

Traject3D User Manual

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Contents

1	Before starting	2
1.1	KNIME installation	2
1.2	R Integration	3
1.3	Python Integration	4
2	Workflow structure	4
3	Data import and pre-processing	5
3.1	Select Directory and match experiment key	5
3.2	Manually Set Output Folder	5
3.3	Select Samples and Frames to Include	5
3.4	Create and Populate ShapeClassification column	5
3.5	Duplicated Tracking Label Correction	6
3.6	Edit Column Names, Create Unique-object_id, Filter Columns, and Normalise	6
4	Feature Analysis	6
4.1	PCA of Replicates	6
4.2	Calculate TimeChunks	7
4.3	Measurements Over Time	7
4.4	Heatmap/Line Plot of Spheroid Number Over Time, Heatmap of Initial Spheroid Number	7
5	User-defined Classification	8
5.1	Find Trajectories	8
5.2	Compare Proportions of Trajectories	8
5.3	Get Representative spheroid per Trajectory	9
5.4	Subsample or use Entire Dataset	9
5.5	Perform tSNE	9
5.6	tSNE Plotting	9
5.7	Perform tSNE using different variable combinations	9
5.8	Get representative outlines for each classification	10
5.9	Colour and Overlay Outlines	10
5.10	ShapeClassification Mean Measurements	10
5.11	Calculate TimeChunks	10
5.12	ShapeClassification Over Time	10
6	Data-Driven Classification	11
6.1	Subsample or use Entire Dataset	11
6.2	Number of States with Varying knn	11
6.3	Identify States	11
6.4	Find Trajectories	11
6.5	Find Trajectories in One Image Sequence	12
6.6	Compare Proportions of Trajectories	12

6.7	Trajectory Timechunk Plots (Frequency Motifs and Transitions)	12
6.8	Get representative spheroid per Trajectory	13
6.9	Compare Proportions of PhenoGraph Clusters	13
6.10	Get representative outlines for each state	13
6.11	Perform tSNE using different variable combinations	13
6.12	Perform tSNE	14
6.13	tSNE Plotting	14
6.14	Colour and Overlay Outlines	14

1 Before starting

The computer you will be running the KNIME analysis from should have either access to, or a local, copy of the directory containing your phase images, and output from CellProfiler. For reference, this directory should have the following structure:

1. DatasetName
 - Experiment__1
 - Data
 - Experiment Key
 - Phase
 - PhaseGrayCystOutlines
 - Experiment__n
 - Output

Experimental replicate folders are named "Experiment__n", where n corresponds to the replicate number (in no particular order). The "Experiment Key" and "Phase" folders should be populated by the user prior to image analysis with CellProfiler, which will in turn populate "Data" and "PhaseGrayCystOutlines" upon completion of the analysis.

Prior to analysis using this KNIME pipeline, the above folders should contain:

- *Data*: Your data as a CSV file named "PhaseGrayCysts", in which each column is either metadata or a measured morphological feature, and each row corresponds to a single object, from a single image. This file is generated by CellProfiler.
- *Experiment Key*: An Microsoft Excel spreadsheet containing relevant metadata including, but not limited to, sample and experiment names, and well IDs. A sample spreadsheet is provided: simply complete metadata columns (named "Metadata_ExperimentName", "Metadata_Plate", "Metadata_Well", "Condition") in the sheet called "Experiment Key", and other sheets will be automatically filled in. The contents of "Metadata_ExperimentName" should be unique to, and "Condition" naming (e.g. cell line and/or manipulation) should be consistent (in terms of case, spelling, symbols or spaces used) between, each experimental replicate.
- *Phase*: Phase images, in RGB (3-channel) TIFF format. Filenames should be in the following format: ExperimentName_Plate_Well_Site_Date_Time. Image and filename formatting is typically set at the point of export from the imaging system utilised.
- *PhaseGrayCystOutlines*: Binary (white on black background) images of the object outlines, as PNGs. There should be one, equivalently named, outline image for each image in the "Phase" folder. These images are generated by CellProfiler.

The default output directory for KNIME analysis results will be the "Output" subdirectory of this folder.

Experiment Key and directory structure templates can be found in the **davebryantlab/Traject3d** Github repository.

1.1 KNIME installation

KNIME can be downloaded from [here](#).

1.1.1 Notes for KNIME workflow

Required KNIME version and extensions:

Name	Version
KNIME Analytics Platform	4.0.2.v201909300912
KNIME Interactive R Statistics Integration	4.0.1.v201908131226
KNIME Core	4.0.2.v201909300912
KNIME Quick Forms	4.0.2.v201909242005
KNIME Math Expression (JEP)	4.0.2.v201909242005
KNIME Python Integration	4.0.0.v201906241606
KNIME Image Processing	1.8.0.201911140609
KNIME SVG Support	4.0.1.v201908131226
KNIME Virtual Nodes	4.0.0.v201905311239
KNIME Distance Matrix	4.0.2.v201909260824
KNIME Data Generation	4.0.0.v201905311239
KNIME File Handling Nodes	4.0.1.v201908131226
Vernalis KNIME Nodes	1.24.2.v201911141223
KNIME Excel Support	4.0.1.v201908131226
KNIME HCS Tools	4.0.0.v201906200802

1.2 R Integration

The KNIME workflow uses an R integration for some steps of the analysis. The workflow was built using R version 3.6.2, which is available [here](#). Instructions for setting up the R integration can be found [here](#).

Windows users: Do not install Rserve version 1.7-3.1 as is suggested in point 1 of the “R packages installation” section of the instructions in the link above. Instead, go straight to point 2 of the section, to install Rserve v1.8-6. More information on installing Rserve can be found [here](#).

All users: In KNIME Analytics Platform, go to File → Preferences. From the list on the left, under “KNIME”, select R. Set the “Rserve receiving buffer size limit” to 0.

The KNIME workflow should automatically download and install any missing R packages that are required for the analysis. If, for any reason, the automatic download and installation is unsuccessful, any missing packages can be installed manually in the R application (externally to KNIME). Traject3D utilises the following packages:

Package	Version
ClassDiscovery	3.3.12
circlize	0.4.10
cytofkit	1.12.0
DescTools	0.99.31
doParallel	1.0.15
factoextra	1.0.6
FactoMineR	2.0
fields	10.0
ggdendro	0.1-20
ggimage	0.2.8
ggnewscale	0.4.1
ggplot2	3.3.0
ggseqlogo	0.1
imputeTS	3.0
MASS	7.3.51.4

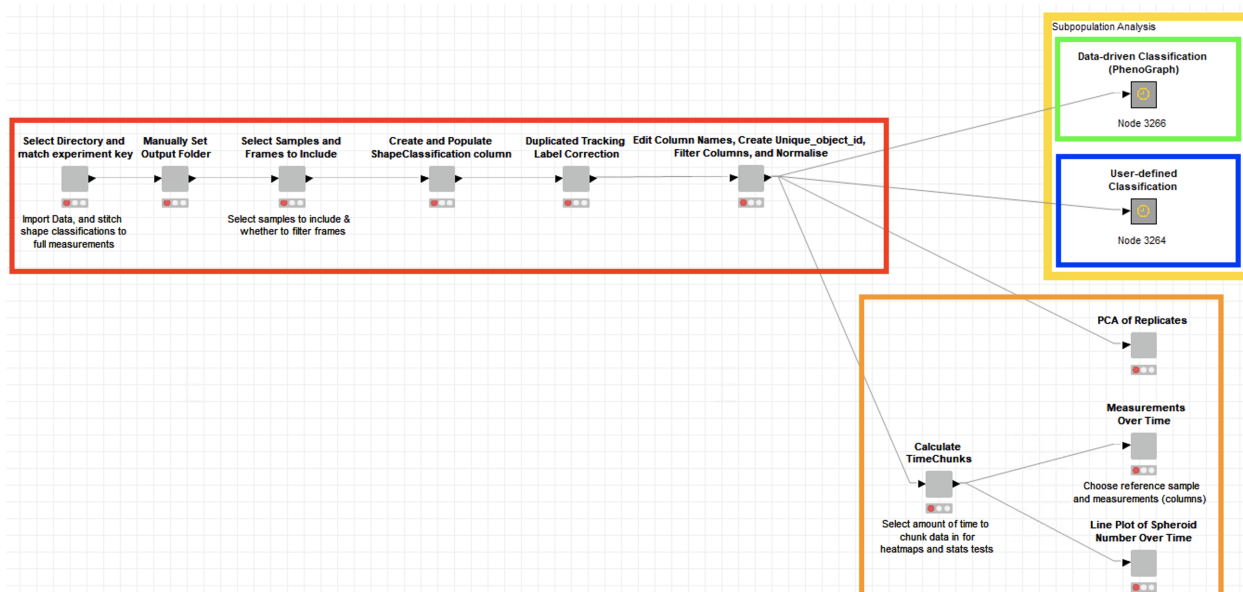
Package	Version
pheatmap	1.0.12
plyr	1.8.5
png	0.1-7
RColorBrewer	1.1-2
reshape2	1.4.3
Rserve	1.8-6
Rtsne	0.15
vcd	1.4-4
viridis	0.5.1

1.3 Python Integration

The KNIME workflow requires Python in order to run the [GeoSketch](#) algorithm for subsampling data. Instructions for setting up the Python integration can be found [here](#). First, follow the "Anaconda Setup" instructions, specifically the "Anaconda Installation" subsection. Preconfigured Python environments are provided on the [davebryantlab/Traject3d](#) Github repository, so creating your own Conda environments as instructed in the documentation linked above is not necessary. Instead, load the provided environments into Anaconda - this can be easily done using the Anaconda Navigator application, instructions for which can be found [here](#) under "Importing an environment". Then follow the instructions for ["Setting up the KNIME Python Integration"](#), and select the provided environments within your KNIME Python preferences.

Package	Version
GeoSketch	1.0

2 Workflow structure



The KNIME workflow is comprised of four parts, each addressed in one section of this manual:

[Data Import and Pre-processing](#)

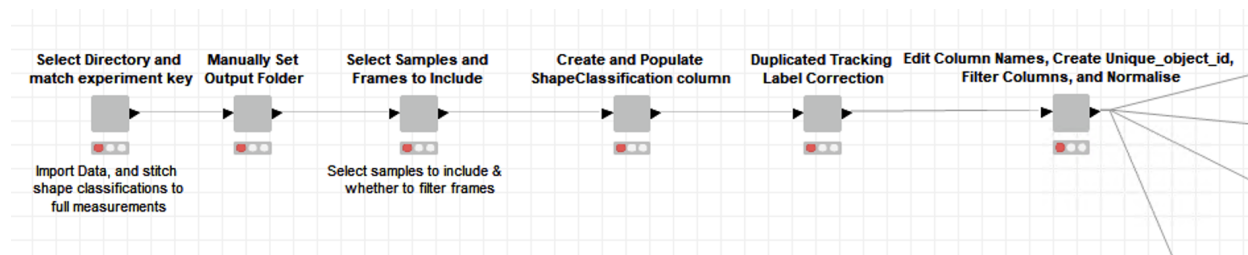
[Feature Analysis](#)

User-Defined Classification

Data-Driven Classification

Tip for those new to KNIME: Double-click on a component/node to configure it.

3 Data import and pre-processing



3.1 Select Directory and match experiment key

This component reads in the CSV files output by CellProfiler and combines them with their respective experiment keys.

Configure the node to set the dataset root directory containing your images, Experiment Keys and CellProfiler outputs. There is also the option to search for and replace a String value in user-specified columns.

3.2 Manually Set Output Folder

This component can override the default output directory.

If you wish to override the default output directory (“Output” subdirectory of your root directory), select “Yes”, and choose an alternative directory for output. Otherwise, select “No”.

3.3 Select Samples and Frames to Include

This component applies sample, frame, and lifetime filtering of objects in the dataset.

To filter out samples: Samples to be retained for analysis should be in the green “Include” box, and others in the red “Exclude” box.

To filter by frame number (timepoint): In order to include all frames, set “Filter by timepoints” to “No”. Otherwise, set it to “Yes”, and set the “Start Frame Number” and “Stop Frame Number” values to the lower- and upper-bound frames of interest, respectively.

To filter by lifetime: Set “Filter objects by lifetime” to “Yes”, and set the maximum and minimum lifetime values.

Lifetime: the number of frames an object was tracked for by CellProfiler.

3.4 Create and Populate ShapeClassification column

If user-defined shape classifications are present, this component parses the classification columns from CellProfiler into a new “ShapeClassification” column. The new column is populated with the classification names as defined in CellProfiler.

Tick the box to indicate that user-defined shape classifications are present in the CellProfiler output.

3.5 Duplicated Tracking Label Correction

In CellProfiler, when a track object splits into two, or more, new objects, the software assigns the tracking label of the parent to the daughter objects. This creates conflicts downstream in the KNIME workflow, whereby multiple objects have the same tracking ID. This metanode ensures that the tracked objects in each Experiment, Plate, Well, and Site combination, each have a unique tracking label. Where duplicate tracking labels are present, the object that is largest at the initiation of the duplication retains this label, and the smaller object(s) is assigned a new tracking label.

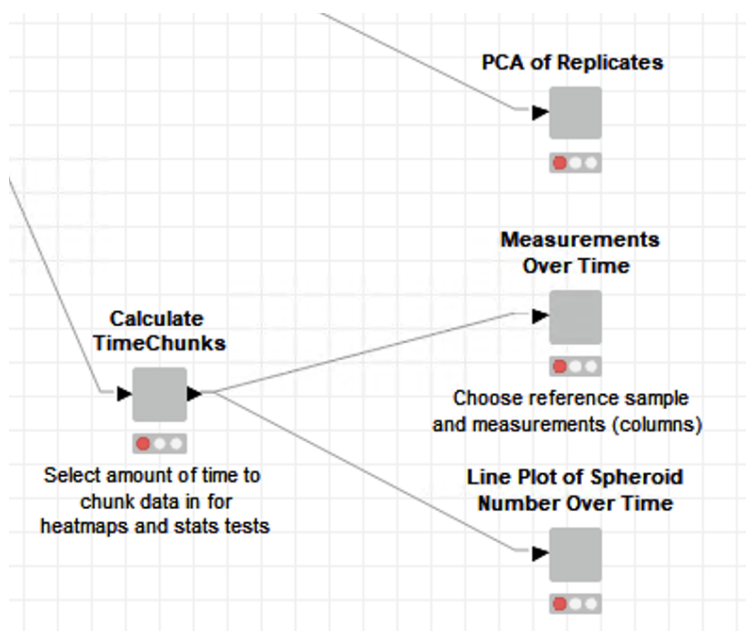
No user configuration required.

3.6 Edit Column Names, Create Unique-object_id, Filter Columns, and Normalise

This component removes unnecessary prefixes from column names. The AspectRatio is then calculated. Unique IDs are created for each tracked object. Finally, the filename of the originating image is extracted, and measurement columns are Z-Score normalised.

No user configuration required.

4 Feature Analysis



4.1 PCA of Replicates

This component performs Principal Component Analysis (PCA) on the dataset replicates, and outputs results for two PCAs: one in which each point is a well, and another in which each point is an experiment. A plot of PCA Loadings is also output for each analysis. Four files are saved in the main Output directory: “PCAofReplicates_perWell.pdf”, “PCAofReplicates_perWell_Loadings.pdf”, “PCAofReplicates_perExperiment.pdf”, “PCAofReplicates_perExperiment_Loadings.pdf”.

No configuration required.

4.2 Calculate TimeChunks

Visualisation of the change in a variable over time can be complex to display when many timepoints are present. To simplify this, data can be presented in grouped segments of time, rather than at individual timepoints. This component calculates time interval chunks for the input data table.

Configure the component to indicate how many timepoints (frames) to include in each timechunk. If chunking is not required, set this value to 1.

4.3 Measurements Over Time

This component generates a heatmap showing change in measurements of area, shape, and movement over time (for a set of user-specified measurements). For each sample, the average value of the measurement is calculated at each of the previously defined time chunk intervals. For the purposes of presentation, resulting values are Z-score normalised per measurement. T-tests are performed to compare samples at each time interval, to the specified reference sample. A Bonferroni adjustment is applied to adjust for multiple testing. Two files are generated in the main Output directory: “MeasurementsOverTime_CONTROLSAMPLEControl.pdf”, “MeasurementsOverTime_CONTROLSAMPLEControl.csv”

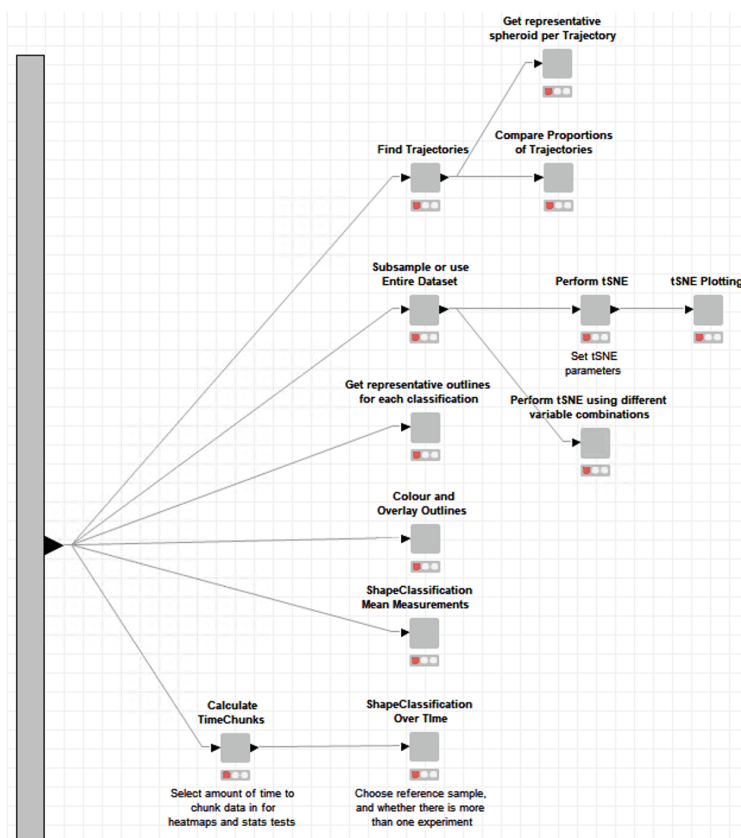
Configure the component to set a reference sample for statistical comparison. Measurements to be plotted in the heatmap should be included in the green “Include” box - all others should remain in the red “Exclude” box. Finally, indicate how the rows (samples) in the heatmap should be ordered: alphabetically or in user-specified ordering. If a user-specified order is to be applied, use the text box to define this order (from top row of the heatmap, to bottom). Sample names should be written as they appear in the Experiment Key, separated by only commas. The easiest way to ensure this is done correctly is by copy and pasting sample names directly from the Experiment Key. **Tip:** Be aware of any leading or trailing spaces in sample names in the Experiment Key, and if present, ensure not to delete them when configuring this text box.

4.4 Heatmap/Line Plot of Spheroid Number Over Time, Heatmap of Initial Spheroid Number

This component outputs the initial counts of spheroids in each treatment/sample per experiment (CSV file), and the relative change from this initial count over time (line plot). Three files are generated in the main Output directory: “InitialSpheroidNumbersPerExperiment.csv”, “SpheroidNumberOverTime.csv”, “SpheroidNumberOverTime_LinePlot.pdf”

No configuration required.

5 User-defined Classification



5.1 Find Trajectories

This component strings the user-defined classifications into a time sequence for each tracked object. After a series of filtering steps to remove objects that were only tracked for a short period of time, the component uses the PhenoGraph algorithm to identify recurring “trajectories” of behaviour change over time. A heatmap of trajectories, as well as the heatmap data are saved in *Output > ShapeClassification > Trajectory*, as “TrajectoryHeatmap.pdf”, and “TrajectoryHeatmap_Data.pdf”

Line plots are output per measurement, of mean values for each identified trajectory: *Output > ShapeClassification > Trajectory > MeasurementAverageOverTime*

Line plots are output per trajectory, of normalised mean Area, Displacement, and DistanceTravelled: *Output > ShapeClassification > Trajectory > AreaMovementPlots*

Configure the component to set a value for the PhenoGraph k -nearest neighbours parameter.

5.2 Compare Proportions of Trajectories

This component compares the proportions of objects that belong to each Trajectory classification. This is done pairwise between reference and non-reference samples. Output files include a heatmap of the fold changes from control and statistical comparisons (PDF), and a CSV file containing the heatmap plot data. Files are saved within *Output > ShapeClassification > Trajectory* as “Trajectory_TotalProportionHeatmap_StatsTest_REFERENCE SAMPLE”, where StatsTest represents: Cochran-Mantel-Haenszel test (CMH), Chi-Squared test (ChiSq).

Configure the component to set a reference sample, and to select which statistical test to perform. Next, indicate whether rows (Trajectories) should be ordered numerically, clustered (dendrogram), or by a user-

specified ordering. If a user-specified ordering is required, input the desired order into the text input, separated by commas but without spaces. If clustered row ordering is selected, the associated dendrogram will be output as a separate PDF file. Finally, indicate whether to order columns (samples) alphabetically, or by a user-specified ordering. If the latter, input the desired order into the appropriate text input - sample names should be written as they appear in the Experiment Key, and separated using only commas (no spaces). The easiest way to do this is by copy and pasting directly from the Experiment Key.

5.3 Get Representative spheroid per Trajectory

This component selects and outputs phase images of x “representative” spheroids for each trajectory. Images are saved as square crops around the selected spheroid, but can also be output as uncropped files. The resulting images are saved in Output > ShapeClassification > Trajectory > RepresentativeSpheroids.

Configure the component to indicate how many representative spheroids should be output per trajectory, and whether uncropped phase images should be saved (in addition to the cropped images).

5.4 Subsample or use Entire Dataset

This component first ensures equal sample size. Then, if the data is to be subsampled, this is done to user specifications. Otherwise all the remaining data is retained.

Configure to indicate whether data is to be subsampled. If so, select which subsampling method to use (Random or GeoSketch [Geometric Sketching]), and input the subsampling depth, as a percentage.

5.5 Perform tSNE

Performs tSNE using Area, Zernike features, Displacement, and Distance Travelled.

Configure the component to set values for perplexity, theta, the number of iterations to perform, and to indicate whether PCA should be performed first.

5.6 tSNE Plotting

After ensuring equal sample size, and subsampling as required by the user, this component produces tSNE plots of the input data table. Plots are produced in which points are: coloured by sample, both in individual plots and all together; coloured by their value for each measurement; for each sample, coloured by point density (one plot per sample); and coloured by user-defined classification. Plots are saved to Output > tSNE, within a folder whose name summarises the tSNE parameters as set by the user.

Configure component to indicate whether to subsample prior to plotting - this value should be lower than the number of objects used for tSNE analysis. Additionally, select a colour scheme, and point size to be used for plotting.

5.7 Perform tSNE using different variable combinations

tSNE is performed (on Area, Zernike features, Displacement, and DistanceTravelled) using combinations of different values for all parameters. Plots for these are coloured by user-defined classification and saved within Output > tSNE. These are intended to aid in selecting the appropriate values to use for best possible visualisation.

perplexity: 25, 50, 100

theta: 0.25, 0.5

iterations: 5000

PCA: TRUE

No configuration required.

5.8 Get representative outlines for each classification

This component selects and outputs a representative outline for each user-defined classification. This is selected from outlines of x objects nearest (in terms of Euclidian distance) to the mean measurements of the group. Outlines are saved in the ShapeClassification > RepresentativeOutlines subdirectory. Plots and the selected representative object outline are subsequently saved in ShapeClassification > RepresentativeOutlines > Plots subdirectory.

Configure to indicate the number of outlines from which to select a representative per classification group.

5.9 Colour and Overlay Outlines

This component overlays object outlines, coloured by their user-defined classification, onto the original phase images. The resulting images are saved in Output > ShapeClassification > OutlineOnPhase.

Configure this component to select an experiment, plate, and site for which to overlay outlines. Next indicate whether this is to be performed for all wells, or a user-defined subset. If the latter, populate the associated text input with the wells of interest, separated by commas but no spaces. Finally indicate whether to process frames for the whole duration of the experiment, or for a user-defined subset of timepoints/frames. If a subset is to be used, fill in the appropriate text input to indicate the frames of interest, again separated by commas but no spaces.

Within the component (right-click, select Component > Open), configure the “Color Manager” node, highlighted by a yellow box, to set the colours to be used for each classification - note the colours that are used for each shape classification in the tSNE plots etc, if you want these to match.

5.10 ShapeClassification Mean Measurements

This component generates a CSV file containing the mean values of measurements for each user-defined class. The resulting file is named “ShapeClassificationMeanMeasurements.csv”, and located in Output > ShapeClassification.

5.11 Calculate TimeChunks

Visualisation of the change in a variable over time can be complex to display when many timepoints are present. To simplify this, data can be presented in grouped segments of time, rather than at individual timepoints. This component calculates time interval chunks for the input data table.

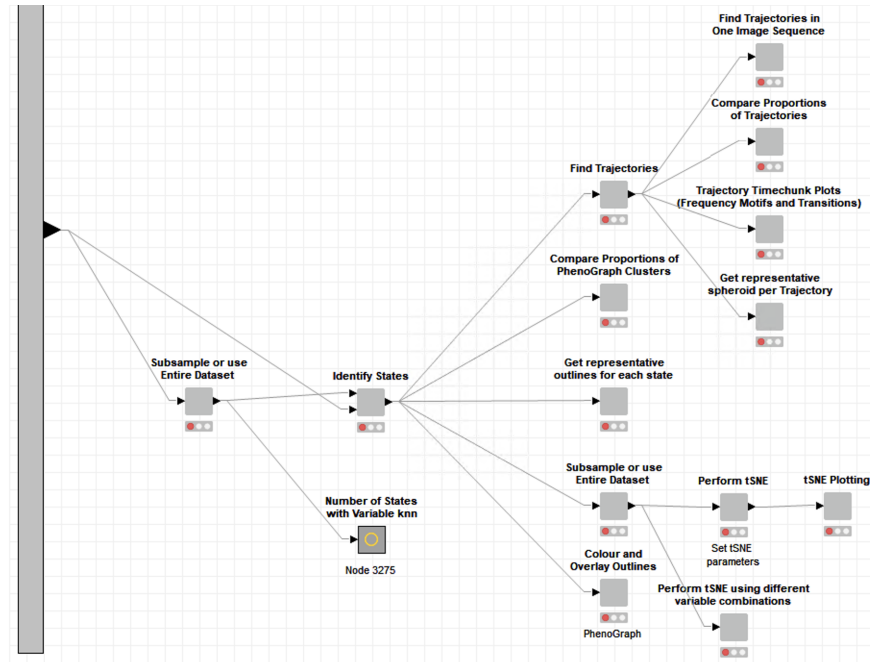
Configure the component to indicate how many timepoints (frames) to include in each timechunk. If chunking is not required, set this value to 1.

5.12 ShapeClassification Over Time

This component compares, between a reference and non-reference samples, the proportions of each user-defined classification at every time chunk interval. A heatmap, and CSV file of results is output to Output > ShapeClassification, named: ShapeClassification_ShapeChangeOverTimeHeatmap_StatsTest_REFERENCE SAMPLE NAME, where StatsTest represents: Cochran-Mantel-Haenszel test (CMH), Chi-Squared test (ChiSq).

Configure the component to set a reference sample for statistical comparison. Then, indicate how rows (samples) in the heatmap should be ordered: alphabetically or by user-specified ordering. If a user-specified order is to be used, use the text box to define this order (from top row of the heatmap, to bottom). Sample names should be written as they appear in the Experiment Key, separated by only commas. The easiest way to ensure this is done correctly is by copy and pasting sample names directly from the Experiment Key. **Tip:** Be aware of any leading or trailing spaces in sample names in the Experiment Key, and if present, ensure not to delete them when configuring this text box.

6 Data-Driven Classification



6.1 Subsample or use Entire Dataset

This component first ensures equal sample size. Then, if the data is to be subsampled, this is done to user specifications. Otherwise all the remaining data is retained.

Configure to indicate whether data is to be subsampled. If so, select which subsampling method to use (Random or GeoSketch [Geometric Sketching]), and input the required subsampling depth, as a percentage.

6.2 Number of States with Varying knn

The PhenoGraph clustering algorithm is performed on the Area, Zernike features, Displacement, and Distance Travelled columns of the input data table. This is done using a varying knn value of 20-100. The number of identified states across knn iterations is output as a line plot (PDF) named “VaryingKNN_LinePlot” within Output > PhenoGraph. This is intended to aid in selecting a knn value for the analysis.

No configuration required.

6.3 Identify States

The PhenoGraph clustering algorithm is performed on the Area, Zernike features, Displacement, and Distance Travelled columns of the input data table. The data previously excluded when ensuring equal sample size and during subsampling is then retrofitted to the identified states, using Euclidian distance. Mean measurements (i.e. Area, AspectRatio) of the identified states are calculated, and output as a heatmap. The heatmap is saved as “PhenoGraph_MeanExpressionHeatmap.pdf” within Output > PhenoGraph .

Configure the component to set a value for the PhenoGraph k -nearest neighbours parameter.

6.4 Find Trajectories

This component strings the state classifications into a time sequence for each tracked object. After a series of filtering steps to remove objects that were only tracked for a short period of time, the PhenoGraph algorithm is used to identify recurring “trajectories” of state change over time. A heatmap of trajectories, as well as

the heatmap data are saved in *Output > PhenoGraph > Trajectory*, as “*TrajectoryHeatmap_knn.pdf*”, and “*TrajectoryHeatmap_Data_knn.csv*”

A CSV file containing the number/percentages of total values plotted in the heatmap, imputed values, and retrofitted PhenoGraph classifications are also saved: “*TrajectoryHeatmap_DataSourceCounts.csv*”

Line plots are output per measurement, of mean values for each identified trajectory: *Output > PhenoGraph > Trajectory > MeasurementAverageOverTime*

Line plots are output per trajectory, of normalised mean Area, Displacement, and DistanceTravelled: *Output > PhenoGraph > Trajectory > AreaMovementPlots*

Configure the component to set a value for the PhenoGraph k -nearest neighbours parameter.

6.5 Find Trajectories in One Image Sequence

This component finds an image sequence in which objects of user-specified trajectories are nearest to each other. The outlines (coloured depending on Trajectory ID) of the objects in the specified trajectories are overlayed onto the phase image. The resulting images are saved in Output > PhenoGraph > Trajectory > OverlayedOutlines.

Populate the text box to indicate the trajectories of interest: separated by commas only (no spaces). Additionally input how many image sequences/movies are to be output.

Note on the algorithm: First, the component determines which Experiment, Plate, Well, and Site combinations (image sequences) contain objects from each of the trajectories defined by the user. Next, Euclidian distance is calculated pairwise between the trajectories of interest; specifically, for each spheroid in Trajectory X, the nearest spheroid belonging to Trajectory Y is identified. For each pairwise comparison, the shortest distance between the pairs of spheroids is retained. Finally, for each image sequence, the pairwise distances are summed and the sequence with the shortest total pairwise distance (smallest sum) is output.

6.6 Compare Proportions of Trajectories

This component compares the proportions of objects that belong to each Trajectory classification. This is done pairwise between reference and non-reference samples. Output files include a heatmap of the fold changes from control and statistical comparisons (PDF), and a CSV file containing the heatmap plot data. These are saved within Output > PhenoGraph > Trajectory as “Trajectory_TotalProportionHeatmap_StatsTest_REFERENCE SAMPLE NAME”. StatsTest represents: Cochran-Mantel-Haenszel test (CMH), Chi-Squared test (ChiSq).

Configure the component to set a reference sample, and to select which statistical test to perform. Next, indicate whether rows (Trajectories) should be ordered alphabetically, clustered (dendrogram), or by a user-specified ordering. If a user-specified ordering is required, input the desired order into the text input, separated by commas without spaces. Finally, indicate whether to order columns (samples) alphabetically, clustered (dendrogram), or by a user-specified ordering. If the latter, input the desired order into the appropriate text input - sample names should be written as they appear in the Experiment Key, and separated using only commas (no spaces). The easiest way to do this is by copy and pasting directly from the Experiment Key. If clustered row and/or column ordering is selected, the associated dendrograms will be output as separate PDF files.

6.7 Trajectory Timechunk Plots (Frequency Motifs and Transitions)

Ensure the “Get representative outlines for each state” component has been completed. This component generates summary plots for each identified Trajectory. Three types of plots are generated for each Trajectory: 1) Frequency motif of states at the defined time chunk interval. 2) Chord diagram of transitions between states, segmented by the defined time chunk interval. 3) Transitions plotted as line segments onto a PCA plot of states (one plot per time chunk). The files are saved in Output > PhenoGraph > Trajectory, in subdirectories named “Frequency Motifs”, and “Transition Plots”.

Configuring this component is required to indicate the time chunk interval. A transition frequency (as a percentage) filter is required for both types of transition plot - transitions that occur at a lower frequency (within a time chunk interval) will not appear in the plots. Finally, a minimum line thickness is required for generating the PCA transition plots.

6.8 Get representative spheroid per Trajectory

This component selects and outputs phase images of x “representative” spheroids for each trajectory. Images are saved as square crops around the selected spheroid, but can also be output as uncropped files. The resulting images are saved in Output > PhenoGraph > Trajectory > RepresentativeSpheroids.

Configure to indicate how many representative spheroids should be output per trajectory, and whether to save uncropped phase images (in addition to the cropped images).

Note on the algorithm: First a consensus sequence (matching the sequence appearing in the state frequency motif) is determined for each trajectory - this is comprised of the most frequent state at each point in time. Next, the sequence of state classifications for each spheroid in a trajectory is determined, which is then compared to the consensus sequence for the trajectory group using a string comparison algorithm. Only mismatches at equivalent timepoints are assessed, while insertion/deletion type alterations are not taken into consideration. This results in a similarity score. Images of x spheroids deemed most similar, in terms of this score, to the consensus sequence are retrieved. The spheroid outlines are overlaid, cropped, and output.

6.9 Compare Proportions of PhenoGraph Clusters

This component compares the proportions of objects that belong to each PhenoGraph (behaviour state/shape) classification. This is done pairwise between reference and non-reference samples. Output files include a heatmap of the fold changes from control and statistical comparisons (PDF), and a CSV file containing the heatmap plot data, are saved within Output > PhenoGraph as “PhenoGraph_TotalProportionHeatmap_StatsTest_REFERENCE SAMPLE NAME”. StatsTest represents: Cochran-Mantel-Haenszel test (CMH), Chi-Squared test (ChiSq).

Configure the metanode to set a reference sample, and to select which statistical test to perform. Next, indicate whether rows (behaviour states) should be ordered alphabetically, clustered (dendrogram), or by a user-specified ordering. If a user-specified ordering is required, input the desired order into the text input, separated by commas without spaces. If clustered row ordering is selected, the associated dendrogram will be output as a separate PDF file. Finally, indicate whether to order columns (samples) alphabetically, or by a user-specified ordering. If the latter, input the desired order into the appropriate text input - sample names should be written as they appear in the Experiment Key, and separated using only commas (no spaces). The easiest way to do this is by copy and pasting directly from the Experiment Key.

6.10 Get representative outlines for each state

This component selects and outputs a representative outline for each identified state. This is selected from outlines of x objects nearest (in terms of Euclidian distance) to the mean measurements of the group. Outlines are saved in the PhenoGraph > RepresentativeOutlines subdirectory. Plots and the selected representative object outline are subsequently saved in PhenoGraph > RepresentativeOutlines > Plots subdirectory.

Configure to indicate the number of outlines from which to select a representative per classification group.

6.11 Perform tSNE using different variable combinations

tSNE is performed (on Area, Zernike features, Displacement, and DistanceTravelled) using combinations of different values for all parameters. Plots for these are coloured by PhenoGraph classification and saved within Output > tSNE. These are intended to aid in selecting the appropriate values to use for best possible visualisation.

perplexity: 25, 50, 100

theta: 0.25, 0.5

iterations: 5000

PCA: TRUE

No configuration required.

6.12 Perform tSNE

Performs tSNE using Area, Zernike features, Displacement, and Distance Travelled.

Configure the component to set values for perplexity, theta, the number of iterations to perform, and to indicate whether PCA should be performed first.

6.13 tSNE Plotting

After ensuring equal sample size, and subsampling as required by the user, this component produces tSNE plots of the input data table. Plots are produced in which points are: coloured by sample, both in individual plots and all together; coloured by their value for each measurement; for each sample, coloured by point density (one plot per sample); and coloured by PhenoGraph classification (data-driven state). Plots are saved to Output > tSNE, within a folder whose name summarises the tSNE parameters as set by the user.

Configure component to indicate whether subsampling should occur - this value should be lower than the number of objects used for tSNE analysis. Additionally, select a colour scheme, and point size to be used for plotting.

6.14 Colour and Overlay Outlines

This component overlays object outlines, coloured by their state classification, onto the original phase images. The resulting images are saved in Output > PhenoGraph > OutlineOnPhase.

Configure this component to select an experiment, plate, and site for which to overlay outlines. Next indicate whether this is to be performed for all wells, or a user-defined subset. If the latter, populate the associated text input with the wells of interest, separated by commas but no spaces. Finally indicate whether to process frames for the whole duration of the experiment, or for a user-defined subset of timepoints. If a subset is to be used, fill in the appropriate text input to indicate the frames of interest, again separated by commas but no spaces.