Linear Analysis of Midgut
LAM v0.3.2

User Manual

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1 Description

Linear Analysis of Midgut (LAM) is a tool for reducing the dimensionality of microscopy image–obtained data, and for subsequent quantification of variables and feature counts while preserving spatial context. LAM's intended use is to analyze whole *Drosophila melanogaster* midguts or their sub-regions for phenotypical variation due to differing nutrition, altered genetics, etc. Key functionality is to provide statistical and comparative analysis of variables along the whole length of the midgut for multiple sample groups. Additionally, LAM has K-D tree-based functions for the estimation of feature-to-feature nearest distances and for the detection of clusters (4.6). LAM also approximates the widths of the samples along the antero-posterior axis and estimates border region locations based on multivariate scoring. The analysis is performed after image processing and feature detection. Consequently, LAM requires coordinate data of the features as input.

The analysis is based on approximation of the samples from end to end through the creation of vectors onto which features can be projected by minimal distance estimation (4.4). The vectors are divided into user-defined number of bins that can be readily compared between samples and sample groups. The feature-specific point of projection along the vector is linked to all relevant input data through feature identifiers, subsequently allowing quantification of cells and their differing characteristics along the length of the sample (4.5). All individual samples' quantification data is joined with their sample groups to find the groups' general characteristics, which can then be compared group-to-group in a statistical analysis. The joining of the data is based on anchoring of the vectors to each other via a user-provided input coordinate (MP) that corresponds to a distinguishable biological segment of the samples, e.g. R3–region (see 4.2.4). In practice, the anchoring means that all samples are inserted into a data matrix where all anchoring points are located at the same index position, with variable lengths of the vector at either side. This is necessary due to the inherent proportional variation within the midgut that leads to a compounding error when moving further along the bins. Consequently, insertion into the matrix guarantees that the samples are in 'focus' at the anchoring site and that the correct biological regions are compared against each other.

LAM has been tested on larval and adult midguts, but usage can most likely be extended to other tissues where linear quantification could be applicable. The analysis is for the most part restricted to planar geometries, and consequently most functionalities of LAM do not offer resolution on the Z-axis. The limitation to XY-coordinates should be sufficient for most research questions regarding the midgut, as it is pseudostratified and has only minute layering of cells.

Notification:

While the positional input data that LAM accepts can indicate any sort of feature that is to be counted and analyzed, **for the purpose of the user manual individual observations will be referred to as cells.** LAM also accepts multiple data folders for each sample, and while each of these folders does not necessarily contain data from one microscopy channel, **these separate data will be referred to as channels for simplicity.** Microscopy channels will be referred to as such.

2 Installation and Dependencies

LAM is developed in Python >=3.7 environment. The LAM-master–folder has setup.cfg that contains required information for installation with setuptools.

Python Virtual Environment

It is recommended to create a dedicated virtual environment for LAM in order to avoid any dependency clashes. This can be done on command line with the following command:

python -m venv <yourenvname>

If Python is not in the system environment variables, full path must be given to python.exe, e.g. "**c:\Program Files\Python38\python -m venv LAMenv**". The virtual environment can be activated with:

Linux: **source** <yourenvname>**/bin/activate**
Windows: <yourenvname>**\Scripts\activate**

LAM and dependencies can then be installed on the environment (when located in the LAM-master–directory where the setup file is) with:

python setup.py install

Anaconda Environment:

The distribution also includes LAMenv.yml that contains names and version numbers of dependencies. The yml-file can be used to create an Anaconda virtual environment, followed by LAM installation in Anaconda prompt:

conda env create -n <yourenvname> **-f** <path\to\environment.yml>
E.g. ***conda env create -n LAM -f C:\User\LAM-master\docs\LAMenv.yml***

conda activate <yourenvname>
E.g. **conda activate *LAM***

python setup.py install

Dependencies

Matplotlib==3.1*, numpy>=1.18, pandas>=1.1, pathlib2>=2.3, scipy>=1.5, seaborn>=0.11.0, shapely>=1.7.0, scikit-image>=0.16.2, scikit-learn>=0.22.1, statsmodels>=0.11.0

3 Usage in brief

Input

LAM accepts feature coordinates and feature-related data such as intensities in csv's and in wide-format as input. The data must be located so that each sample has a separate folder named as "<group>_<sample>", e.g. "holidic_sample1". In the sample-folders each dataset is to be in its own folder named as "<group>_<sample>_<channel>_<xyz>", e.g. "holidic_sample1_DAPI_stats". The coordinate data for each dataset must be in a file named "Position.csv" with columns "Position X", "Position Y", "Position Z", and must also include dataset-unique identifiers [0, N] for features in a column named 'ID'. All additional data to include must be defined in *AddData*-variable in settings.py or in the GUI's *Other*-window. If using anchoring of samples, i.e. samples are to be merged at a specific point and not bin-to-bin, the user must provide the anchoring points similarly to other datasets, but with the folders named according to the value in *MPname*-variable ("*MP label"* in GUI, default "MP"). Additionally, you can provide self-made vector coordinates for each sample to "./Analysis Data/Samples/<sample group>_<sample name>/" named as "Vector.csv" or "Vector.txt".

Analysis in GUI

LAM can be launched from command line with command ***lam-run*** if installed using setup.py. LAM, or alternatively by executing "*LAM-master/src/run.py*". First, provide path to the directory with the sample data by clicking the *directory*-button at the top of the GUI. If the path is valid, LAM should update all sample groups and channels (i.e. object-datasets) next to the *Detect*-button. In the upper part of GUI are the primary settings: **Process**, **Count**, **Distance**, **Plots**, and **Stats**. Ticking the boxes enables related options. The **Process**-setting creates vectors for all samples, **Count** projects datasets and counts objects, **Distance** performs feature-to-feature distances and clustering, and **Plots** and **Stats** enable plotting and statistical calculations. Some related settings are hidden behind the "*Other/Plots/Stats*"-buttons at the bottom of the GUI.

If LAM has been provided vector-files, the **Process**-setting can be left unticked. The **Count**-setting must be performed, but the rest are optional. Once **Count** has been performed once, the optional primary settings can be run at will unless changes have been made to underlying data. All output by LAM will be placed to "*Analysis Data"*-folder that is created to the root of the work directory. Most produced data can be found in "*Data Files"*-folder, but data of each sample's each object-dataset can be found in the *Samples*-folder.

Vector creation in LAM

For the automatic vector creation, the samples must have been oriented along the x-axis of the image so that either end of the intestine has the lowest x-values (typically anterior). As a rule, the *Median vector creation* functions best for samples that have no backwards movement along x-axis, while *skeleton vector creation* functions for curved intestines. Intestines that loop over itself should not be used for analysis. When **Process** is checked, you can use **Create vectors**-button to open creation window. Select vector type '*Median*' initially, press *Loop*, see "Analysis Data/Samples/vectors.png" to select valid vectors, and then press **Keep.** Change creation settings and loop until all samples have vectors.

4 Usage in full

LAM is used by using console command **lam-run** or by executing src/run.py. The execution by default opens the graphical user interface (GUI) which allows the use of all functionalities of LAM. Most analysis settings are handled through settings.py, but certain arguments can be parsed from command line: use either **lam-run -h** or **python run.py -h** for help or refer to docs/CommandLine_Args.txt. When executing from command line, the arguments described as toggles will switch the default value in settings.py to the opposite Boolean value, e.g. argument -C switches clustering from default value False to True.

The workflow is as follows:

0. (Object detection on microscopy images) (user)
1. Organizing files for input (user)
2. Creation of sample-specific vectors (automated or user)
3. Gathering of data and projection onto vector
4. Determination of sample width
5. Calculation of cell numbers and additional data
6. Finding nearest cells and clusters
7. Border region detection
8. Calculation of statistics
9. Plotting

The analysis requires a specific folder and file hierarchy, all of which need to be named respecting a convention in order to provide information for the program (see 4.2.1). The sample vectors that are used to reduce the dimensionality of data can be either made by the user or alternatively by LAM (see 4.4). The rest of LAM's functionalities are performed automatically in accordance to user-defined settings.

4.1. Image pre-processing

To assure the best functioning of LAM, certain pre-processing steps are required before object detection and subsequent data feeding to LAM. Of these, the masking and orienting of the microscopy images is paramount. The vector creation also functions best with straight midguts; inordinate curvature may cause premature end of vectorization or other artifacts. The samples are to be masked so that no excess features are present, i.e. the image contains only intensities from the midgut. When using automatic creation of vectors, the samples should also be oriented so that either end of the midgut has the smallest X-coordinate value. This orientation must be the same between samples of an experiment, e.g. all samples have the anterior end at the lowest X-axis values. The orientation is necessary in order to create proper vectors and to provide comparable quantification. LAM also includes a script to rotate feature coordinate csv's around the origin, if necessary (comp/rotator.py). When providing user-made vectors, the coordinates must be provided in the same orientation, e.g. from anterior end to posterior, but the rotation of samples does not matter.

Notification:

*Required **sample size** for each sample group depends on multiple factors including the strength of the effect caused by the experiment, but also how variable the response is. Exact sample size is difficult to determine beforehand, but **we recommend at least 10 samples per group**.*

4.2. Input

Originally, LAM was designed to be an extension to Imaris–analyses and consequently exported data files from Imaris's feature detection and other tools are in a format that is immediately usable by LAM. However, LAM only requires that the data is numerical and in "Position.csv" with certain column labels. The main functionality of LAM, i.e. the locational quantification of cells, requires only XY-coordinates of cells within a csv-file with all variables on columns and each cell on separate row, i.e. in wide-format. For finding nearest cells and clusters, the Z-coordinate of the cells is also required. Any additional data that is to be analyzed must be defined in the settings ('*AddData*') and can either be located within the same csv-file with the positional data or separately. All samples are not expected to have data from all microscopy channels; LAM only adds the data to analysis if it is found.

4.2.1. File organization and naming

LAM expects to find all input data in a certain hierarchy of directories and with specific filenames that contain necessary information (Fig. 1). As LAM scans the directory for files and folders without user-defined samples, sample groups, or channels, the recommendation is not to have any unrelated files or folders within the input data-directory, as this can break the analysis. LAM automatically gathers the names of the samples, groups, and channel-names from the paths of the files.

The full naming convention for the paths in the analysis is as follows:

<group>_[<descriptor>_<sample>]_<channel>_<xyz>,

where group denotes the sample group, descriptor and sample are used to identify individual samples (e.g. date of imaging and sample name), and channel is an identification for a data folder. The descriptor and sample can also be replaced with just one identifier. The "xyz" at the end can be any string of text and is not used by LAM; it is present for convenience, as e.g. Imaris-exported data includes "_Statistics" at the end.

Within the analysis directory, the files and folders for the analysis must be organized in the manner shown by Fig. 1. All samples must be located at the root of the directory in folders named as <group>_<descriptor>_<sample>, e.g.

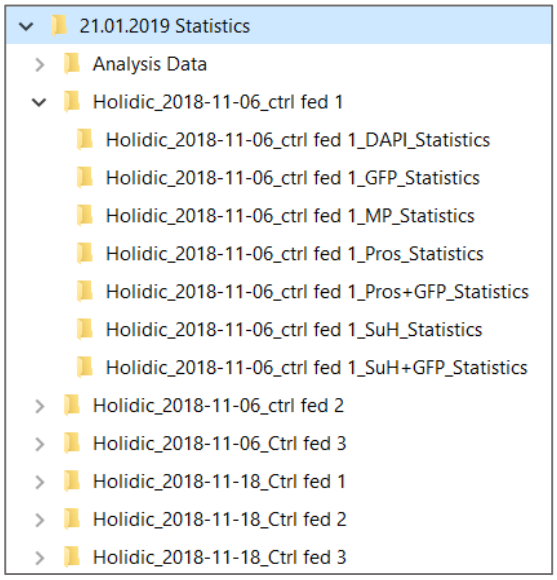

Fig. 1. Analysis directory hierarchy. *All sample folders are to be in the root of the analysis directory. Within the sample folders, each channel must have its own folder containing all channel-specific data files. Additionally, the folders must be named consistently and according to a convention.*

Notification:

Underscore '_' is used as a delimiter for information and is consequently reserved for LAM. *Any use of underscore by the user will likely interfere with the analysis. Naming should be restricted to letters [a-z, A-Z], numbers [0-9].*

control_09-06-2019_sample1. Within the sample folders, each sample must have a separate folder for each channel it has data for, named with the before mentioned name extended with <channel>_xyz, e.g. *control_09-06-2019_sample1_DAPI_Stats*. Each data file that relates to the specific channel and sample must be found within these channel folders.

4.2.2. Data file column labels

The datafiles require specific columns (Fig. 2). In the Position.csv, the coordinates of cells should be marked with column labels 'Position X', 'Position Y', and 'Position Z'. Additionally, each cell should have its own unique identification number in a column labeled as 'ID'. This cell ID is used to identify cells *within* a channel and must be shared between all data files of the channel to correctly associate their data. If the data files contain e.g. metadata-headers, the *'header_row'*-setting can be changed to point at the correct row with column labels, with row numbering starting from zero. LAM expects all input datafiles to have the same header row index.

All data specific to a sample must be in its sample-specific folder at the root of the analysis directory. Similarly, all data related to one channel must be in the specific channel-folder of the respective sample folder.

Position X,Position Y,Position Z,Unit,Category,Collection,Time,ID,		
4735.07,390.268,0.7,um,Spot,Position,1,0,		
4741.71,397.542,0.7,um,Spot,Position,1,1,		
4726.84,399.752,0.7,um,Spot,Position,1,2,		
4705.06,405.75,0.7,um,Spot,Position,1,3,		
4698.85,411.068,0.7,um,Spot,Position,1,4,		

Fig. 2. Example of Position.csv column labels and data in Imaris–format. *LAM requires the positional coordinates in columns labeled with 'Position X', 'Position Y', and 'Position Z'. Additionally, the file must contain 'ID'–column where each cell of the channel has a unique identifier that is used for merging data. Of these column labels, the unit, category, and collection are not used by LAM.*

4.2.3. Additional data

LAM considers all data in addition to the positional coordinates and ID as '*additional data*', e.g. area, volume, and intensities. These data files must be defined within the *'AddData'*–dictionary variable in *settings.py*, or alternatively in the *'Other'*-window of GUI. Additional data can be in the Position.csv, but its location must be given in the variable. In the *AddData*-dictionary, the key (in bold below) defines the column label for the data:

*AddData = {"**Area**": ["Area.csv", "Area, $\u03BCm^2$"]}.*

The values given to the key, in order, are the name of the data-containing file, and the unit of the variable. Any special characters should be given in Unicode and between '$'-signs.

When inputting a variable that has multiple types, e.g. intensities from each microscopy channel, LAM expects the column labels to have an identifier separated by underscore, e.g. "Intensity Median_Ch=1" or "Intensity Median_ch2". The identifiers need not be defined, as LAM collects all files that contain the key-string while using the identifiers to separate the data from each other. The intensity channels can however be defined in order to change them to a form that is more informative, e.g. from Ch=4 to DAPI, using the *'replace file ID'*–option in GUI's Other–window (*replaceID* and *channelID* in settings.py).

4.2.4. Sample anchoring (MP)

On some experimental setups the size proportions of different regions may alter, e.g. when comparing starved and fully fed midguts. In these cases, more accurate results can be obtained by dividing the data into multiple analyses (see 4.5 split & combine). Alternatively, a user-defined coordinate (MP = measurement point) at a distinguishable point can be used to anchor the individual samples for comparison. For example, MP at R2/3-border of each sample causes them to be lined at a specific bin, with each sample having variable numbers of bins on either side (Fig. 3). The proportional variation however likely leads to a compounding error as distance from the MP grows. When MP is not used, the samples are lined at bin 0 and compared bin-by-bin. The MP-input is given similarly to channel data, i.e. as a separate directory that contains "Position.csv" for a single coordinate, the MP. The anchoring point data folder is to be named after *MPname*-variable (default '*MP'*). The anchoring functionality is controlled by the '*useMP'*-setting.

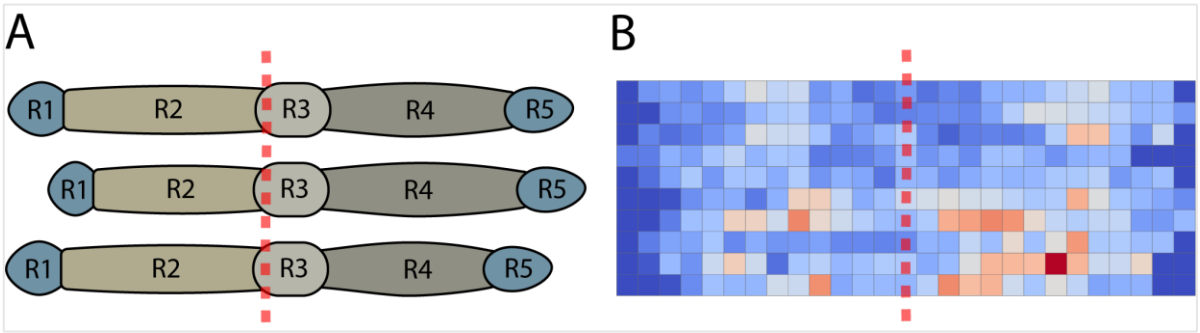

Fig. 3. Sample anchoring using Measurement Points. *Due to possible proportional variation between sample groups, LAM has the option to anchor samples based on user-given coordinates. The anchoring coordinate is called a measurement point, or MP. When using MPs, the samples are lined so that every MP is located at same bin index. As a result, the samples are in 'focus' at the given positions and comparison between samples and sample groups gives biologically relevant data.* ***a)*** *MP marked by the red dashed line. Each sample has the same number of bins, i.e. 100% of user-defined bins, but after sample anchoring the total length of the dataset can be greater than 100% due to difference in anchoring point locations between samples.* ***b)*** *Heat maps of anchored data with each row representing one whole-midgut sample. MP marked by the red dashed line. Any compounding error from proportional variation is smallest near the MP-bin.*

4.3. Primary functionalities

LAM has several primary functionalities:

- **Process**
- **Count**
- **Distance**
- **Plots**
- **Stats**

The ***Process***-setting creates the vectors, while ***Count*** projects cells from all found channels and counts their numbers. The output files from **Count** are required for the rest of the functionalities. Making of plots and statistical calculations are controlled via the ***Plots*** and ***Stats*** settings. The ***Distance*** setting is used for the calculation of distances to nearest cells and for finding clusters of cells. In the GUI, clicking any of these primary settings will enable or disable input for the related settings.

NOTE: using **Count** *clears the 'Analysis Data'–folder which holds many LAM–created data files. Move any earlier data you may want to keep.*

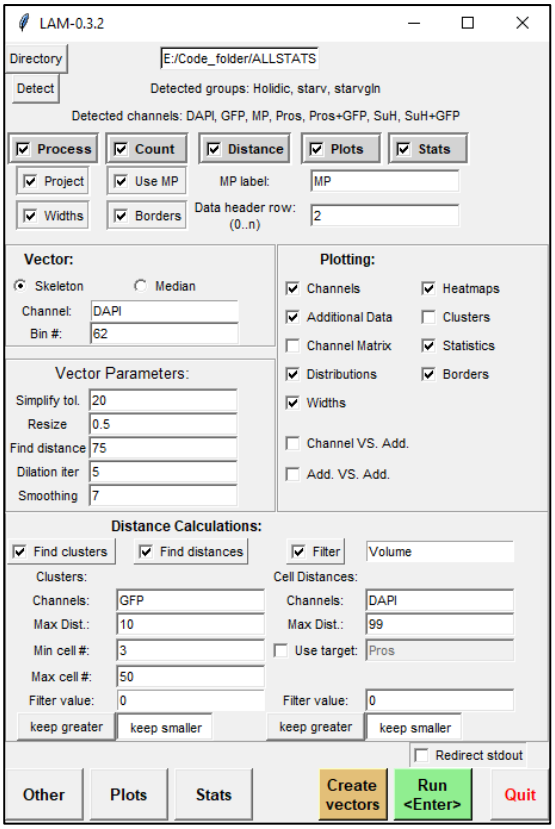

Fig. 4. LAM graphical user interface. *The five primary functionalities of LAM (dark boxes) control which settings are enabled. Use of 'Count' requires that either 'Process' has been run or user has provided self-made vector-files. The three other functionalities require that 'Count' has been performed.*

4.3.1. Default values

The default values for the GUI (and the analysis) are stored in settings.py and can be altered at will. Any changes to the settings within the GUI will revert to the default values when the program is run again.

4.4. Vector creation

The vector creation of LAM is limited to planar geometries by the *Shapely* package, i.e. only XY-coordinates are used. The limitation should not cause variation in the counting of cells as the midgut is pseudostratified and there is little Z-coordinate difference between cells, assuming the sample is flat when imaged. The vectors are created based on positional data of the 'vector channel', defined by the given value in *'Channel'*-setting (*'vectChannel'* in settings.py). The creation begins from cells with the lowest X-coordinates, and consequently all samples are expected to be oriented the same in the coordinate system. In case of wrongly oriented samples, a coordinate system-rotation script (rotator.py) can be found in the 'comp'-folder of LAM.

The vectors for the samples can be created automatically in two ways: by a running median or by skeletonization. Both methods have their own benefits and drawbacks (4.4.1). The whole analysis can be performed in one run; however, the best and most accurate results can be obtained by subjecting the samples to multiple rounds of vector creation and keeping the well-fitting vectors for further analysis.

A typical workflow starts by selecting only the ***Process***-setting and pressing "*Create vectors"*-button at the bottom of the GUI to create vectors for all samples in loops with varying creation parameters. After each round of vector-creation, the quality of the vectors can be verified from the vector plot located at *'./Analysis Data/Samples'*. Next, select all samples with fit vectors in the "*Create vectors"*-window and press "Keep" to drop the samples from further creation loops. After, the remaining samples can be subjected to another loop with altered vector creation parameters, again keeping the fit vectors, and looping until all samples have valid vectors. Any unfit vectors can also be directly modified by changing any offending coordinates or by adding new ones to the vector.csv in the "Analysis Data/Samples"-directories. Alternatively, the user can generate the vector themselves and place it in vector.csv (or .txt) (4.4.2).

4.4.1. Vector types

Both vector creation types share two settings: the vector channel and simplify tolerance (Fig. 5). The vector channel designates the data channel that is used for creating the vector. Best approximation of the sample is achieved by using a channel that is densely populated by cells. Consequently, the recommendation is to use data files containing nuclear label data (e.g. DAPI) or similarly dense features.

Different methods of vector creation can lead to different kinds of artifacts in the approximation. The artifacts can often be removed with vector simplification, i.e. the straightening of the vector by moving its points. The *'Simplify tol.'*–setting gives the maximal distance of the movement.

Median vector

The median vector–creation performs ideally with fully straightened samples. However, steep curvature in the sample or any backwards movement on X-axis will cause problems for this vector type. The vector creation is simple; it is based on calculating the middle Y-axis coordinate between the top-most and bottom-most cells in each 'median bin'. The number of the bins is defined by the *'Median bins'*-setting in vector parameters (Fig. 5a). The edges of the median bins are defined by equidistant points between the lowest and highest X-coordinates of the dataset's cells. The left-most bin is assumed to be the first bin, meaning that the vectors are created from left to right (smallest X-value to largest), and the samples should be oriented to account for this. To assure the best quality of sample approximation for the vector, the number of median bins should be set high. The median bin number is ultimately limited by the density of cells on the channel that is used for vector creation; too many bins might lead to bins with no cells, in which case the previous bin's value is copied. The empty bins can consequently lead to stair-like increases in the median, but this can be remedied with vector simplification, set by *'Simplify tol.'*.

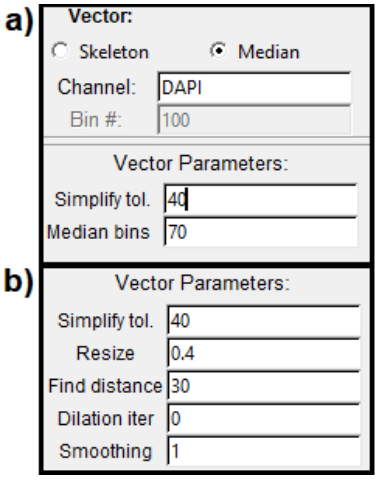

*Fig. 5. Vector creation settings. **a)** In the upper frame are the general settings for vector creation and in the lower frame the median vector-related settings. **b)** All settings related to skeleton vector creation.*

Skeleton vector

The skeleton-method offers better approximation for highly curved samples, especially if the sample has any back and forth movement on the X-axis. The downside is that some settings may sample-specifically

cause branching in the skeleton, resulting in a broken vector. Recommendation is to ascertain the quality from the vector plot; there is no guaranteed warning when a vector is faulty. When using skeleton vectors, LAM produces an additional plot of the original binary image and the resulting skeleton to each sample's folder. This skeleton plot can be useful in determining how the creation settings should be changed to get a fit vector.

The creation settings for the skeleton vector are *'resize', 'find distance', 'dilation iter.',* and *'smoothing'* (Fig. 5b). The optimal settings are highly dependent on the shape of each sample. Most important of these are *'resize'* and *'smoothing'* (Fig. 6). Both settings remove roughness from the edges of the sample and consequently reduce branching of the skeleton. However, in samples where loops in the midgut bring the intestinal walls close together, these settings can cause the different segments to merge. In these cases, it is necessary to have a lesser size decrease and less smoothing. When any resizing would lead to merging of midgut segments, increasing the iteration number of binary dilation (*'dilation iter.'*) may help in making the midgut uniform in the transformed binary image and consequently reduce skeleton branching.

The transformation of the skeleton into a vector is performed by following pixels beginning from the smallest X-coordinate. From the start point, the creation algorithm adds new points to the vector by scoring pixels based on distance and the direction of the existing vector. Due to the scoring method, the vector creation can at times follow the wrong pixels if there is any branching in a steep curve or if the correct path takes a sharp turn. In these cases, it is necessary to reduce the branching with for example increased smoothing in order to get a fit vector. In case of a difficult sample, the user can provide self-created vector-file.

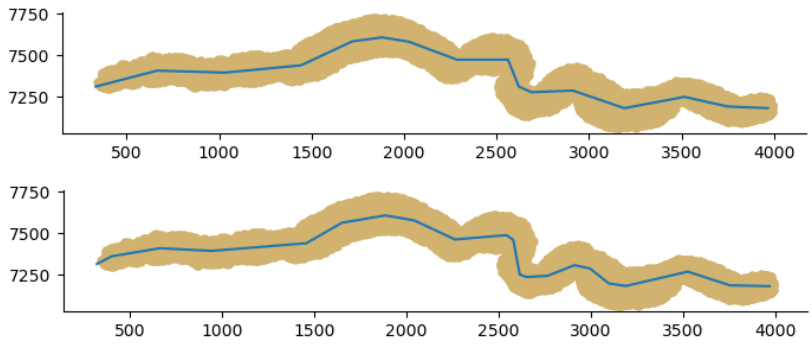

Fig. 6. Effects of resizing and smoothing on skeleton vector creation. *On the top values of resizing and smoothing are 0.5 and 7, respectively. The settings cause fusing of segments in the latter half of the sample, subsequently leading to errors in object projection. On the lower image, using a lesser reduction in size (0.8) and lesser smoothing (3) leads to a much improved, nearly optimal vector.*

4.4.2. User-generated vectors

When opting to use self-created vectors, the coordinates must be given in the same order for all samples, e.g. from anterior to posterior. The number of the vector-defining coordinates is arbitrary. A convenient method for self-drawn vectors is to use one of Fiji ImageJ's line tools on the microscopy image and then

saving as XY coordinates. The user-generated vectors must be placed similarly to LAM-created vectors, in the vector.csv's (or txt) located at respective sample-folder in *'. /Analysis Data/Samples'.*

The vectors can be provided in either csv- or txt-format. Exported ImageJ line tool XY-coordinate can be directly used as vector files when named as 'Vector.txt'. The txt-files must only contain the X and Y coordinate values on columns in respective order and with no header, separated by tabulator ('\t').

4.5. Cell projection and counting

After vector creation, all channel datasets are projected onto the vector if the ***Count*** setting is selected. For counting, the most critical value is *'Bin #*' located within the *vector*-frame in the GUI (*projBins* in settings.py). The bin number determines the length of LAM-created data arrays and consequently affects plotting and statistical analysis. All vectors are divided into the same number of bins. The interval of the bins is uniform within a sample but is variable between samples due to length differences in vectors. Consequently, the binning functions well between sample groups that have similar proportions of different parts of the midgut. If proportional variance exists, the samples can be cropped so that the binning corresponds to the biological proportions (see below, split & combine).

When anchoring the samples at some specific point along the midgut, e.g. R3-region, the *'use MP'*-option can be selected and input given to *'MP label'* to collect the coordinates of the anchoring point for each sample. When using *MP*, each sample must have the anchoring coordinate located in Position.csv similarly to other channel data, with the data-folder named after the '*MP label*'-variable. When '*use MP'* is not selected, the samples are aligned bin to bin.

The projection is done by minimal distance estimation and consequently the cells are counted as belonging to the bin interval that is nearest to them. Data on the projection can be found within csv's named by respective channels at *'./Analysis Data/Samples/'*. The new data columns created by the projection are *'VectPoint'*, *'NormDist'*, and *'DistBin'*, which are the XY-coordinates of the point of projection along the vector, the normalized distance [0, 1] along the vector, and the projection's bin number, respectively.

Cropping samples and combining subsets (split & combine)

The simplest way to divide samples into comparable subsets of data is to provide cut-point coordinates for each sample in a similar manner to a *MP* and then use the script *split_dataset.py* found in 'LAM-master/comp'-folder. Alternatively, the script can be provided with a csv-file that contains bin-numbers of cut-off points for each sample. One way to do this is to run border detection for anchored whole midgut samples and determining the cut-off points for each sample from the output files. The border detection requires LAM-created width-data and feature-to-feature distances on the vector-creation channel (default DAPI).

In practice, if proportional variance exists at R4-5 region between the sample groups, each sample's data could include as a channel an individual coordinate point that corresponds to R3/4 border. After using **Count** on the full dataset, the projected border-coordinate can be used to split the sample's data and

vector using the *split_dataset*-script. The script also provides bin length suggestions for each subset of the split data in order to preserve a more standardized biological length of each bin. Afterwards, each subset can be analyzed separately to compare region-specifically. As a note, when analyzing the subsets with LAM, the 'header_row'-setting must be set to zero, as data produced by LAM contain no headers.

Another way of doing the analysis after splitting is to only perform ***Count*** for each of the data subsets, and then combining the sets with the companion-script *combineSets.py*. Combining the datasets in effect rejoins all data while retaining the refined binning of the subsets for further analysis, e.g. statistics and plotting of the full-length midgut. Note that after using **Count** on the subsets, all cells have already been projected before combining, and consequently the subsequent ***Count*** for the whole data set should be performed with *'project'* set to False in order to preserve projection data of each subset. Combining the data breaks the equality of bin lengths within a sample but will align the sample groups in a way that is more biologically relevant if done properly.

4.6. Distance calculations

The two functionalities controlled by the ***Distance***-setting, i.e. the computation of feature-to-feature nearest distances and the detection of clusters, function based on K-Dimensional tree calculations. Only the smallest distance is used when computing nearest distances, but when finding clusters, neighborhoods of cells are grouped to form 'seeds' that are later merged based on shared cell ID's to form the final clusters. Both functionalities are controlled individually, but they share settings (Fig. 7). Both can be performed on multiple channels within the same analysis, set by *'Channels'*. The *'Max Dist.'* limits how distant cells are considered neighbors. The cells can also be filtered by a variable of choice such as volume, to include cells with either smaller or larger value than the value set in *'Size incl'*. In case of feature-to-feature distance calculation, the distance can be set to be calculated against a specific target channel, defined by *'Use target'*. With this setting, the user can for example find nearest Pros-positive cells for each GFP-positive cell. When finding clusters, the user can define cell number limits for what is considered a cluster by modifying the *'Min cell #'* and *'Max cell #'*–settings.

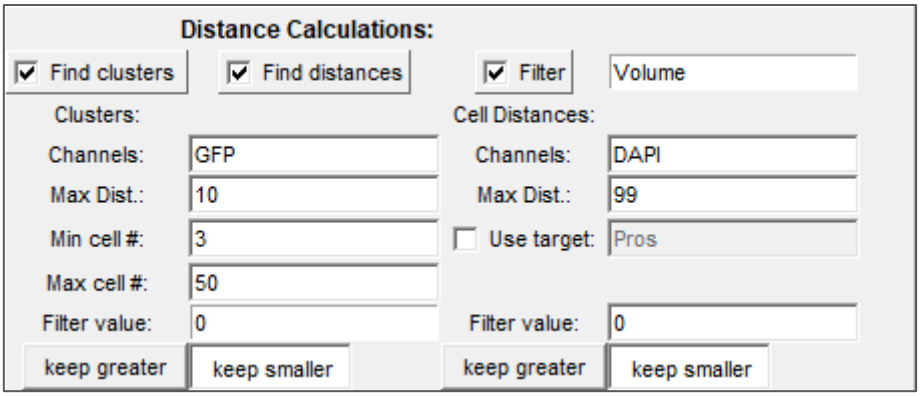

Fig. 7. The settings for distance calculations. *The distance calculation performs two separate functionalities, i.e. the detection of clusters and finding of nearest cells. Settings on left are for clustering and on the right for nearest distances. The "keep greater/smaller"–buttons define how to filter the dataset; either analyze cells with variable (here volume) value greater or smaller than the value given in* 'Filter value'.

Both functionalities create new data columns to the channel csv's in the *'./Analysis Data/Samples/'*–folders. The 'Find distances' creates two columns, labeled *'Nearest_ID'* and *'Nearest_Dist'*, which are the ID of the nearest feature and the distance to it, respectively. The 'Find clusters' creates only one column labeled 'ClusterID', which is a unique identifier that is shared between members of same cluster.

4.7. Width estimation

The estimation of sample width is performed on the vector channel ('vectChannel') and is controlled by the 'Widths'-checkbox in the GUI or the 'measure_width'-setting in settings.py. Additionally, LAM can create plots of the widths by checking the 'Widths'-box in the Plotting-frame of GUI or by setting 'plot_width' to True in settings.py. The width is calculated by following the vector bin-by-bin and summing the average distances to the most distant decile of cells on both hand sides of the vector. The width estimation uses twice the size of bins than the other functionalities to provide increased resolution for border detection (4.8). The estimation creates two output files: the "Sample_widths.csv" that contains non-anchored values, and "Sample_widths_norm.csv" that contains sample values anchored to the MP.

4.8. Border detection

For the most part border detection is controlled from settings.py, but there is an ON/OFF checkbox at the upper frame of GUI and the '*Borders'*-checkbox found in the 'Plotting'-frame which controls the creation of border plots. The settings.py has a border detection subsection that has settings for what variables are used for scoring and what are the scoring weights of the variables: 'border_vars' and 'scoring_vars', respectively. In the 'scoring_vars'-setting, adding extensions "_diff" or "_std" to the ends of the variable names causes LAM to compute either differentials or standard deviations of the variable for every bin along each sample's vector. On default settings, the border detection requires LAM-created width data (0) in addition to feature-to-feature distance data on the channel provided to the 'border_channel'-setting (default channel is the vector creation channel) (4.6). The default values are intended for border detection on whole-midgut DAPI-data. All found border regions of each group can be added to other plots via the Boolean 'add_peaks'-setting. Additionally, if 'select_peaks'-setting is True the user will be asked to define which peaks will be plotted, otherwise all found peaks are added. The border detection algorithm will as a default produce a "All-Border_Scores"-plot into "Analysis Data/Plots/Borders" that can be used in determining each sample groups' valid peaks.

The border detection outputs two data files: "Borders_peaks.csv" that contains the peaks of the border scores and their prominences for each sample group, and "Borders_scores.csv" that contains the border score of each bin of each sample.

4.9. Statistics

With the selection of **Stats**–setting, LAM can compute two different kinds of statistics, called total statistics and versus statistics. The total statistics create comparisons of sample group data regarding total cell numbers on each channel and averages of additional data in each bin of sample groups. The versus statistics are comparisons of binned data of control group and a test group. The statistics are

calculated channel-specifically for cell counts and additional data, e.g. for GFP cell counts and for the areas of the GFP cells etc. Additionally, the statistical analyses are performed for any LAM-calculated data such as clusters and feature-to-feature distances. For statistical plots to be created, both ***Stats*** and ***Plots***–primary settings must be selected. The calculated statistical data can be found in *'./Analysis Data/Stats'*-folder.

The versus statistics are calculated with Mann-Whitney-Wilcoxon U-test with corrections for continuity and multiple testing. The total statistics are done similarly, but without multiple test correction as each group is pooled. The versus statistics can be performed either bin-by-bin or with a sliding window with adjustable leading and trailing edge lengths (stats-window of GUI). On channels with low count numbers, increasing the size of the window leads to greater number of non-zero observations per test, consequently increasing its power at the cost of resolution.

4.10. Plotting

Plots are drawn by selecting the ***Plots***–setting and all wanted plots from *'Plotting'*. All plotting excluding statistical plots can be performed in a separate run from the other functionalities of LAM; the plots are created from the data files that are found in the analysis directory. To obtain statistical plots, the ***Stats***–setting must also be selected. Similarly, the 'borders'–plot option also requires 'border detection' to be in use.

The LAM-included plotting options are:

Additional Data – Bin-by-bin line plots of additional data, including LAM-created cell-to-cell distances. The line is formed from each sample's averages at the respective bin marked by X-axis. The band around the line indicates the standard deviation of the averages. Output files named e.g. "Additional Data – GFP"

Borders –Creates a file that contains plots regarding scores and found borders for each sample group. The 'Raw group score"-plot shows average scores of samples in the groups. To obtain the 'Smoothed mean scores'-plot, the raw scores are fitted with a curve that is promptly subtracted from the raw values to simulate more local changes in the score, which are then rescaled to [0, 1]. By setting '*plot_samples'* to True in settings.py, LAM also creates individual sample score plots. The "Normalized variables" contains normalized values of each used variable from which a fitted curve is subtracted and resulting deviances are multiplied by the scoring weights to provide the 'Raw scores'-plot. The 'Smooth score differential'-plot contains the smoothed differential of the sum of raw scores in order to detect regions where score shifts rapidly. Output to subfolder 'Borders'.

Channels – Bin-by-bin line plots of counts for each channel, with bins of the full data-matrix on X-axis and cell counts on Y-axis. Output file is named "Channels – All".

Channel Matrix – A grid of channel count data against each other, with channel count distribution on the diagonal axis. Each scatter marker on the grid represents the average cell counts of the samples in one bin on the respective channels. The bands around the regression lines indicate standard deviation. Output file: "Channels – Matrix".

Clusters – Creates three kinds of plots based on computed clusters: heatmaps and line plots of counts of clustered cells, and positional plots of clusters within samples. On the positional scatter plots, the tan-colored markers are all cells on the channel, and each found cluster has an individual randomized color. Requires data from *Distances / Find clusters*. Output files created to "Clusters"-subfolder.

Distributions – Probability density distributions of bin values of channel counts and additional data, including LAM-created cell-to-cell distances. Output files named e.g. "Distributions – Additional GFP Data".

Heatmaps – Heat maps of bin-by-bin cell count averages of sample groups ("Heatmaps – Groups") and cell counts of samples on all channels ("Heatmaps – Samples").

Statistics – Two types of plots: total and versus plots. Total statistics creates violin plots with each sample group for every channel and additional data type. The versus statistics are plotted as bin-by-bin box plots of two sample groups, with P-value significance marked either with stars or negative log2 line plot, and with a possibility for coloring of significant bins. Additionally, the 'observations'-setting in the Plots-window can be selected to plot individual observations on top of the box plot. The Y-limit of the negative log2 line can be changed in the GUI's Plots-window or by changing *'ylim'* in settings.py. The stars from one to three indicate significances of 0.05, 0.01, and 0.001, respectively. Color fill of significant regions is based on the same significances but begins from the P-value defined by *'alpha'* (GUI's Stats window). The whiskers of the boxplots extend to 1.5 times interquartile range and scatters mark data points beyond this. Output to "Plots/Stats".

Widths – Creates a line plot that contains the average width of each sample group along the antero-posterior axis. The band around the average line is the standard deviation. Output file is named "Widths – All".

Channel VS. Add. – A bivariate density estimate of the channel counts and additional data, using averages of each bin of a sample group. Plots created for all possible combinations and may consequently create many plots depending on settings and data. The plotting combinations can be restricted by inputting only wanted data variables in GUI's Plots–window under the *'versus plots'*-section (*'vs_chans'* and *'vs_adds'* in settings.py), e.g. to DAPI and Intensity Mean-Ch=3. The plots represent density estimation of one sample groups values of two variables. The marginal plots are distributions of the variable on the opposing axis line. Output to "Versus"-subfolder.

Add. VS. Add. – As in *'Channel VS. Add.'* but with additional data type against another type.

5 Output Files

LAM creates myriad of output files depending on used settings. All created files can be found in *'./Analysis Data'* and its subfolders. The format of created plot-files can be changed within the *'Plots'*-window in the GUI or by *'saveformat'* in settings.py.

Collections of the used channels, samples, and sample groups can be found at the root of the *'Analysis Data'*–folder. The folder also contains the following subfolders for storing various data:

Data Files – For storing collections of data from samples. As a baseline, the files have either samples or sample groups in the columns and the projection bins in order as rows. The exception to this are 'Total Counts.csv' with the total cell numbers for each channel on each row, the 'MPs.csv' with the bin number of each sample's MP on the row, and 'Length.csv' with the length of each samples vector on the row.

The rows of files with the 'All_'-prefix contain cell counts of each bin of each sample. The data with 'Norm_'-prefix is the same data as with 'All_', but with MP anchoring. The rows of 'Avg_'–prefixed files contain the average values of the variable for all cells that fall into the bin. Similarly, the rows of files with 'ChanAvg_'–prefix contain the average channel cell counts of the sample groups for each bin. The 'Clusters' and 'ClNorm' contain the numbers of clustered cells without and with anchoring, respectively.

Plots – Contains all plots created by LAM, except for vector-related plots that are in 'Samples'-subfolder.

Samples – Stores LAM–created and –edited sample-specific data. Within each sample's folder can be found the vector.csv and channel-specific csv's. The channel csv's contain all collected input data for the sample, in addition to LAM–created columns. The created columns are:

- Projection – *'VectPoint'*, *'NormDist'*, and *'DistBin'* are the XY-coordinates of the point of projection along the vector, the normalized distance (0–1) along the vector, and the projection's bin number, respectively.
- Clustering – *'ClusterID'* indicates the given identification number to the cluster that the respective cell belongs to.
- Distance – *'Nearest_XYZ'*, *'Nearest_Dist'*, and *'Nearest_ID'* are the coordinates of the nearest cell, the distance to it, and its ID, respectively.

The root of the *'Samples'*–folder also contains the plot of sample vectors if automatic vector creation was used, and within the subfolders can be found the skeleton plots if skeleton vector creation was used.

Statistics – Contains all files with statistical data. Each file contains the data of bin-to-bin or windowed tests of the control group against another group. The test variable is indicated after the '='–sign in the file name, i.e. *"Stats_<test groups> = <channel> (<additional data>)".*

Column labels:
- U Score – The MWW U–score of the compared populations
- Corr. <Greater/Lesser> – Corrected P-value that control group is <Greater/Lesser>
- P <Greater/Lesser> – Non-corrected P-value that control group is <Greater/Lesser>
- Labels with 'Two-sided' indicate the probability that there is a significance in either way

Additionally, the Statistics–folder contains the 'Total Count Stats.csv' with the two-way statistics of each channel for the test groups.

6 Definitions

6.1. Top Frame

GUI	*Settings.py*	Description
Borders	*Border_detection*	Calculate border region scores for samples and groups
Count	*process_counts*	Projection, anchoring, and counting of data on all channels
Data file header row	*header_row*	The expected row of column labels in user given data files
Distance	*process_dists*	Calculation of cell-to-cell distances and detection of clusters
Directory	*workdir*	Path to analysis directory where sample data is located
MP label	*MPname*	The name of the csv data files containing anchoring point locations
Plots	*Create_Plots*	Creation of any/all plots
Project	*project*	Whether to project features onto vector during **Count**
Process	*process_samples*	Creation of vectors for samples
Stats	*statistics*	Calculation of statistics
Use MP	*useMP*	Whether to use user given anchoring points
Widths	Measure_width	Estimate widths of each sample

6.2. Vector Frame

GUI	*Settings.py*	Description
Bin #	*projBins*	Number of bins onto which data is projected on all vectors
Channel	*vectChannel*	The channel on which vector creation is based on
Skeleton / Median	*SkeletonVector*	The type of vector to be created

6.3. Vector Parameters Frame

GUI	*Settings.py*	Description
Dilation iter.	*BDiter*	Iterations of binary dilation (2x2) on resized and image-transformed position coordinates
Find distance	*find_dist*	The maximum coordinate distance of next pixel (coordinate) of vector when creating from skeleton
Median bins	*medianBins*	The number of medians that are calculated for the sample when creating a median vector
Resize	*SkeletonResize*	Binary image resizing factor when creating skeleton vector
Simplify tol.	*simplifyTol*	Tolerance of coordinate adjustment for vector simplification
Smoothing	*SigmaGauss*	Sigma for Gaussian smoothing of binary image

6.4. Plotting Frame

GUI	*Settings.py*	Description
Add. VS. Add.	*Create_AddVSAdd_Plots*	Plot additional data against additional data
Additional Data	*Create_AddData_Plots*	Plot additional data averages per bin
Borders	*Create_Border_Plots*	Plot border scores of sample groups
Channel pair plots	*Create_Channel_PairPlots*	Create plot matrix of all channels against all channels
Channel VS. Add	*Create_ChanVSAdd_Plots*	Plot channels versus additional data
Channels	*Create_Channel_Plots*	Plot box plots of channel data
Clusters	*Create_Cluster_Plots*	Create box plots and heat maps of clustered cells
Distributions	*Create_Distribution_Plots*	Plot distribution densities for all channel and additional data
Heat maps	*Create_Heatmaps*	Plot sample and sample group heatmaps of channel counts
Statistics	*Create_Statistics_Plots*	Plot statistical box plots of all data

6.5. Distance Calculations Frame

Compared to cell-to-cell distances, the variables specific to clustering algorithm have the prefix 'Cl_' in settings.py.

GUI	*Settings.py*	Description
Channels	*Distance_Channels, Cluster_Channels*	The channels to which distances are calculated
Filter size	*N/A*	Whether to filter cells based on volume
Find clusters	*Find_Clusters*	Whether to perform clustering of cells
Find distances	*Find_Distances*	Whether to find cell-to-cell distances
Greater / Smaller	*incl_type, Cl_incl_type*	The direction of size filtering, includes cells of either smaller or greater size
Max cell #	*Cl_max*	Maximum cell number that is considered to be a cluster
Max Dist.	*maxDist, Cl_maxDist*	Maximum distance between cells to be considered neighbors. In clustering Max Dist is a hard distance limit for inclusion into a cluster.
Min cell #	*Cl_min*	Minimum cell number of a cluster
Size incl.	*Vol_inclusion, Cl_Vol_inclusion*	The volume limit of filtering
Use target	*use_target + target_chan*	Calculate cell-to-cell distances from one channel to the target channel

6.6. Other Window

GUI	*Settings.py*	Description
Column label / csv-file / Unit	*AddData*	Gathering of additional data. The label of the column where data is located / the name of the file where data is / unit of the data for plotting
Distances i. col	*incl_col*	Define variable for filtering during **Distance** functionalities
File descriptor / Change to	*channelID*	Replace these ID's found in file names / the proper names of the ID's
Replace file ID	*replaceID*	Whether to replace channel ID's found in file names

6.7. Plots Window

General settings:

GUI	Settings.py	Description
Drop outliers	*Drop_Outliers*	Whether to drop data point outliers based on **Std. dev**
Pairplot jitter	*plot_jitter*	Create jitter for pair plot scatters for easier visualization of discretized data
Plotted add. data	*vs_adds*	Define additional data to be plotted in versus plots
Plotted channels	*vs_channels*	Define channel data to be plotted in versus plots
Save format	*saveformat*	The saving format of all plots
Std dev.	*dropSTD*	The standard deviation limit for **Drop outliers**

Statistical Plotting:

GUI	Settings.py	Description
Sign. color	*fill*	Do color fill for statistically significant regions
Sign. stars	*stars*	Include significance stars to statistical plots
Neg. log2	*negLog2*	Create negative log2 significance line for statistical plots
y-limit	*ylim*	Y-axis value limit for **Neg. log2** in plot
Observations	*observations*	Plot individual observations (DEPRECATED)

6.8. Stats Window

GUI	Settings.py	Description
Alpha	*alpha*	The P-value limit for the rejection of null hypothesis
Control Group	*cntrlGroup*	Name of the control group
Group vs. Group	*stat_versus*	Create control group versus sample group statistics
Total Statistics	*stat_total*	Create statistics of total cell counts
Trailing / Leading window	*trail / lead*	Number of included bins in front and behind current index position when using **windowed statistics**
Windowed statistics	*windowed*	Statistics done in a sliding window defined by **trail** and **lead**

6.9. Redirect stdout

GUI	*Settings.py*	Description
Redirect stdout	*non_stdout*	Redirects output to a separate window

6.10. Non-GUI settings

Settings.py	Description
border_channel	Define channel name for border detection
plot_samples	Create border plots for individual samples
border_vars	Define variables that are collected for border detection
scoring_vars	Define scoring weights for border detection variables
add_peaks	Add detected border regions to other plots
select_peaks	Only subset of peaks will be plotted by 'add_peaks'
seaborn_style	Change plot style. Can break plots.
seaborn_context	Change plot element sizes. Can break plots.
Palette_colors	Change sample group plot colors. Groups given color in alphabetical order.

7 Command line arguments

usage: run.py [-h] [-p PATH] [-o OPTIONS] [-b BINS] [-v CHANNEL]
[-g CONTROL_GROUP] [-H HEADER] [-M] [-m MP_NAME] [-G] [-F]
[-f DISTANCE_CHANNELS] [-C] [-c CLUSTER_CHANNELS]
[-d CLUSTER_DISTANCE] [-B] [-W] [-r] [-D]

Perform LAM analysis from command line. Args described as toggle alter the default value in settings.py to the opposite Boolean. All settings that are not altered through parsed arguments will use default values from settings.py.

optional arguments:

-h, --help	show this help message and exit
-p PATH, --path PATH	Analysis directory path
-o OPTIONS, --options OPTIONS	option string: r (process), c (count), d (distance), l (plots), s (stats)
-b BINS, --bins BINS	Sample bin number
-v CHANNEL, --channel CHANNEL	Vector channel name
-g CONTROL_GROUP, --control_group CONTROL_GROUP	Name of control group
-H HEADER, --header HEADER	Header row number
-M, --measurement_point	Toggle useMP
-m MP_NAME, --mp_name MP_NAME	Name of MP
-G, --GUI	Toggle GUI
-F, --feature_distances	Perform feature-to-feature distances
-f DISTANCE_CHANNELS, --Distance_channels DISTANCE_CHANNELS	f-to-f distance channels
-C, --clusters	Perform feature clustering
-c CLUSTER_CHANNELS, --cluster_channels CLUSTER_CHANNELS	Clustering channels
-d CLUSTER_DISTANCE, --cluster_distance CLUSTER_DISTANCE	Clustering max distance
-B, --borders	Toggle border detection
-W, --widths	Toggle width calculation
-r, --no_projection	Projection to false
-D, --force_dialog	Force no user input

Examples:

python src\run.py -p C:\experiment\DSS -o cls -b 62 -MGD

python src\run.py --options rcd --control_group ctrl -F -f GFP -f DAPI -C -c GFP --borders

8 Companion Scripts

The LAM-master folder includes several companion scripts in the 'comp'-folder that perform functionalities related to LAM. These are:

- ChannelPositionPlots.py

Create plots of cell locations on different channels.

- combineSets.py

Used to combine LAM-created data sets from cropped samples.

- rotator.py

Rotate coords around origin to properly orientate samples for LAM vector creation.

- ImarisFileConverter.ijm

Fix broken Aurox confocal images for the Imaris file converter & stitcher

- ManualVectorPlots.py

Create vector plots from vector files

- FileMove.py

Move and rename image files after splitting channels and focal planes (ImarisFileConverter.ijm)

- split_dataset.py

Split all data and vectors in a dataset based on cut point channels

- stitcher-grandma-pro.py

Stitches tiff-images in a level-plane, i.e. without jumps in z-stack.

Refer to files of individual scripts for more information.

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9 Test Data

The distribution includes a small test data set that can be used to try LAM. The data consists of three sample groups with four samples each. The sample groups are Holidic, Starved, and StarvGln, i.e. fully-fed, fed with starvation media, and fed with starvation media with glutamine supplementation. The samples contain data from DAPI, Prospero, and Su(H) stainings, and additionally GFP lineage labelling via Esg-Flip out –system.

It should be noted that four samples per sample group is not enough for a proper analysis, and any results from these samples are only directional.

10 Troubleshoot

As a first rule, when you get errors and/or modify files you should always restart LAM between runs. You should also remove all folders from "Analysis Data" except for the Samples-directory that holds the vector files. Critical errors are often caused by missing data due to faulty vectors and projections. You should always check the LAM-created vector-plot in the Samples-folder, or alternatively when giving user-created vectors, use the companion-script ManualVectorPlots.py to look for any offending vectors. You can also look at files in the "Data Files"-folder to determine samples with faulty data, e.g. samples where most values are zero or that extend much longer at either end of the dataset. If you can't figure how any faulty data comes to be, easiest way to handle it is by removing any offending samples. When removing samples, you should remove the sample from both the root of the analysis directory and from the "Samples"-folder of "Analysis Data", and then restart LAM.

- *The resulting cell counts have peaks at the ends of the bin range*

Either the vectors are not optimally created, or the dataset has cell detection artifacts at the ends of the midgut. Make sure that both ends of the vector extend approximately to the ends of the sample. For example, when creating vectors through skeletonization, resizing of the transformed binary image may lead to incorrect approximation of the sample. As a result, the skeletonization produces a truncated vector and causes overrepresentation of cells in the last bins. Alternatively, check that cell detection was done properly.

- *The vector ends abruptly when created through skeletonization*

Certain patterns in the sample may cause branching in the skeletonization. As the algorithm follows the skeletonized pixels to create the vector, sometimes these branches can confuse the algorithm into a dead-end. Increasing *'find distance'* can allow the vectorization to jump back to the correct skeleton. Alternatively, trying different resizing or greater smoothing can also solve the problem. The best solution can be determined by looking at the skeleton plot in *'./Analysis Data/Samples/'.*

- *The vector jumps in an unexpected manner when created through skeletonization*

Sometimes the algorithm that finds the next pixels of the binary image is fooled by branching of the skeleton. If the vector jumps into a branch that has already been passed, reducing *'Find distance'* can solve the problem.

- *LAM will not run because system cannot find path at start up*

Modify *'workdir'* variable in settings.py to point at the analysis directory. Double check that the path is written correctly and is surrounded by r"", for example r"C:\experimentDirectory".

- *LAM outputs a ValueError when normalizing data*

This happens when data is missing for some reason, e.g. a faulty vector for a sample or missing MP projection when using measurement points. Look at "./Analysis Data/Data Files/" for the error–producing channel's file with prefix "All_", e.g. "All_DAPI.csv" and find samples with abnormal values. Easiest way to handle the error is to remove the offending samples from the analysis by removing the sample from

the root of the analysis directory **and** from "./Analysis Data/Samples/". If data of all samples seems fine, there is likely an error with MP-projection. Check "MPs.csv" and look for samples with abnormal or missing values.