# Single Cell Phenotyping and Survival Analysis: An ImageJ toolset for survival and cumulative event analysis in longitudinal live-imaging experiments.

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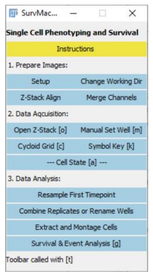
**Abstract:** Single-cell survival analysis is a useful approach for understanding factors that contribute to cell health, particularly in cultured neurons. However, this type of analysis can be difficult to perform due to the relatively large number of data acquisition and analysis steps necessary to produce results. The Single Cell Phenotyping and Survival Analysis toolset provides a simplified approach for survival and cumulative event analysis in cultured cells**.** Implemented entirely with in NIH ImageJ, the toolset contains a guided methodology to track and phenotype single cells in time lapse imaging experiments and to subsequently produce survival and cumulative event plots as well as montaged images following each tracked cell through time. The output figures produced are comparable to those produced by existing dedicated survival analysis software and include basic statistical comparisons. In addition, several methods for data transformation and migration are included in order to aid in expected analyses. In this work, the Single Cell Phenotyping and Survival Analysis toolset is presented and applied to survival and phenotype tracking in human induced pluripotent stem cell-derived cortical neurons.

**Introduction:** Longitudinal single-cell survival analysis is a well-established in-vitro technique that has been used to study the influence of varied exposures and phenotypes on subsequent cell survival. This technique provides improved sensitivity and specificity compared to population-based averages. In addition, sub-classification allows for the use of near-adjacent cells as closely matched controls. Cultured neurons are well suited to single-cell survival analysis because these cells tend to survive for long periods of time, are relatively immobile in culture, and can exhibit neuron-specific phenotypes such as protein aggregation that may predispose to cell death. Although there are several published works implementing single cell longitudinal-analysis workflows in neurons, most rely on machine-learning techniques to phenotype and track neurons and require strict control of culture conditions and a relatively high-level of programming knowledge to implement (1-6). Furthermore, downstream visualization and analysis of survival curves requires additional data handling that can be a barrier to implementing these experiments. **The Single Cell Phenotyping and Survival Analysis toolset provides a simplified alternative approach for survival and cumulative event analysis in cultured cells.**

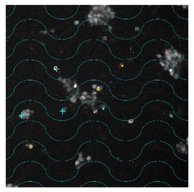
**Single Cell Phenotyping and Survival Toolset:**

1. Prepare Images



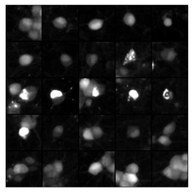

-Z-Stack Alignment
-Merge Channels

2. Track Cells



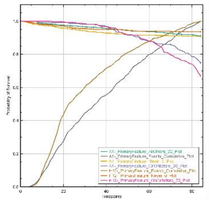
-Optional Stereology
-Manual Tracking:
-Cell Location
-Cell Survival
-Two Cell Features
-Censoring

3. Extract and Montage



-Tracked Cell Z-Stacks
-Sorting by Survival
-Sorting by Feature
-Customizable

4. Survival or Event Analysis



-Kaplan-Meier or Event
-Group by Feature or Well
-Pairwise or Multiple Plots
-Pairwise Logrank Tests
-Optional t0 Reassignment
-Customizable Plots

**Figure 1. Overview.** Tools are provided to 1) prepare stacked and un-stacked time-lapse images for counting (example toolbar shown), 2) perform single cell tracking and phenotyping with or without the use of a stereology grid to select tracked cells (annotated image with Cycloid Grid shown) , 3) extract tracked cell images and create sorted time-lapse montages, and 4) to perform cell survival and cumulative event analysis and subsequent plotting.

**Single cell tracking: Individual cells in time-lapse images are manually tracked** using an optional stereology grid. Cell positions and up to two phenotypes are recorded for each timepoint. Cell death is also recorded when it occurs. Tracked cells can be censored if they leave the field of view or become otherwise obscured.

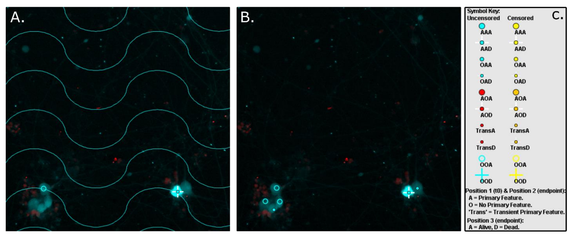



**Figure 2. Cell Tracking.** Example output log images with all tracked cells indicated by symbols corresponding to cell survival and primary feature status. A) Utilization of stereology grid to select tracked cells. B) Alternative approach tracking all cells in the same image. C) Three letter Symbol Key: In Position 1 (t0) and Position 2 (endpoint) A=Primary Feature Present and O=No primary Feature Present at the beginning and end of the tracked period respectively. Trans=transient primary feature (not observed). In Position 3 (endpoint), A=Alive and D=Dead at the end of the tracked period. Left column corresponds to coloring for uncensored cells. Right column corresponds to coloring for censored cells.

**Visualization of tracked cells: ROIs surrounding individual cells can be extracted and montaged** using the per-timepoint cell position, phenotype, and survival data generated during cell tracking with sorting by pre-defined cell phenotypes or cell lifespan. In the example below, individual human induced pluripotent stem cell-derived neurons (iPSC-neurons) are followed over 140 hours using a fluorescent dye to monitor tau aggregation (Cyan) and a far-red dye to detect cell death (red) (Oakley et al., 2021).

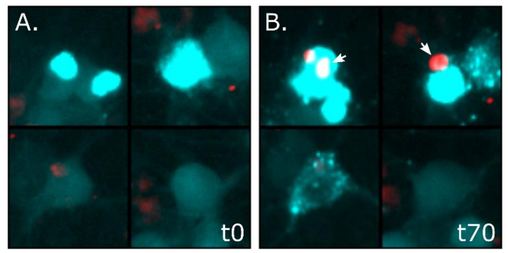



**Figure 3. Time-lapse Montage of Tracked Cells.** Extracted and montaged cells tracked and sorted by occurrence of cell death. A) represents four cells at time 0 (t0). B) represents the same 4 cells at timepoint 70 (t70). Arrowheads highlight fluorescence signal from nuclear dye (red) indicating cell death and at t70. Fluorescent protein aggregation (the tracked primary feature in this experiment) is shown at t0 for the top two cells, later in the experiment for the bottom left cell, and never for the bottom right cell**.**

**Data analysis: Kaplan-Meier survival curves and cumulative event frequency plots are generated using cell phenotype, survival, and censoring data** comparing groups of cells defined by recorded phenotypes or well in multi-well plate culture formats. Statistical analysis is performed when comparing two groups by calculation of the Chi-squared logrank value. These data can additionally be plotted as risk of death as opposed to probability of survival.

Below, single iPSC-neurons from two cell lines are tracked and monitored for the onset of induced tau aggregation (yellow=PSEN1 mutant and blue=Control) (data from Oakley et al., 2021). Survival plots are prepared comparing cells that developed tau aggregation early during the experiment (≤50 hours) to all other cells. These data show a survival deficit in neurons that develop tau aggregates (Fig 4A) and accelerated tau aggregation in PSEN1 mutant cells (Fig 4C).

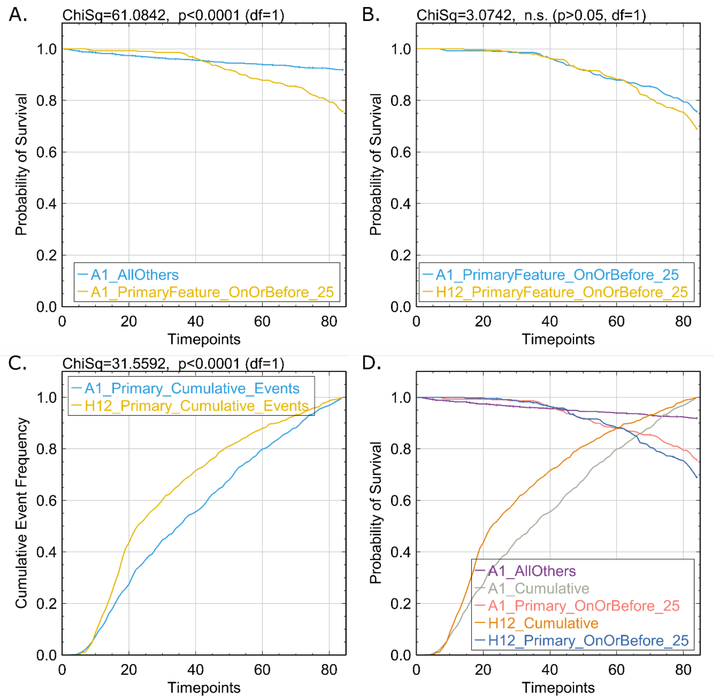



**Figure 4. Survival and Cumulative Event Analysis.** Plots generated using the "Survival & Event Analysis" component of the toolset comparing survival and events between cells grouped by a tracked Primary Feature and a well designation (A1 vs H12). A) Plot restricted to cells in well A1, comparing survival between cells that develop the Primary Feature on or before timepoint 25 compared to all other cells in the well. B) Plot comparing cells in well A1 to those in H12, restricted to cells that develop the Primary Feature on or before timepoint 25. C) Comparison of Primary Feature cumulative events between cells in well A1 and H12. D) Multiplot showing combined data from panels A-C. Chi-squared values and p-value categories are auto-generated for pairwise comparisons. Plot colors, size, and labels are user customizable. Data from Oakley et al., 2021.

**Data resampling: A data resampling function was developed to begin survival analysis after the timepoint of group assignment** (Fig 5). This corrects a bias where comparison of survival between two groups defined by a phenotype that accumulates with time can introduce artificially high early survival probabilities in the group that develops the time-dependent phenotype (Fig 5A).

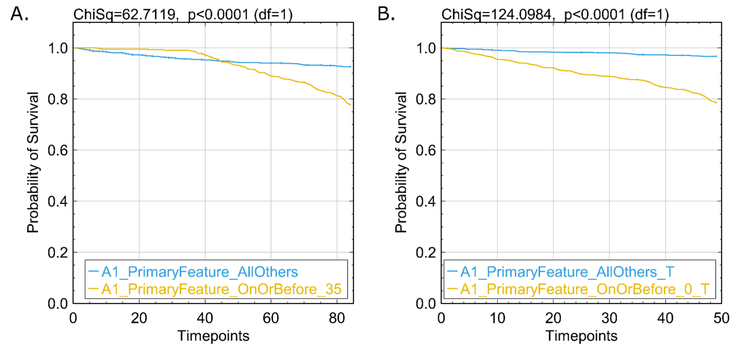



**Figure 5. Resampling of Tracked Cell Data.** Use of resampling function to reassign Primary Feature categories at an arbitrary t0. A) depicts single cell survival data comparing cells that develop a Primary Feature on or before timepoint 35 (t35), to all other cells in the same well (A1). B) depicts the same data after resampling of t0 to t35 and reassignment of groups to represent the presence of the Primary Feature at the new t0. This procedure removes an early bias towards increased survival in cells that develop the Primary Feature. Data from Oakley et al., 2021.

**Validation of data analysis methods: In order to validate the data visualization and analysis components of the ImageJ toolset, results were compared to an existing R package used for survival and cumulative event analysis** (survminer). Survival plot results were identical (Fig 6a), and p values were comparable to this package.

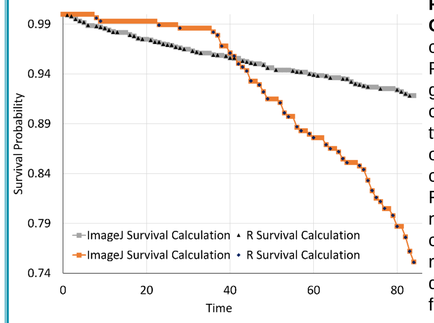



**Figure 6. Comparison with R Survminer-Generated Survival Plots.** Survival probabilities calculated with the NIH ImageJ Single Cell Phenotyping and Survival Toolset match those generated with an established survival probability calculation program run in R (survminer). The two curves represent cells with a Primary Feature on or before timepoint 24 (orange) vs. all other cells (grey) in one well. n=283 cells with Primary Feature on or before 24 (2 censored cells) and n=1315 cells in the "All Others" category (31 censored). Data are combined from 4 biological replicates. Censored cells are included in the data analysis, but censor crosses are omitted from plots. Data from Oakley et al., 2021.

**Conclusions:**

- **The Single Cell Phenotyping and Survival Analysis toolset for NIH ImageJ provides a simple platform for single cell *in vitro* survival analyses.**
- **Multiple image formats and file name structures are accepted.**
- **The toolset allows visualization and comparison of groups defined by a morphologic cellular phenotype or well position.**
- **The graphical and statistical outputs are comparable to existing software, but do not require any programming knowledge to utilize.**
- **The toolset is available at: https://github.com/DHOAKLEY/Single-Cell-Phenotyping-and-Survival-Analysis**

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