using Matlab's mex function.
3. Install all provided debian packages in the LSR directory.
4. Install the gstreamer codecs and mplayer.

Video Considerations

- Videos of various resolutions and frame rates can be analyzed.
- MPEG-4 ASP (lavc) AVIs were used for our study, but any video format that can be read by MatLab is suitable.
- Video pixel aspect ratio should be 1:1
- 96 and 24 well plates have been tested; plates of other sizes (matching the formula 3×2^n) will also be recognized by the software.
- The software recognizes wells as circular areas; use of plates with square or other shaped wells will require modification of the code.
- The plate should be dark in color and dominate the field of view. The wells should be the only lighter colored areas in the image (example below).



Analyzing video recordings:

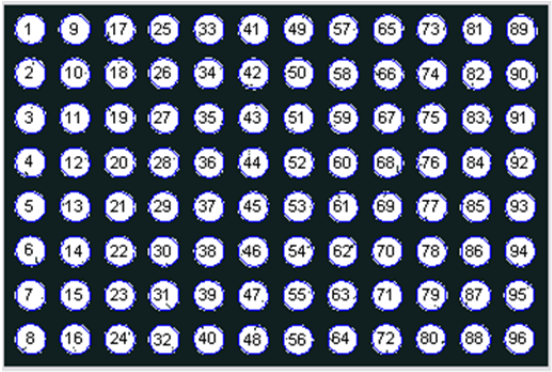
Measurement and analysis of larval movement takes place in two stages:

(i) Video tracking. This is carried out by the ***LSRtrack*** function. Each larva is located in every frame of the video and the coordinates written into a *.MAT file.

(ii) Analysis. This is carried out by the ***LSRanalyze*** function. Mean velocity, active velocity and % time moving are calculated and written into tab-delimited spreadsheets, graphical outputs are drawn and can be saved in different formats from within MatLab.

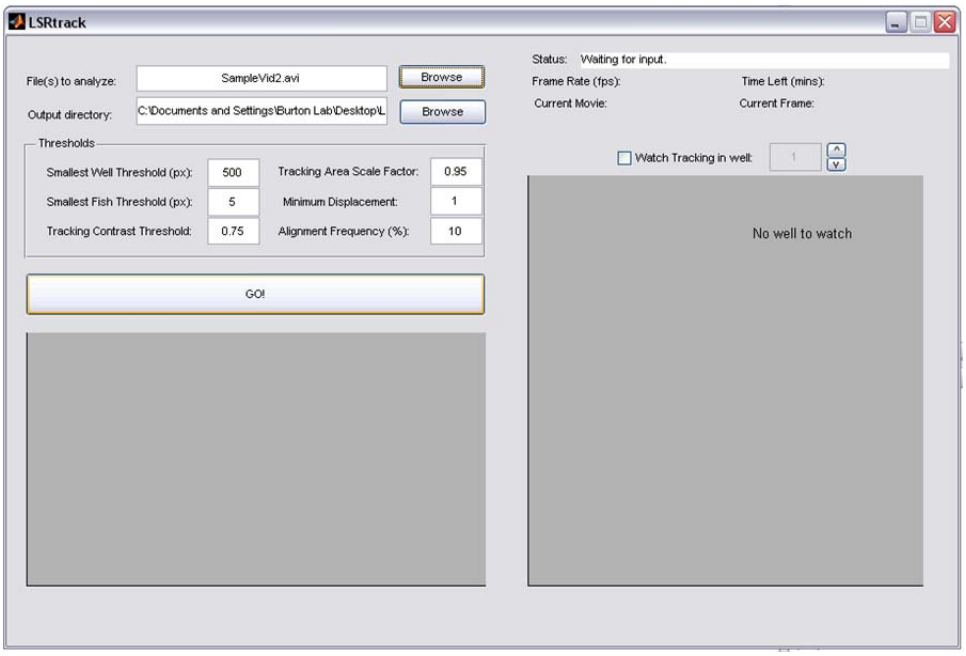
Well numbering

Wells are automatically assigned identifier numbers during tracking in order allow grouping during analysis and other functions as listed below. By default, wells are numbered consecutively from top to bottom in columns from left to right:



Tracking videos using LSRtrack

1. Open Matlab
2. Select the directory containing the LSR software.
3. Type **`LSRtrack <Enter>`** in the MatLab command window.
4. An window like the one below will appear:



5. File to analyze: click the browse button to select the videos that will be tracked. Multiple videos can be selected by holding down the <Ctrl> key (a sample video is included in the LSR-windows.zip folder, which can be used to test the software installation).

6. Output directory: click the browse button to select the output directory to which the tracking output files will be written.

7. Other parameters can be adjusted as the video is being tracked but only valid numbers will be accepted.
    a. Smallest Well Threshold (px): The minimum well size in pixels. Any light colored object smaller than this will not be considered a well.
    b. Smallest Fish Threshold (px): The minimum larval size in pixels. Any darker colored object smaller than this will not be considered a larva.
    c. Tracking Contrast Threshold: A value between 0 and 1 that specifies the threshold of pixel darkness for object tracking. Increasing the value increases the sensitivity and vice-versa.
    d. Tracking Area Scale Factor: A value between 0 and 1 that scales the well area to the tracking area. This is useful in reducing well edge noise, but it should be kept as close to 1 as possible (0.98 recommended for 24-well).
    e. Minimum Displacement: The smallest pixel movement required for measurement. For example, MD=1 means the larval centroid must be displace more than one pixel from the last frame to register as having moved. Optimization of this parameter is dependent on the hardware set up for video recording. If the larvae are recorded at low

magnification or if there is little stochastic pixel noise, the threshold can be set lower to be certain of not rejecting movement as noise; conversely if the larvae are shown at high magnification so that movements appear larger, or there is prominent pixel noise, the value can be increased to discriminate better between movement and noise. MD can be optimized empirically for an individual set up by checking "watch tracking in well # …". The marker showing the larval centroid in the displayed well changes color from red to yellow when a movement is registered. MD should be adjusted so that the marker does not change color without the larva moving, but changes promptly as a movement commences. Quantitative validation of the threshold can also be carried out as described in the supplementary figures of the paper.

f. Alignment Frequency: How often the plate image is generated and the wells are aligned to it. If the alignment of plate and camera are not absolutely constant during the recording, for example vibration from adjacent equipment, decreasing this value will result in better tracking performance.

8. When the input file, output directory and tracking parameters have been selected, click on "Go!" to start tracking. ***LSRtrack*** will automatically detect the frame rate of the video.

9. Progress indicators and monitoring:
    a. Status: Displays the current tracking status.
    b. Frame rate, Time Left, Current Movie, Current Frame: These provide dynamically updated estimates of the progress of the tracking process.
    c. Watch tracking in well #: Checking the box will activate a panel that shows live tracking in the selected well. This is useful for adjusting parameters to optimize tracking but slows performance. After appropriate values have been selected for thresholds and scale factor, it is recommended that this option is turned off.

10. When video tracking is complete, files are written to the destination directory:
    a. *.dist file: A matrix of the distances (rows) moved between frames for each fish (columns).
    b. *.jpg: The background image of the plate that is subtracted from each frame leaving only fish objects to track.
    c. *.mat file: The main data output that is used for further analysis.

Analyzing *LSRtrack* output using *LSRanalyze*

1. Open Matlab
2. Select the directory containing the LSR software.
3. Type **`LSRanalyze(options) <Enter>`** in the Matlab command window.

    Options are as follows:
    a. Specify well groupings using cell syntax: {[group1],[group2]…[groupN]}
    b. End with the string 'Yes' to exclude data points that lie outside 2 Standard Deviations from the mean (the default is that these are included).

Examples:

`LSRanalyze({[1:24],[25,27,29],[30:33,35,37]})` Analyzes the video with 3 groups of wells: 1 through 24; 25,27, and 29; and 30 through 33, 35, and 37. It will keep statistical outlier wells.

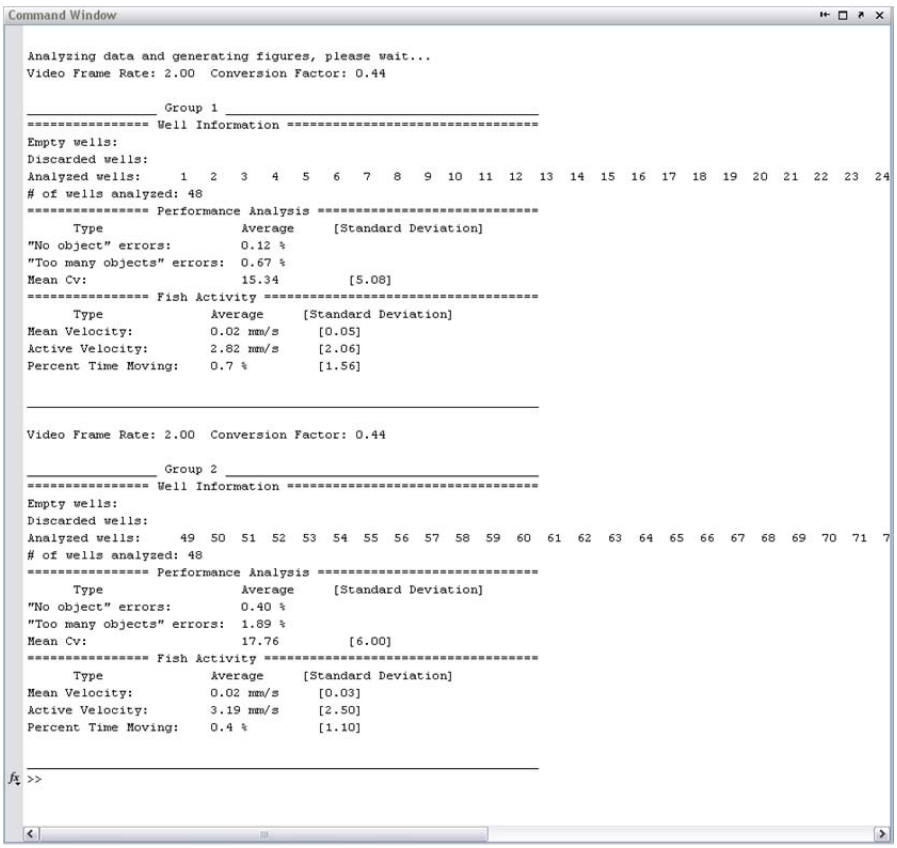
`LSRanalyze({[1:48],[49:96]},'Yes')` Analyzes two groups, removing outliers first.

`LSRanalyze()` Treats the whole plate as one group and does not remove outliers.

LSRanalyze will automatically detect the frame rate of the video being analyzed, provided the frame rate is encoded in the video; this can be written into the video file using AVIdemux. It will also measure the sizes of the wells in pixels and use the mean well diameter to scale pixels into mm for pre-coded mean values 6, 12, 24, 48 and 96-well plates. If different plates are used, the well dimensions should be changed in the ***LSRanalyze*** code in line 48.

4. A window will open for selection of the file to analyze. Navigate to the *.MAT file and select it.

5. After the data are analyzed, a summary of the analysis for each group will appear in the command window:

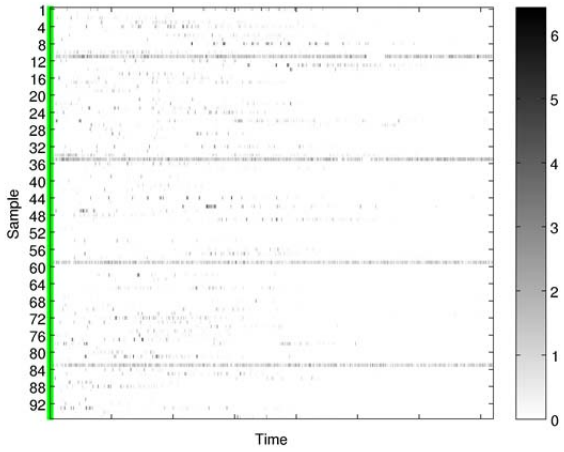


The summary lists:

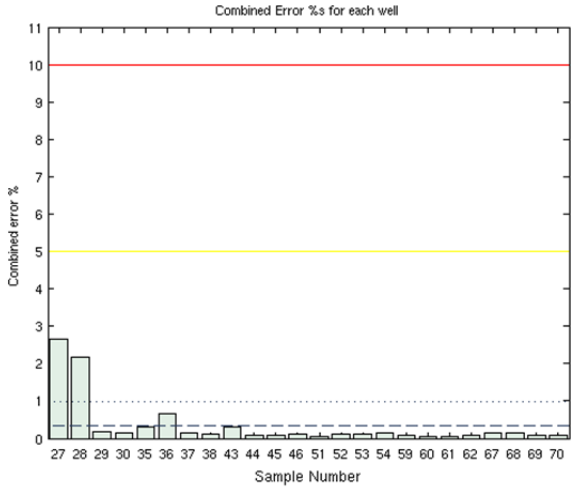
a. Empty wells: Lists numerical identifiers of wells that do not contain an object in more than 50% of the video frames. These are excluded from analysis.
b. Discarded wells: Lists numerical identifiers of wells that returned 'too many objects' or 'no objects' errors greater than 5% of video frames (this value can be changed in the code). These wells are excluded from analysis.
c. Analyzed wells: Lists numerical identifiers of remaining wells, which are included in the analysis.
d. Number of wells analyzed: The total number of wells (whose numerical identifiers are listed in c.), which are included in the numerical analysis.
e. 'No object' errors: % of frames from all wells included in the analysis where no larval object was found.
f. 'Too many objects' errors: % of frames from all wells included in the analysis where more than one object was identified in the well.
g. Mean Cv: Mean coefficient of variation of Vm. Each well has Cv calculated as SD(Vm)/Vm. The value shown in this field is the mean Cv of all wells in the group that were included in the analysis.
h. Mean Velocity: VM for each well = total displacement during recording in mm / total time of recording in seconds. This field displays the mean V_M of all wells in the group that were included in the analysis.
i. Active Velocity: Mean V_A of all wells in the group that were included in the analysis.
j. Percent Time Moving: Mean T% of all wells in the group that were included in the analysis.

6. After the summary is displayed, graphical outputs are generated:

a. **Intensity-time map**: shows movements in each individual well as an intensity map over time. The green dots alongside the left vertical axis indicate which wells were selected for analysis.



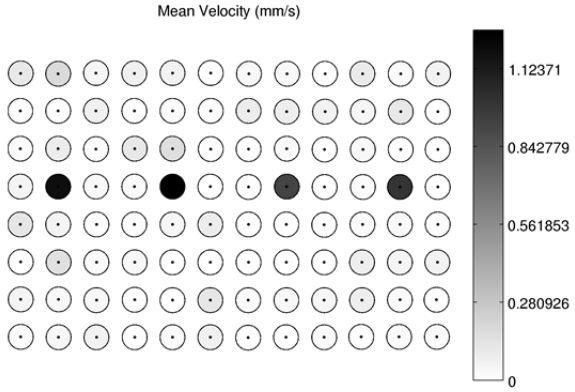
b. **Error report**: shows the combined error (% of time 'no objects' or 'too many objects' were found). The red line indicates the maximum possible combined error and the yellow line indicates maximal individual error before a well is excluded from analysis.



c. **Coefficient of variation over time:** shows how the coefficient of variation changes over the duration of the recording for each group.

d. **Mean velocity over time:** shows how the mean velocity changes over the duration of the recording for each group.

e. **Intensity figures:** depict relative values for V_M, V_A and T% as color intensities superimposed on a map of the plate. Black dots correspond to the center of each well, and shading represents the value of V_M, V_A or T% for each well. In these figures, white represents the lowest value of V_M, V_A or T%, and black the highest value; wells with intermediate values are shaded such that their gray scale density is proportional to their value:



7. Analyses (VM, VA, T%) by group are written to a tab-delimited spreadsheet. The files is named [video name]_GROUP.xls and can be opened using Excel, OpenOffice or other spreadsheet programs.

8. Analyses (VM, VA, T%) for each individual zebrafish larva are written to a tab-delimited spreadsheet. The files is named [video name]_INDIVID.xls and can be opened using Excel, OpenOffice or other spreadsheet programs.

9. A *.MAT file containing all variables used during analysis by ***LSRanalyze*** is also saved. The file is called [video name]_ANALYSIS.MAT. This feature is useful for debugging and developing new features.

Generating additional figures using MatLab

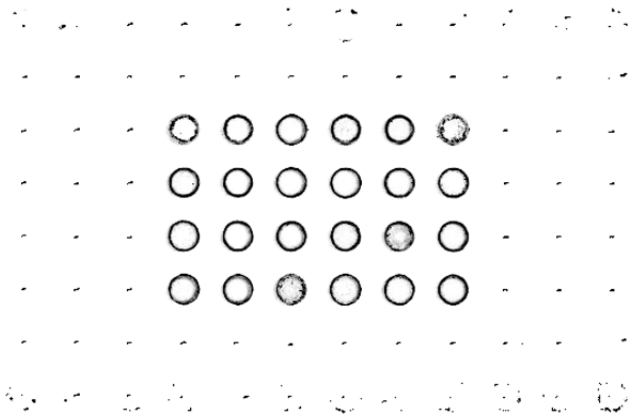
Additional graphical outputs can generated from the *.MAT files produced by ***LSRtrack***. The following examples are illustrated using a 96-well plate in which the central 24 wells were occupied by 7dpf larvae.

To generate additional graphical outputs, open the tracking *.MAT file in Matlab (named [VIDEO NAME].MAT)

1. To generate a frequency-weighted density map of the coordinates occupied by the larva over the duration of the recording, type the following commands:

```
figure;
colormap(flipud(gray(128)));
image(heatMap);
daspect([1 1 1]);
axis off;
```

An example is shown below:



2. To generate a figure illustrating the vectors traversed by each larva over a defined segment of the recording type the following:

```
plotPathOverlay(fishCoords(x:y,a:b,:));
```

x and y are the well numbers that will be plotted. The range can be specified in the format **`(x:y,`** or individual wells can be specified in the format **`([1,2,3:18],`**. **`(:)`** plots all wells.

a and b specify the starting and ending frame numbers that will be plotted.

An example is shown below:

```
plotPathOverlay(fishCoords([27:30,35:38,43:46,51:54,59:62,67:70],1:100,:));
```