

KNIME Pipeline User Manual

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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

The “Experiment Key” and “Phase” folders should be populated by the user prior to analysis with CellProfiler, which will in turn populate “Data” and “PhaseGrayCystOutlines” upon analysis completion.

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/MethodsPaper2020](#) Github repository.

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

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

Name	Version
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 uses R version **version**, which can be downloaded 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 these instructions. 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, select R under KNIME. 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. **Check that cytofit does this** The following packages are utilised:

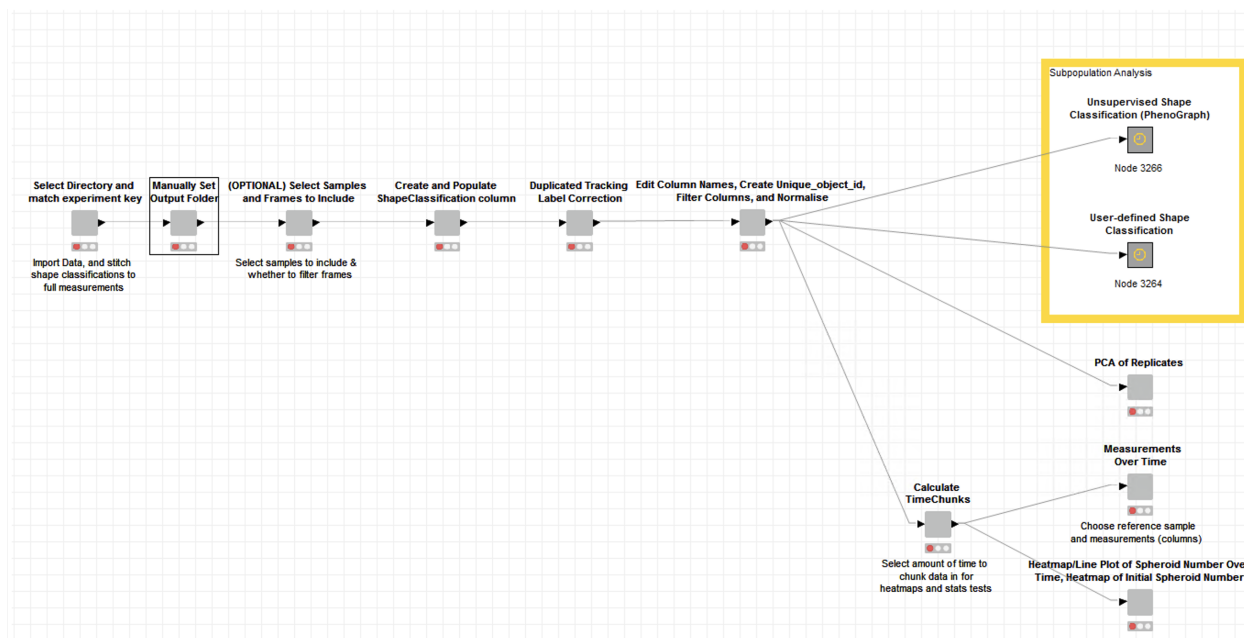
Package	Version
ggplot2	
reshape2	
ggnewscale	

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 “Quickstart” and “Anaconda Setup” instructions, and download the Python environments provided on the **davebryant-lab/MethodsPaper2020** Github repository. Load these environments into Anaconda. Then follow the “Setting up the KNIME Python Integration” instructions, and select the provided environments within your KNIME Python preferences.

Package	Version
GeoSketch	

2 Workflow structure



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

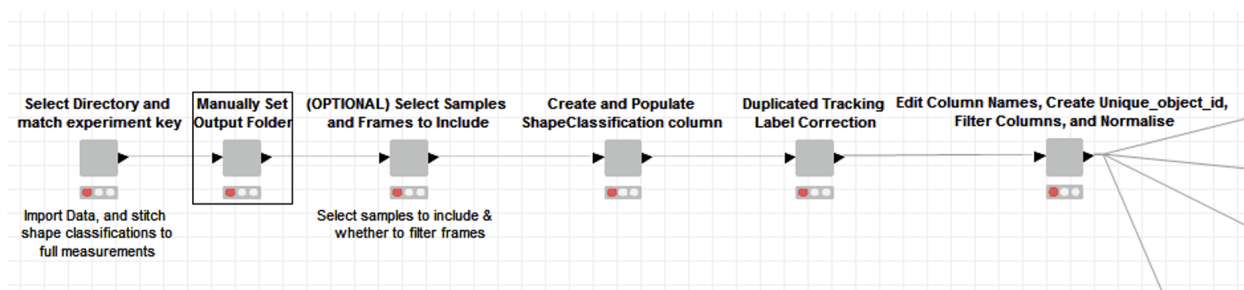
Data Import and Pre-processing

Data Summary Files

User-Defined Shape Classification

Unsupervised Shape Classification

3 Data import and pre-processing



3.1 Select Directory and match experiment key

This metanode 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. **Get description from Dave for the rest of the user inputs**

3.2 Manually Set Output Folder

This metanode 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 metanode 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 metanode 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.

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 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.

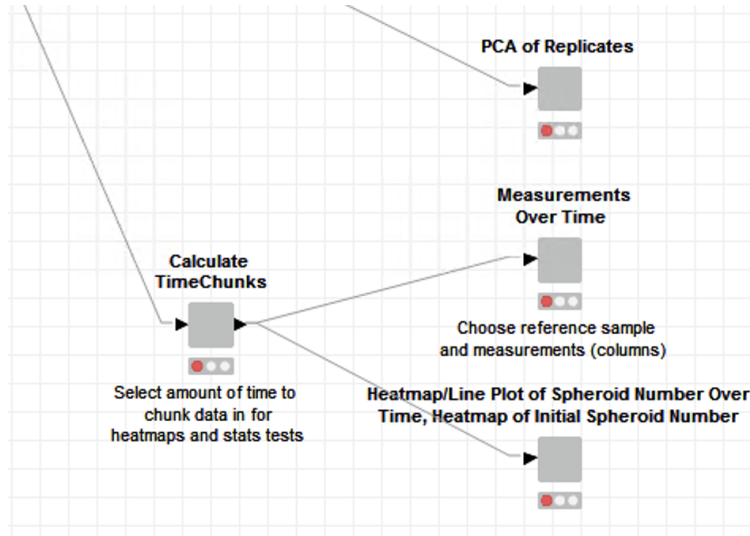
3.6 Edit Column Names, Create Unique-object_id, Filter Columns, and Normalise

This metanode 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 Data summary files

think of a better name for this section



4.1 PCA of Replicates

This metanode 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 metanode calculates time interval chunks for the input data table.

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

4.3 Measurements Over Time

This metanode 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. A 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”

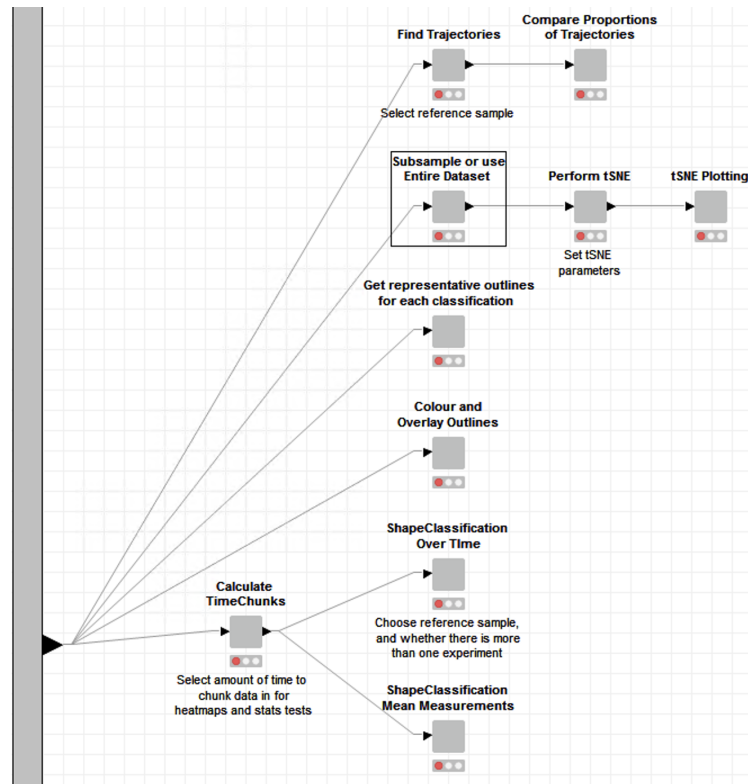
Configure the metanode to set a reference sample for statistical comparison. Measurements to be plotted in the heatmap should be included in the green “Include” box - all others should remain in the red “Exclude” box. Finally, indicate how rows (samples) in the heatmap should be ordered: alphabetically or in 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.

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

This metanode outputs the initial counts of spheroids in each treatment group/sample per experiment (CSV file), and the relative change 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 Shape Classification



5.1 Find Trajectories

This metanode strings the user-defined shape 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 metanode 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 metanode to set a value for the PhenoGraph k -nearest neighbours parameter.

5.2 Compare Proportions of Trajectories

This metanode 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, are saved within Output > ShapeClassification > Trajectory as “Trajectory_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 (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. 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 Find Trajectories in One Image Sequence

5.4 Trajectory Timechunk Plots (Frequency Motifs and Transitions)

5.5 Get Representative spheroid per Trajectory

5.6 Subsample or use Entire Dataset

This metanode 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 is to be used (Random or GeoSketch [Geometric Sketching]), and input the depth, as a percentage, at which to subsample.

5.7 Perform tSNE

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

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

5.8 tSNE Plotting

After ensuring equal sample size, and subsampling as required by the user, this metanode 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 metanode 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.

5.9 Perform tSNE using different variable combinations

5.10 Get representative outlines for each classification

This metanode selects and outputs a representative shape (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 how many outlines are to be output per behaviour state. The single representative outline for the group is selected from these.

5.11 Colour and Overlay Outlines

This metanode 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 metanode 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. 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 metanode (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.

5.12 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 metanode calculates time interval chunks for the input data table.

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

5.13 ShapeClassification Over Time

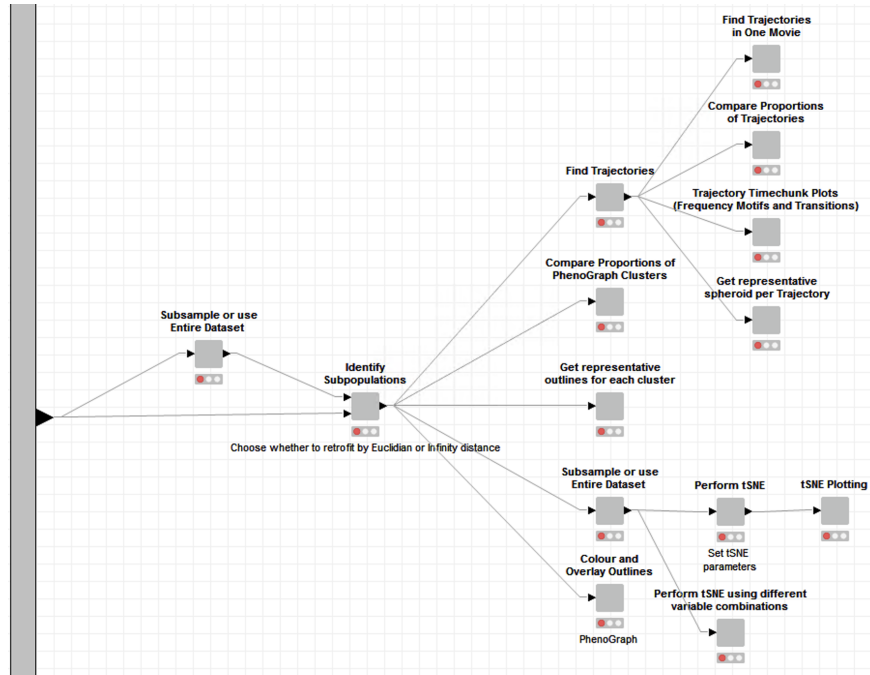
This metanode compares, between a reference and non-reference samples, the proportions of each subpopulation at every time chunk interval. A heatmap, and CSV file of results is output to Output > ShapeClassification, named: ShapeClassification_ShapeChangeOverTimeHeatmap_StatsTest_REFERENCE SAMPLE NAME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

Configure the metanode 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.

5.14 ShapeClassification Mean Measurements

This component generates a CSV file containing the measurement means of each (user-defined) subpopulation. The resulting file is named “ShapeClassificationMeanMeasurements.csv”, and located in Output > ShapeClassification.

6 Unsupervised Shape Classification



6.1 Subsample or use Entire Dataset

This metanode 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 is to be used (Random or GeoSketch [Geometric Sketching]), and input the depth, as a percentage, to which to subsample.

6.2 Identify Subpopulations

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

Configure the metanode to set a value for the PhenoGraph k -nearest neighbours parameter. **include link to PhenoGraph paper for how to choose knn?**

6.3 Find Trajectories

This metanode strings the shape 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 metanode 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 > PhenoGraph > Trajectory, as “TrajectoryHeatmap.pdf”, and “TrajectoryHeatmap_Data.pdf”

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

6.4 Find Trajectories in One Movie

This metanode 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), but no specific ordering is required. Additionally input how many image sequences/movies are to be output.

Note on the algorithm: First, the metanode determines which Experiment, Plate, Well, and Site combinations (image sequences) contain spheroids 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.

6.5 Compare Proportions of Trajectories

This metanode 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, 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 metanode 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. 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.

6.6 Trajectory Timechunk Plots (Frequency Motifs and Transitions)

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

Configuring this metanode 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.

6.7 Get representative spheroid per Trajectory

This metanode 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 the metanode 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).

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 behaviour state at each point in time. Next, the sequence of behaviour 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 specific 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 overlaid, cropped, and output.

6.8 Compare Proportions of PhenoGraph Clusters

This metanode 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.

6.9 Get representative outlines for each cluster

This metanode selects and outputs a representative shape (outline) for each identified behaviour 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 how many outlines are to be output per behaviour state. The single representative outline for the group is selected from these.

6.10 Subsample or use Entire Dataset

This metanode 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 is to be used (Random or GeoSketch [Geometric Sketching]), and input the depth, as a percentage, at which to subsample.

6.11 Perform tSNE using different variable combinations

tSNE is performed (on Area, Zernike features, Displacement, and DistanceTravelled) using different 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 metanode to set values for perplexity, theta, the number of iterations to perform, and indicate whether PCA should be performed first.

6.13 tSNE Plotting

After ensuring equal sample size, and subsampling as required by the user, this metanode 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. Plots are saved to Output > tSNE, within a folder whose name summarises the tSNE parameters as set by the user.

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

Configure this metanode 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. 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.