

Intermediate Image Analysis

Jeremy Pike

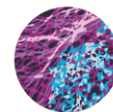
Image analyst for COMPARE

Course website:

<https://jeremypike.github.io/intermediate-image-analysis/>

IN PARTNERSHIP:

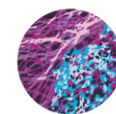
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COMPARE
CENTRE OF MEMBRANE PROTEINS AND RECEPTORS

A brief tour of selected image processing and analysis tools

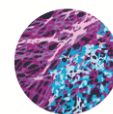
- Deconvolution
- Deep learning-based denoising
- Image segmentation
- Colocalization analysis
- Tracking



Section 1: Deconvolution

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The Point-Spread Function (PSF)

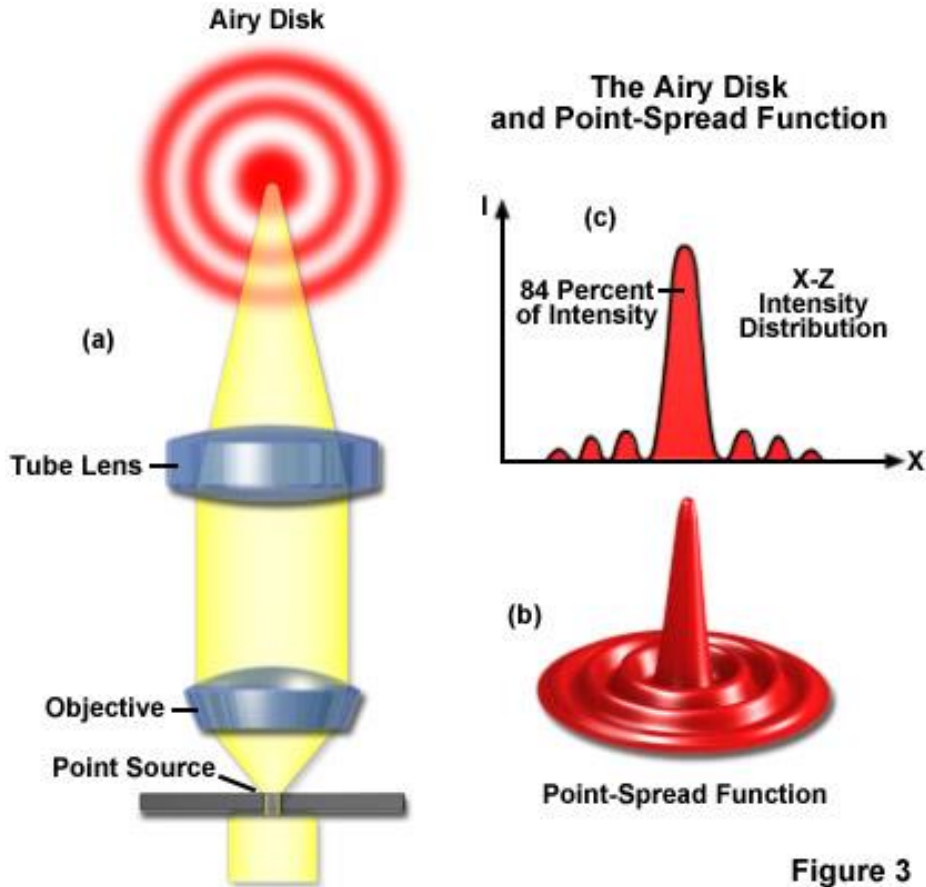
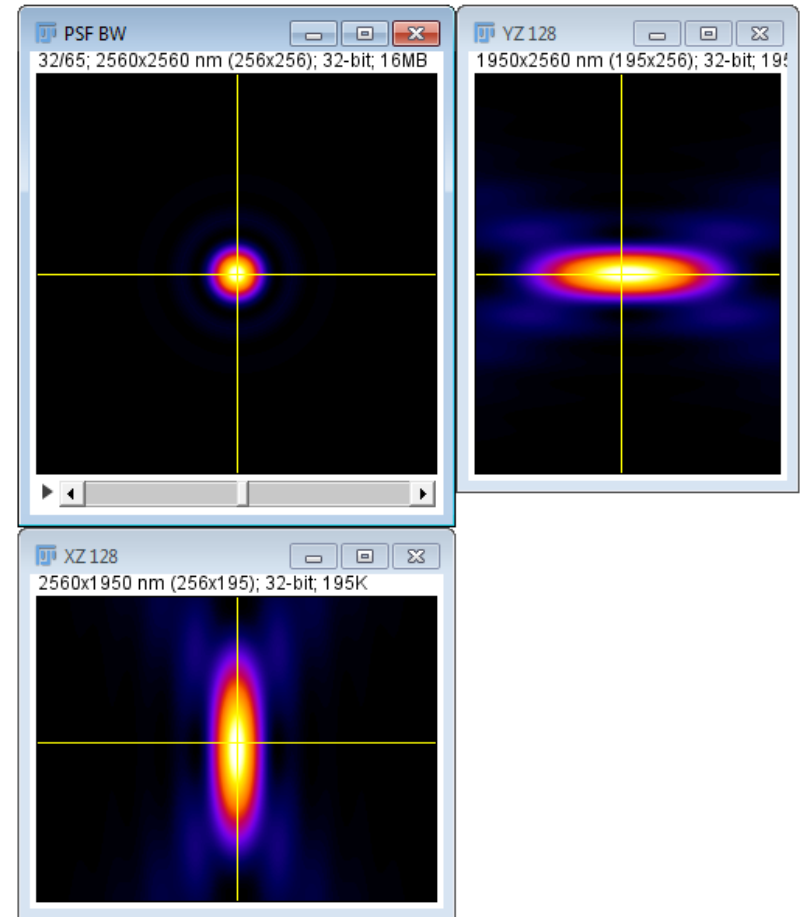
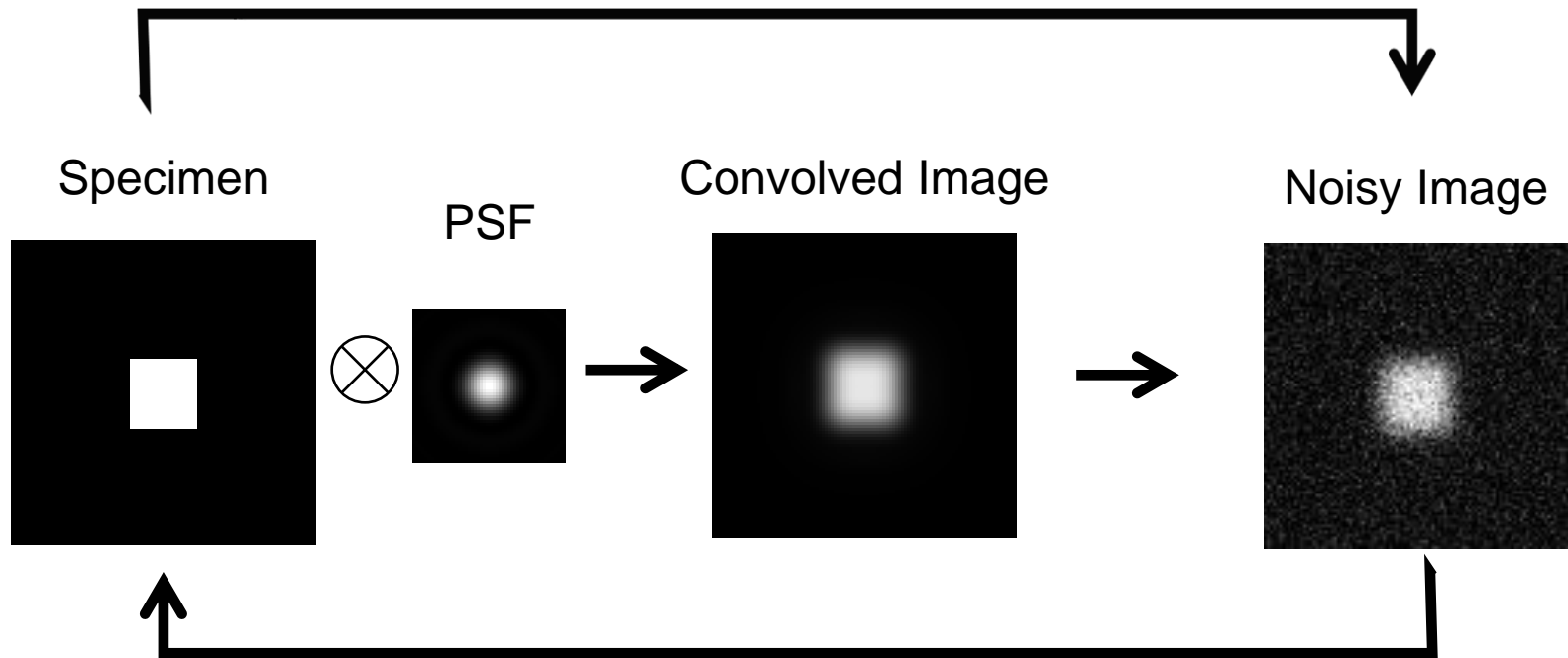


Figure 3



The Forward and Inverse problem in Fluorescence Microscopy

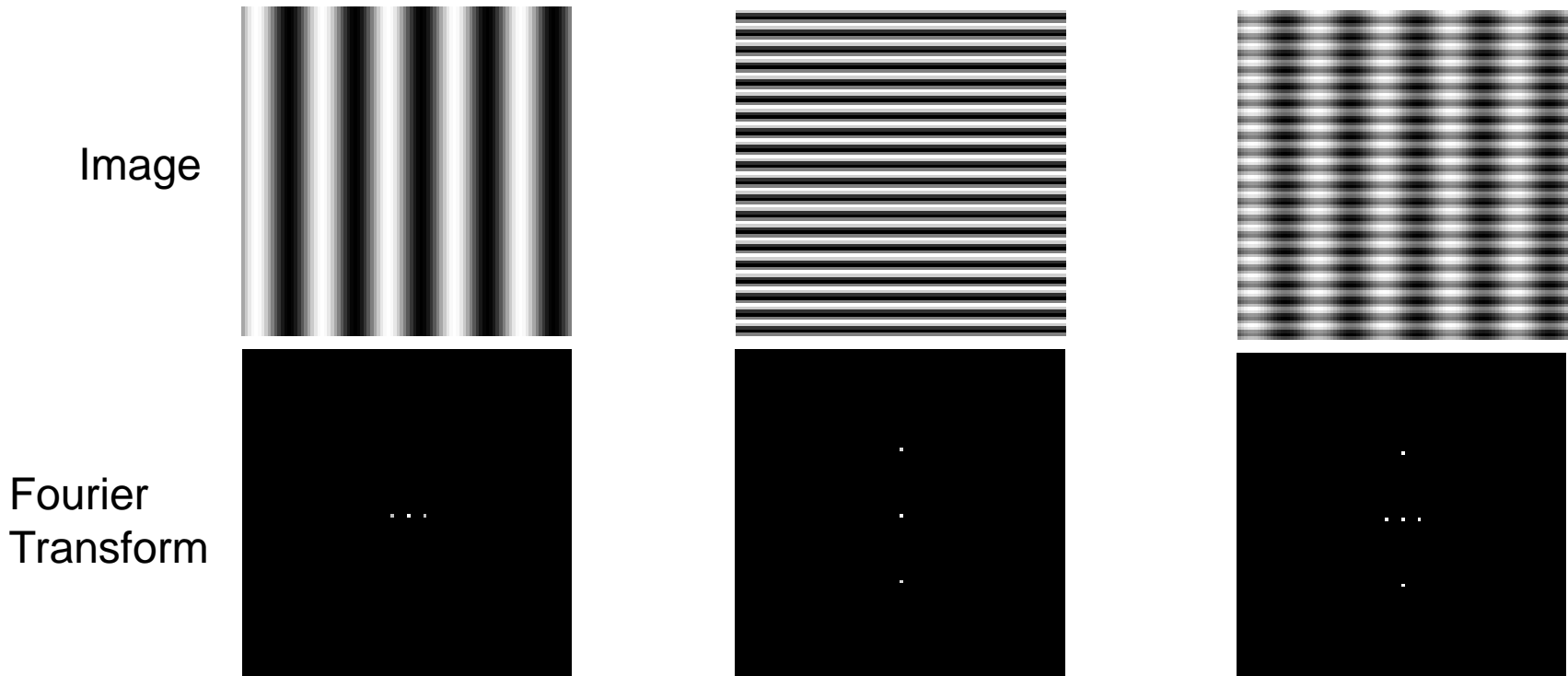
Forward Problem: Predict the observed image from the specimen



Inverse Problem: Predict the specimen from the observed image

The Fourier Transform

- An arbitrary signal can be expressed as the sum of sine waves
<http://www.falstad.com/fourier/>
- Each sine wave has a amplitude and phase
- For images a 2D discrete transform is used



Deconvolution by Inverse Filtering

- Convolutions are simply multiplications in Fourier space!

$$I = I_0 \otimes PSF$$
$$FT(I) = FT(I_0) \times FT(PSF)$$

- We want to find the true sample signal, I_0 , given the observed image, I , and a PSF.
- In Fourier space this seems simple:

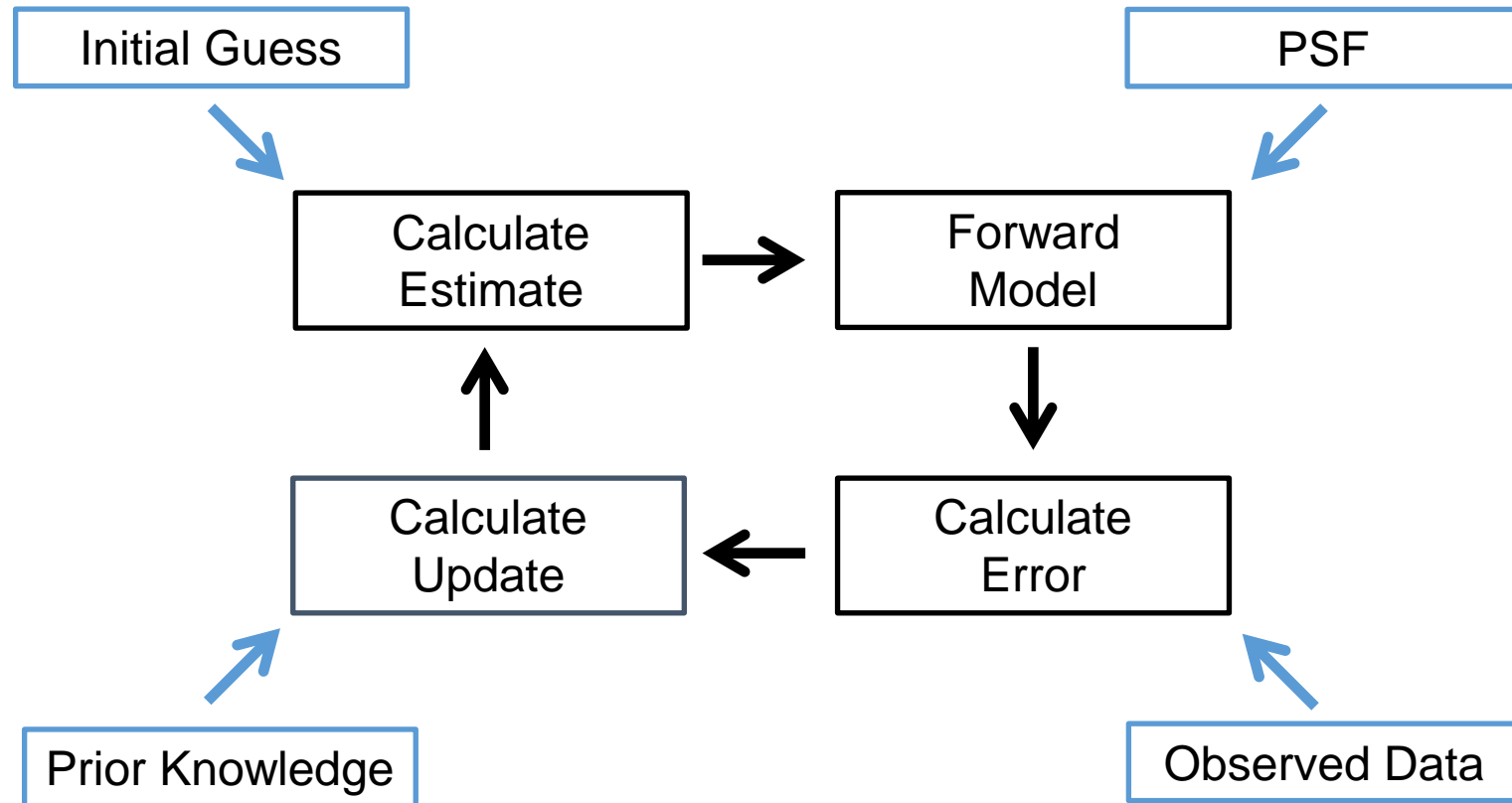
$$FT(I_0) = \frac{FT(I)}{FT(PSF)}$$

- But what about where $FT(PSF) \approx 0$?
- In regularized inverse filtering approach a small number can be added to the denominator:

$$FT(I_0) = \frac{FT(I)}{FT(PSF) + constant}$$

Iterative Deconvolution

- The inverse problem is ill-posed: working backwards is difficult and there is no unique or stable solution
- One approach is to iteratively improve an initial guess using the PSF and the forward problem



Selected Tools for Deconvolution and PSF Generation

DeconvolutionLab

An excellent ImageJ plugin implementing various algorithms

<http://bigwww.epfl.ch/deconvolution/deconvolutionlab2/>

Sage, Daniel, et al. "DeconvolutionLab2: An open-source software for deconvolution microscopy." *Methods* 115 (2017): 28-41.

PSF Generator

Open Java package for PSF generation with interfaces for ImageJ, Icy and Matlab

<http://bigwww.epfl.ch/algorithms/psfgenerator/>

H. Kirshner, F. Aguet, D. Sage, M. Unser, 3-D PSF Fitting for Fluorescence Microscopy: Implementation and Localization Application, *Journal of Microscopy*, vol. 249, no. 1, pp. 13-25, 2013.

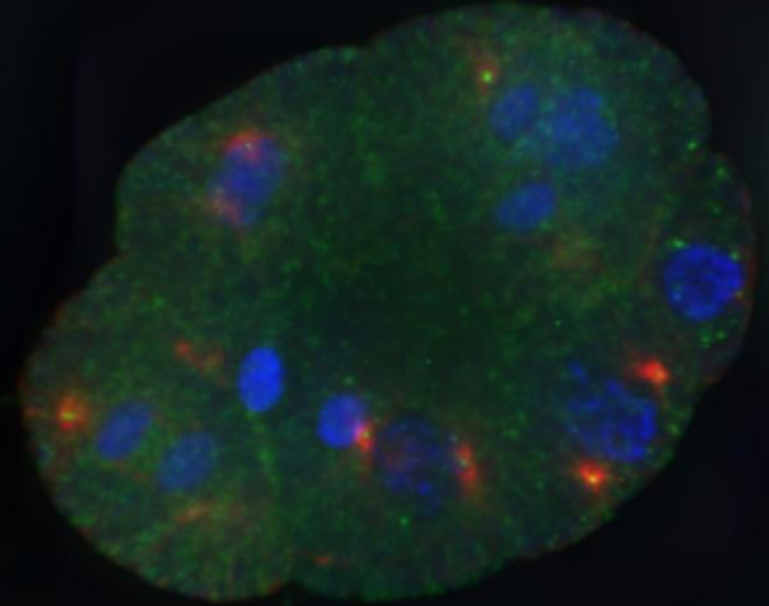
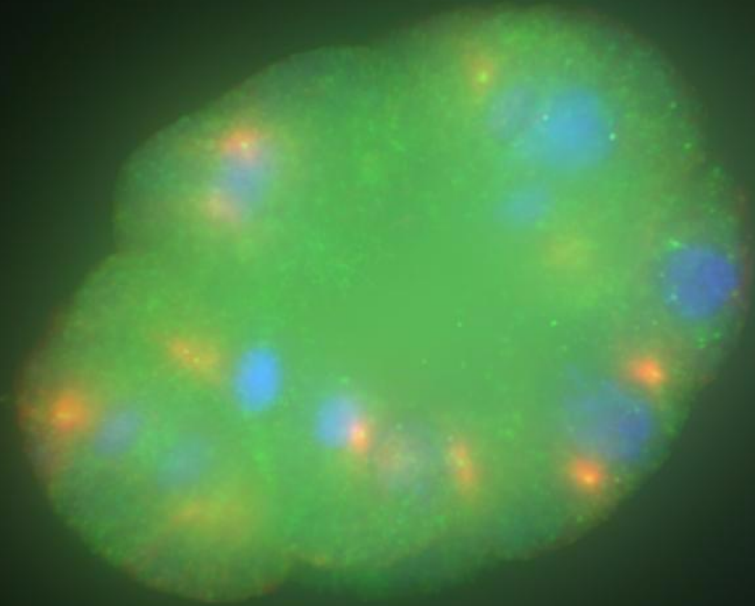
A. Griffa, N. Garin and D. Sage, Comparison of Deconvolution Software in 3D Microscopy. A User Point of View, Part I and Part II, *G.I.T. Imaging & Microscopy*, vol 1, pp. 43-45, 2010.

Huygens (Scientific Volume Imaging)

Commercial software with tools for PSF generation, measurement and deconvolution.

<https://svi.nl/HuygensProfessional/>

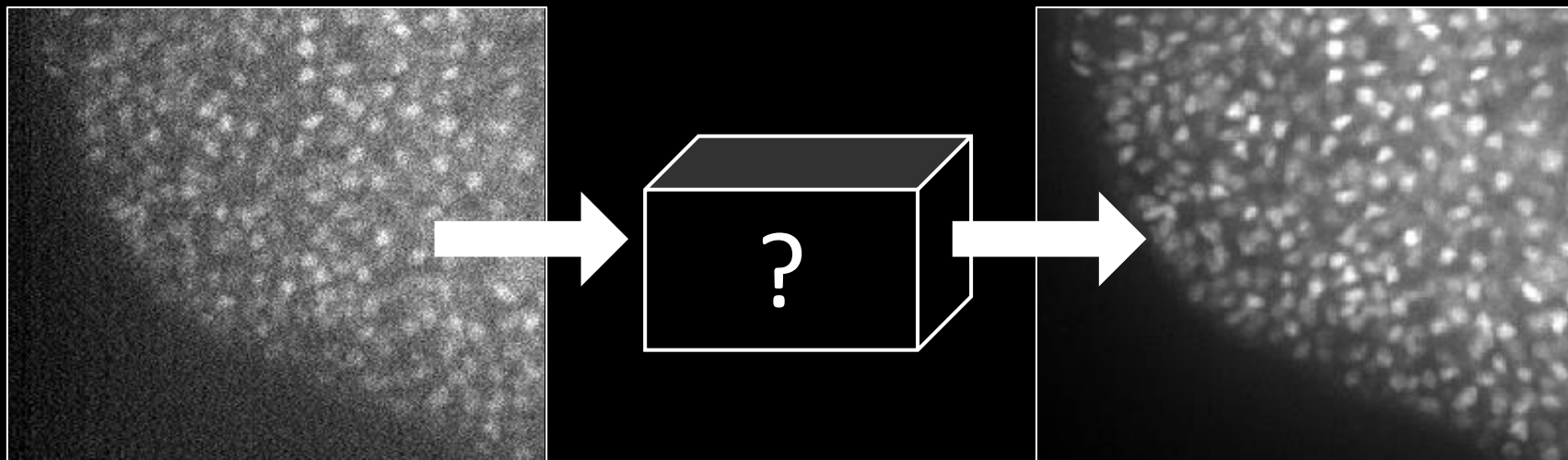
Richardson-Lucy Deconvolution using DeconvolutionLab



Section 2: Deep learning-based denoising

**Slides by Dr Alexander Krull
Lecturer in Data Science and AI, UoB**

The Problem of Noise



Low exposure:

- Gentle 😊
- Noisy 😞

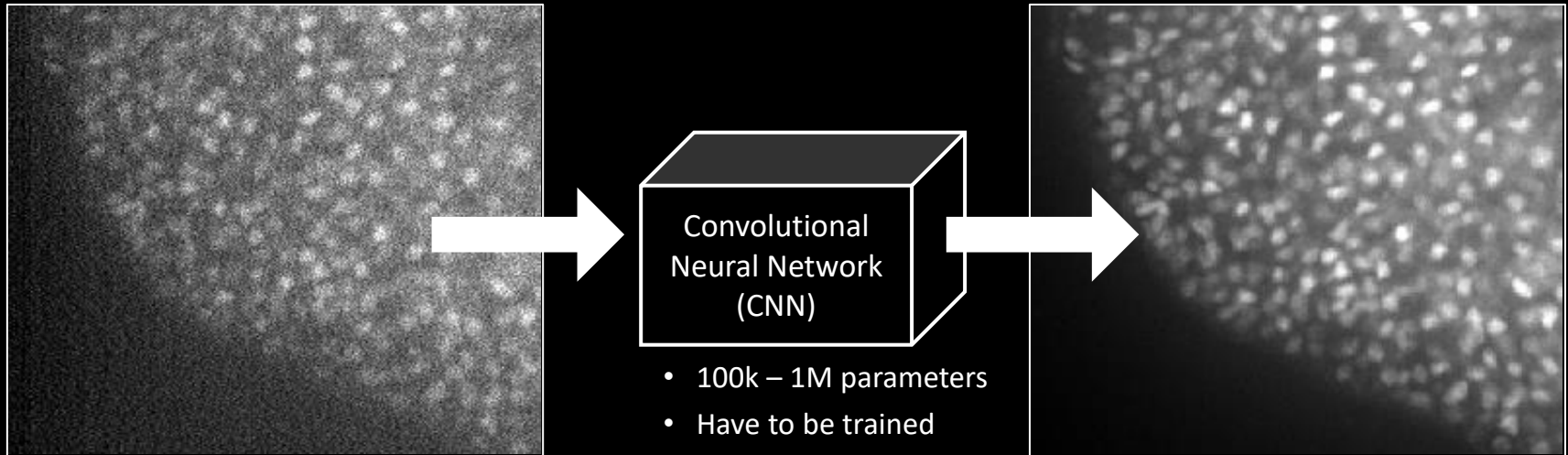
High exposure:

- Damaging 😞
- Clean 😊

Traditional Supervised Training

You need clean data.

Deep Learning for Denoising



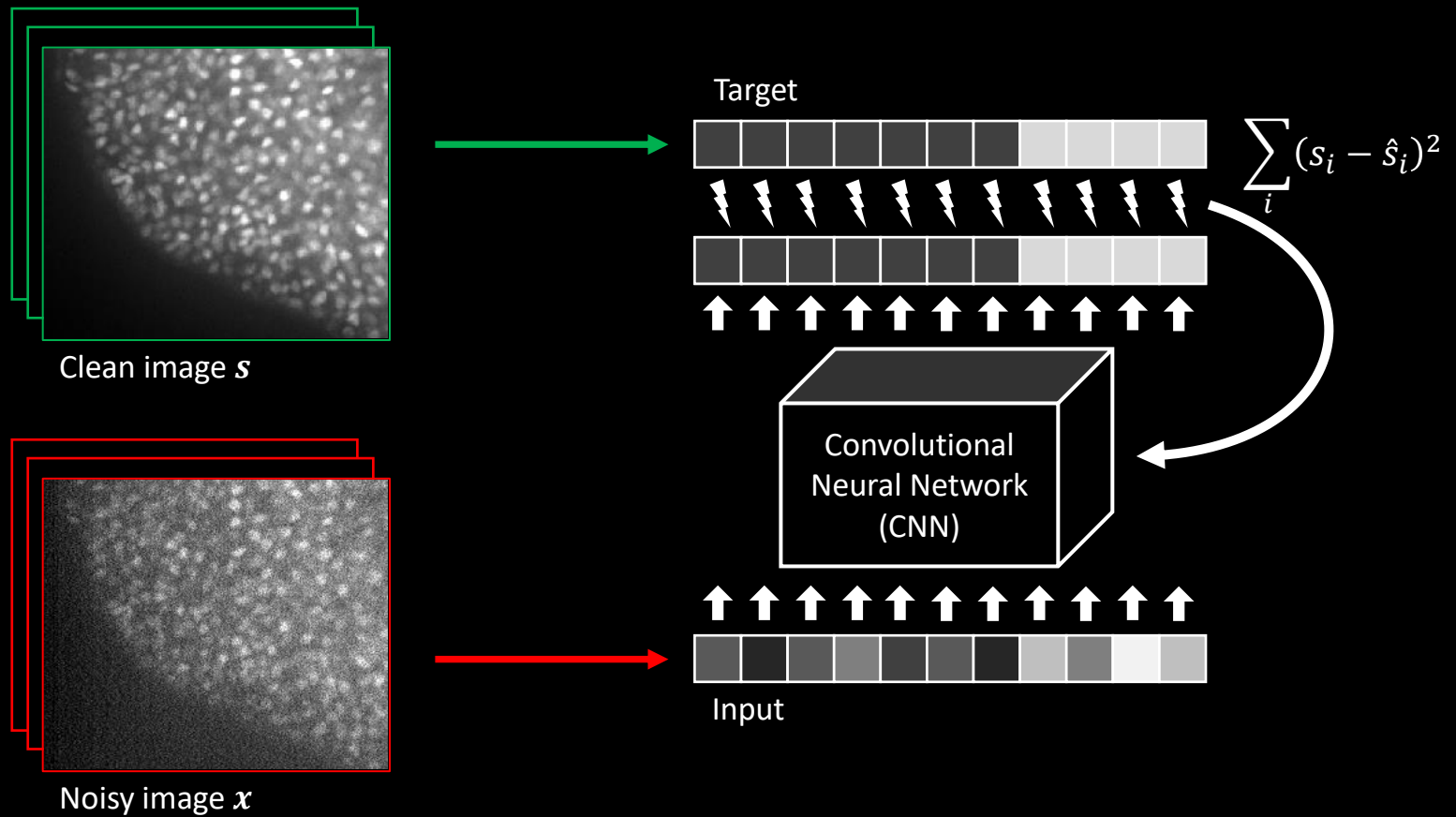
Low exposure:

- Low photo toxicity 😊
- Low bleaching 😊
- Noisy 😞

High exposure:

- Strong photo toxicity 😞
- Strong bleaching 😞
- Less noise 😊

CARE – Traditional Supervised Training

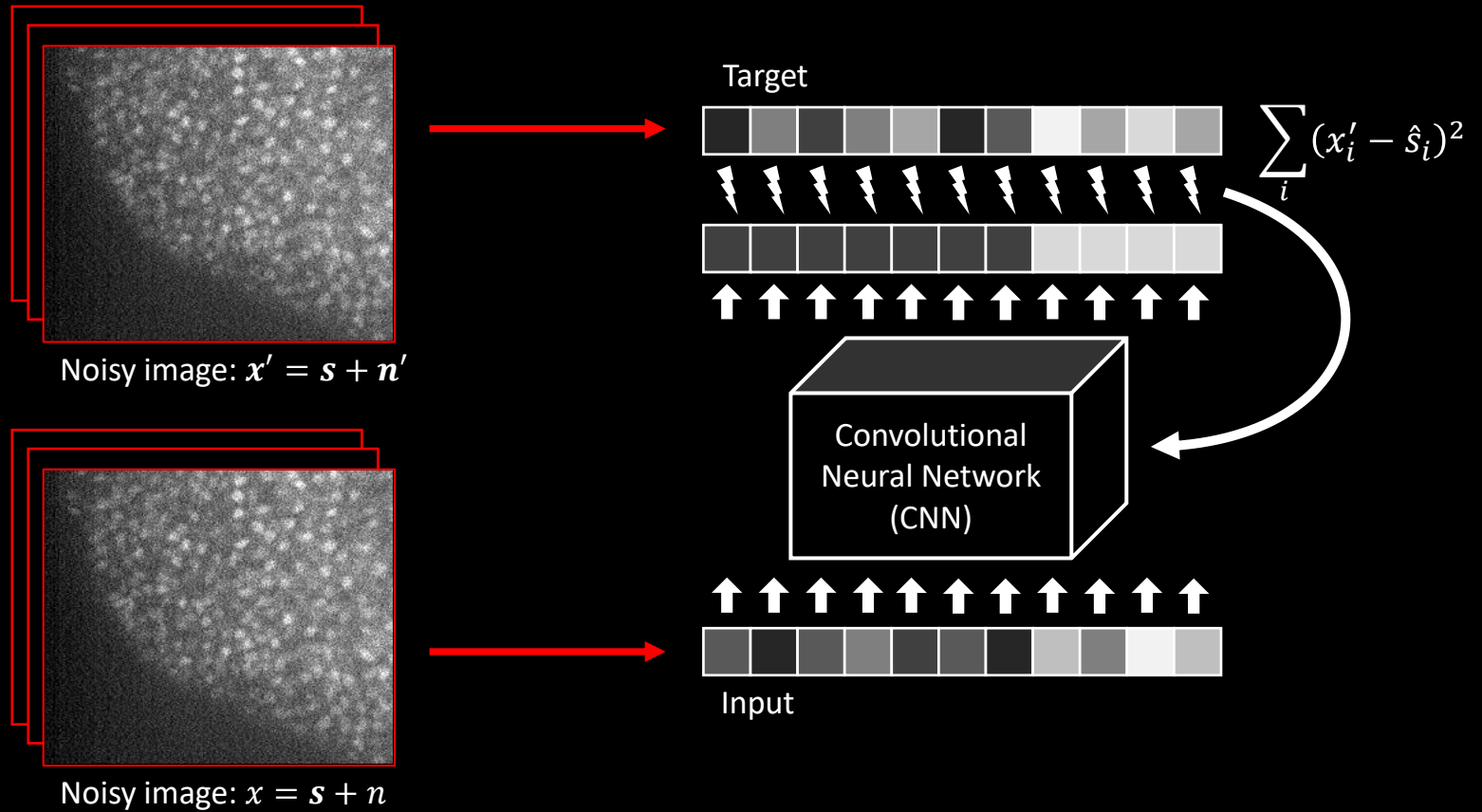


Noise2Noise

You only need noisy data!

Lehtinen et al. 2018

Noise2Noise training

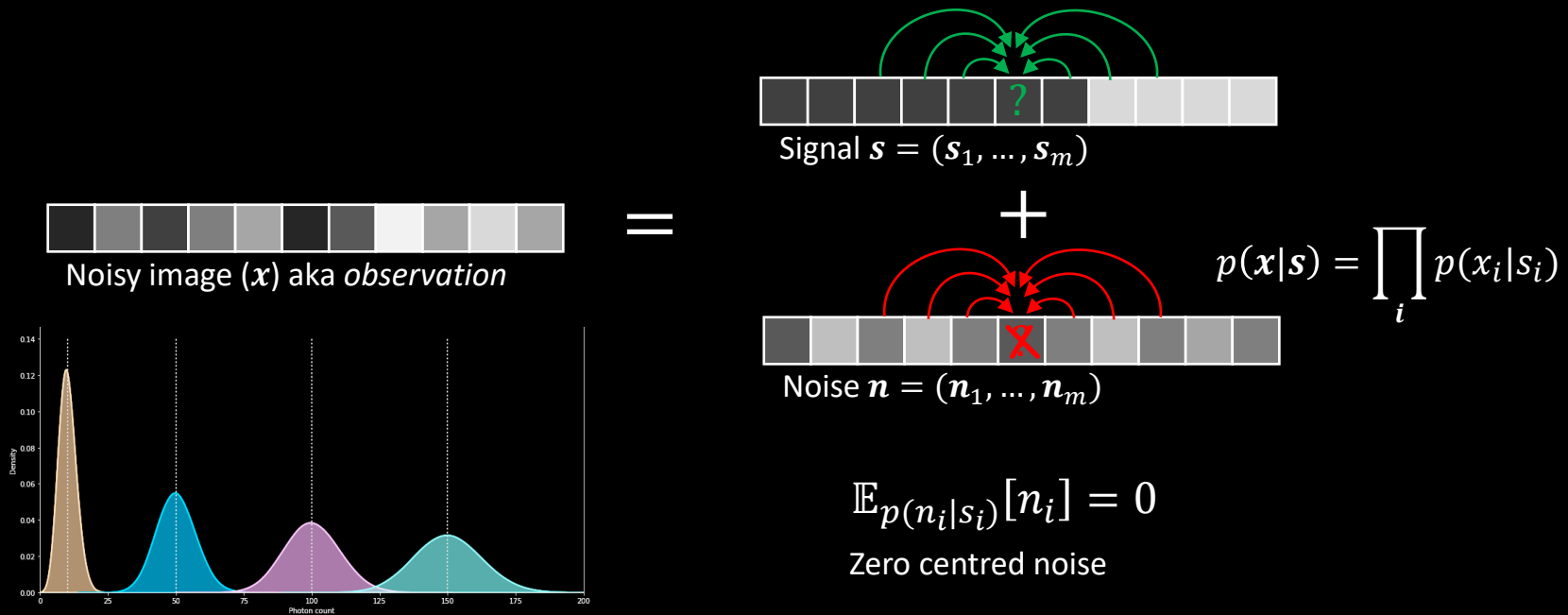


Self-Supervised Denoising: Noise2Void

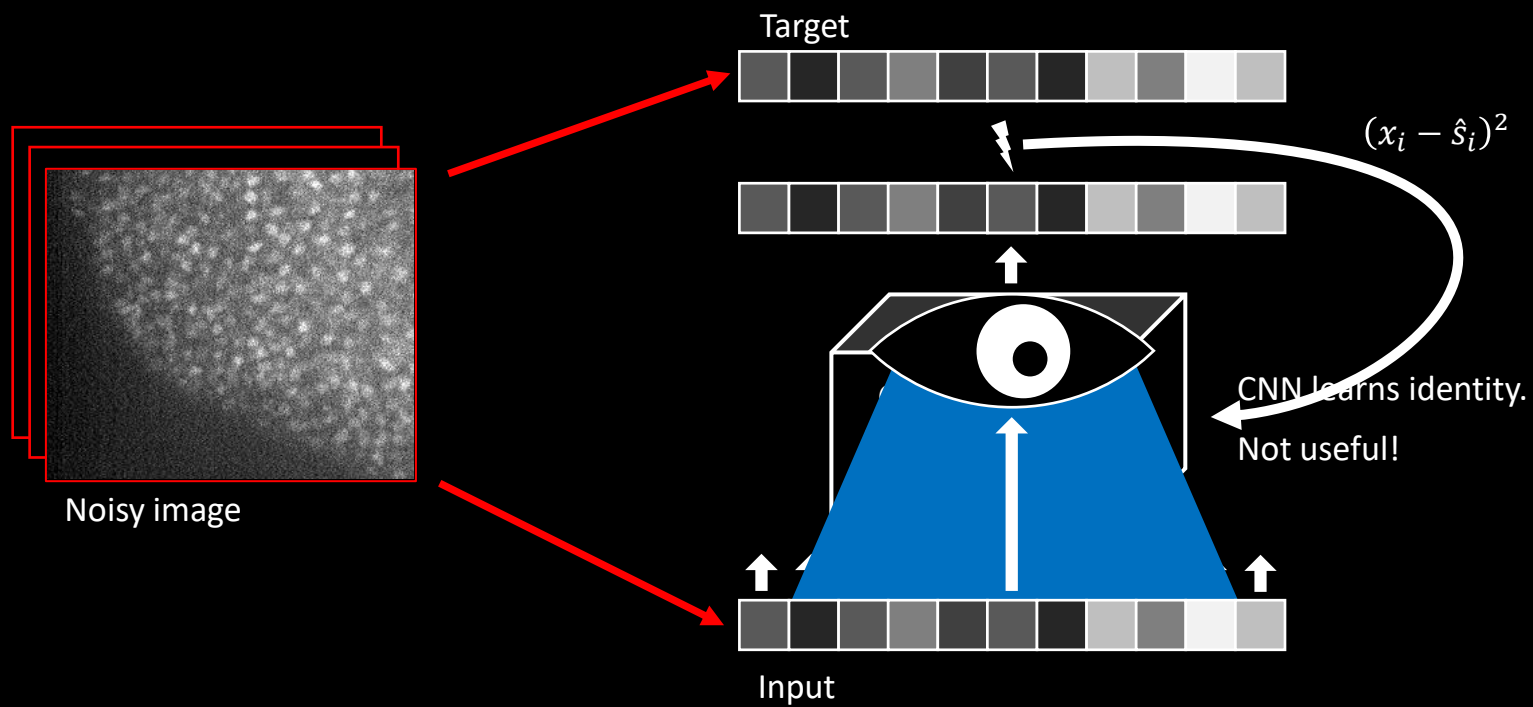
You only need individual noisy images!

Krull, Buchholz, and Jug 2019

Noise2Void – Assumptions



Noise2Void

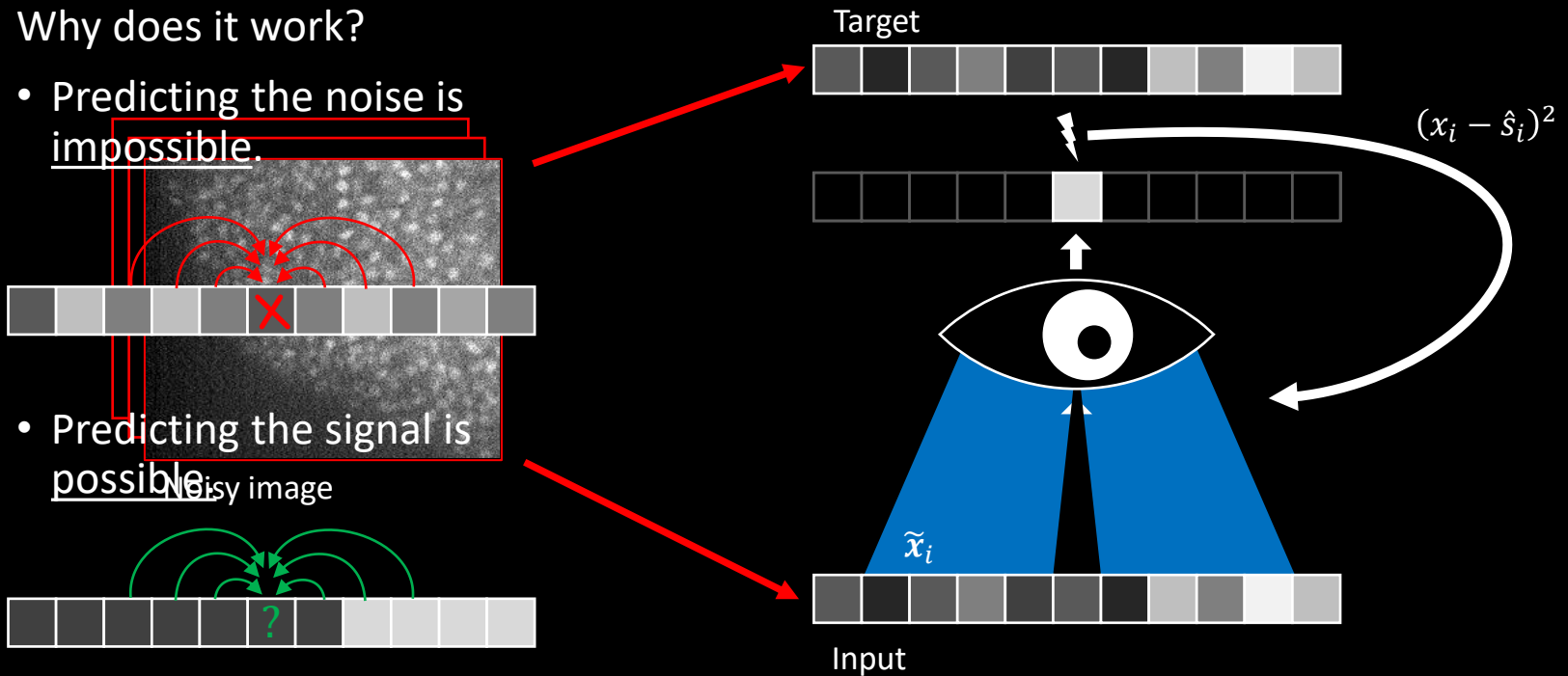


Noise2Void - Blind Spot Network

Why does it work?

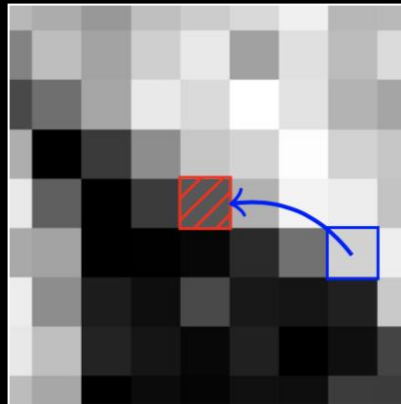
- Predicting the noise is impossible.

- Predicting the signal is possible.

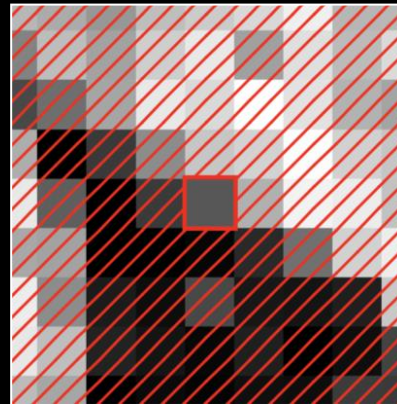


Noise2Void - Blind Spot Implementation

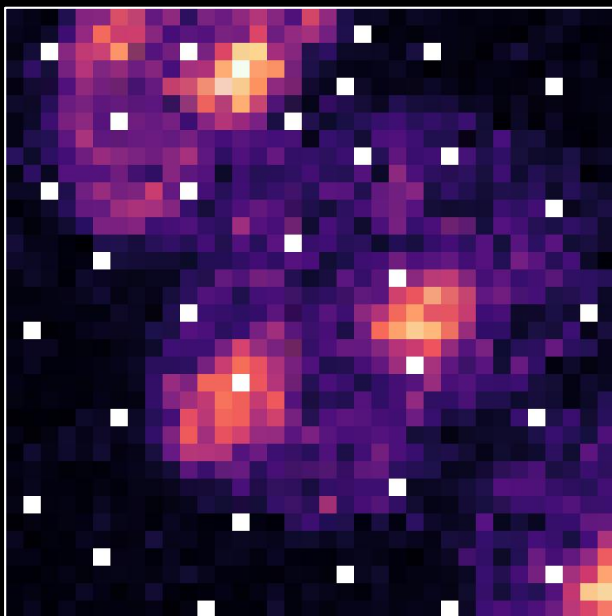
input



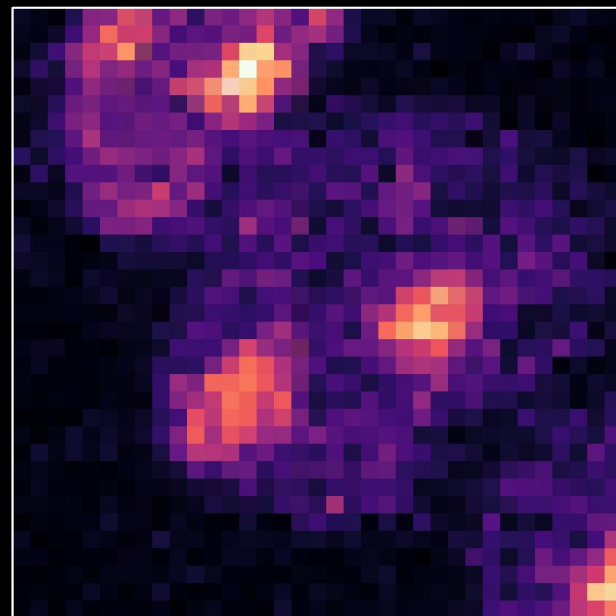
target



Noise2Void - Blind Spot Implementation

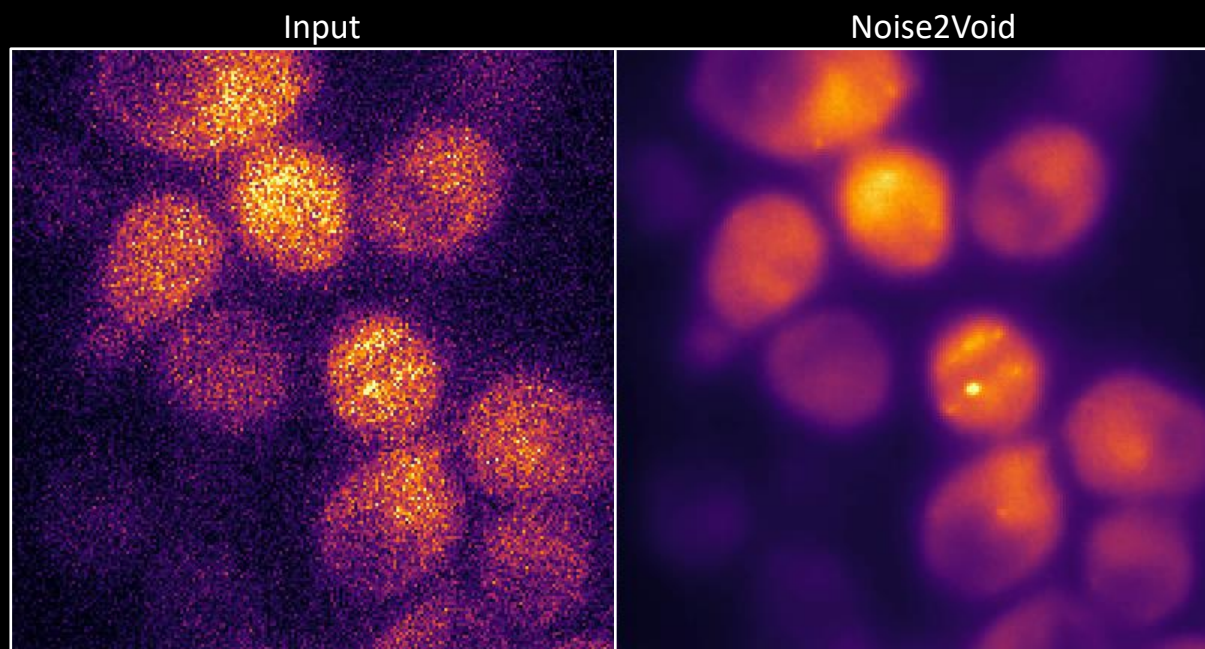


Input



Target

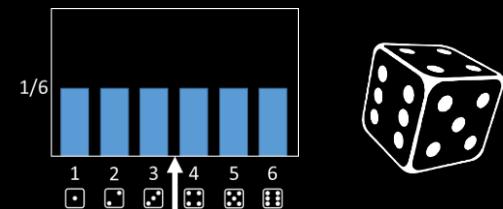
Noise2Void - Results



Data by Stephanie Heinrich

Summary

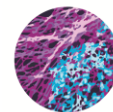
- Traditional supervised training:
 - Tries to map noisy image to clean image.
 - Impossible: map to expected value of clean image.
 - Downside: requires clean images during training.
- Noise2Noise training:
 - Requires no clean data.
 - Tries to map noisy image to noisy image.
 - Impossible: also map to expected value of clean image.
 - Downside: Still requires image pairs.
- Noise2Void training
 - Requires no clean data or image pairs
 - Tries to predict intensity for a given pixel based on the local neighborhood only
 - Impossible: also map to expected value of clean image
 - Downside: Cannot not remove structured noise



Section 3: Segmentation

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Image Segmentation: The Filtering Approach

- Apply a customised series of filters and morphological operations
- The last step is typically a threshold (either global or local)
- Pros:
 - Simply to implement
 - Easy to understand
 - Fast
- Cons:
 - Workflows are specialized: do not work on different problems / datasets without modification
 - Large number of parameters to set and tune
 - Tendency to produce convoluted workflows with many steps

The Filtering Approach

Example: Gaussian Blur and Otsu Threshold

- Convolve image with Gaussian filter to reduce local variation and noise

$$I = I_0 \otimes G\sigma$$

- Otsu thresholding assumes there are two classes (signal and background) and maximises the intra-class variance.

Otsu, N (1979), "A threshold selection method from gray-level histograms", IEEE Trans. Sys., Man., Cyber. 9: 62-66.

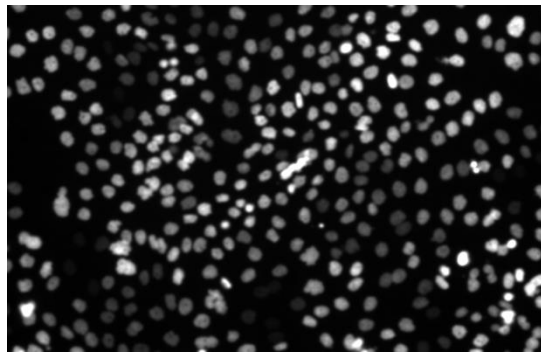
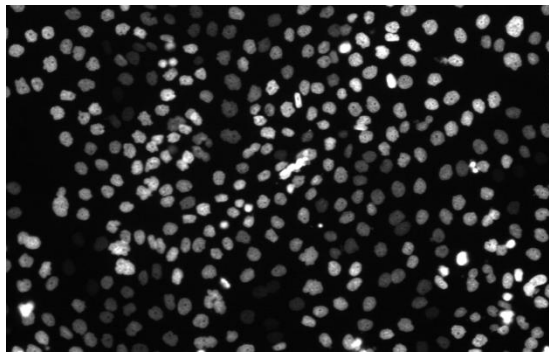


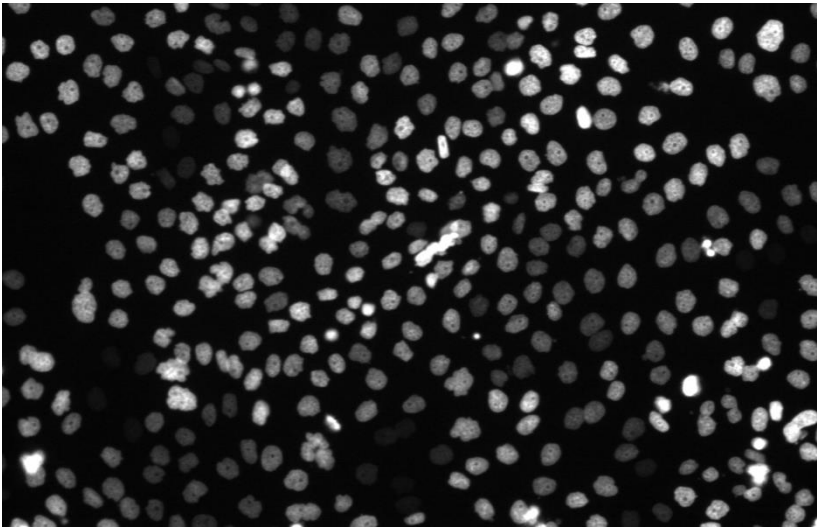
Image Segmentation: The Model Based Approach

- How well does the segmentation explain the observed data given a specific model.
- Incorporate knowledge about the target(s) and/or the forward problem
- Iteratively update segmentation until convergence
- Pros:
 - Can be flexible. Simply change the data model but not the algorithm!
 - Does not require training
 - Well reasoned
- Cons:
 - Can be slower than a filter based approach
 - Hard for a non-specialist to understand and set parameters

The Model Based Approach

Example: Region Competition

- Model based segmentation for an unknown number of objects. Three different models can be used:
 - Piece-wise constant objects
 - Piece-wise smooth objects
 - Convolution with PSF of piece-wise constant objects



<http://mosaic.mpi-cbg.de/?q=downloads/imageJ>

J. Cardinale, G. Paul, and I. F. Sbalzarini. Discrete region competition for unknown numbers of connected regions. IEEE Trans. Image Process., 21(8):3531–3545, 2012.

Image Segmentation: The Supervised Machine Learning Approach

- Teach the algorithm to perform image segmentation using an annotated training set
- Pros:
 - Flexible and adaptable to different problems
 - Can produce excellent results for complex problems (eg tissue segmentation)
 - Not many parameters to set/optimize
- Cons:
 - Training data and expert manual annotation required. Bias?
 - The classifier can be hard to understand and justify
 - Typically slower than a filter based approach

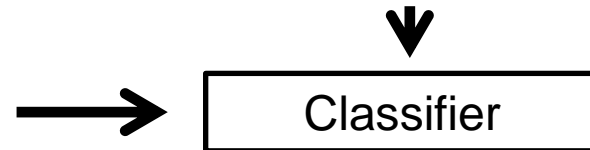
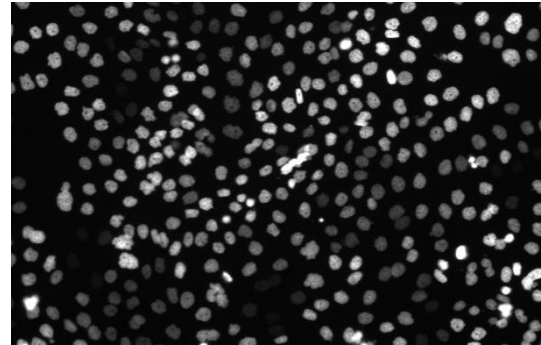
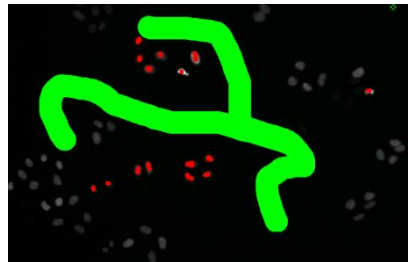
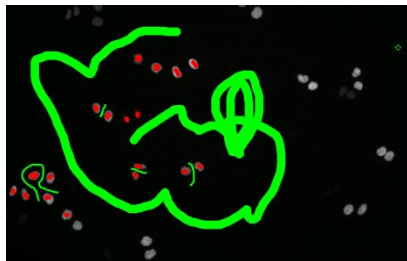
Sommer, Christoph, and Daniel W. Gerlich. "Machine learning in cell biology—teaching computers to recognize phenotypes." J Cell Sci 126.24 (2013): 5529-5539.

The Supervised Machine Learning Approach

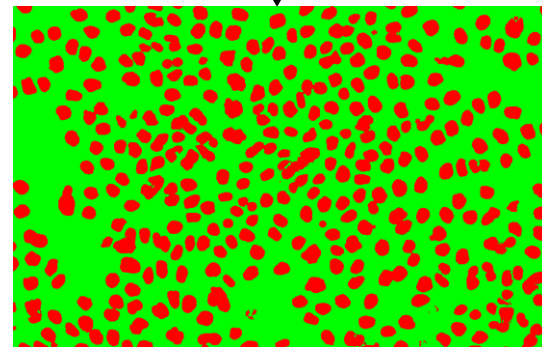
Example: Pixel Classification with ilastik

- Ilastik is an interactive learning toolkit for segmentation, object classification and tracking

Training phase



Classifier

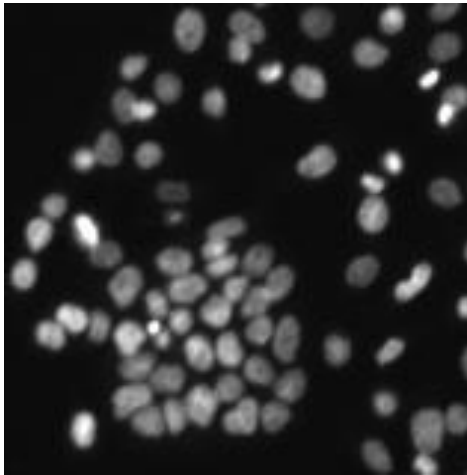


<http://ilastik.org/index.html>

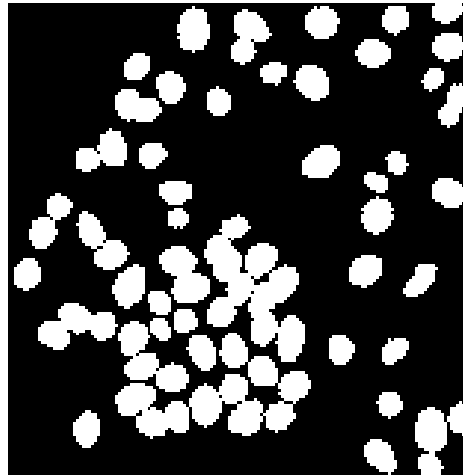
Sommer, Christoph, et al. "Ilastik: Interactive learning and segmentation toolkit." 2011 IEEE international symposium on biomedical imaging: From nano to macro. IEEE, 2011.

Instance segmentation

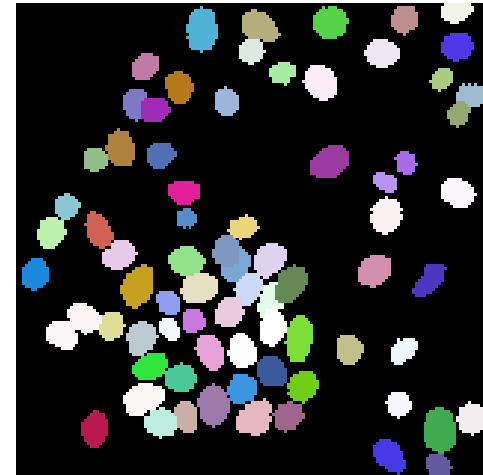
Data



Semantic segmentation
2 or more classes

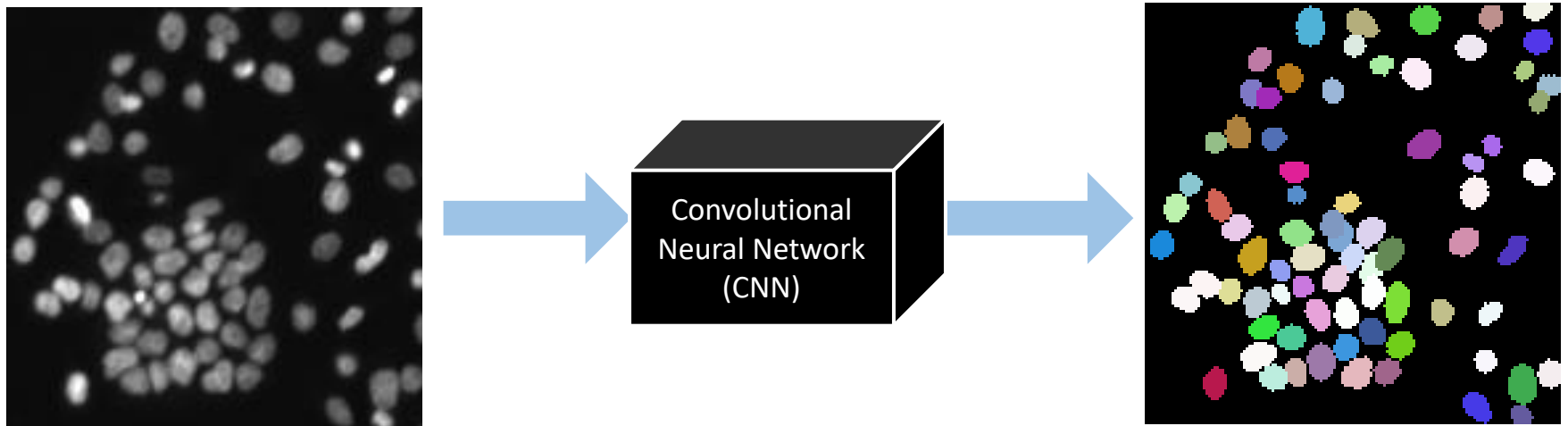


Instance segmentation
Individual objects



- Often interested in per object (e.g nuclei or cell) measurements
- Instance segmentation hard in crowded environments and simple postprocessing steps (e.g. watershed) from semantic segmentation fails

Deep learning-based instance segmentation

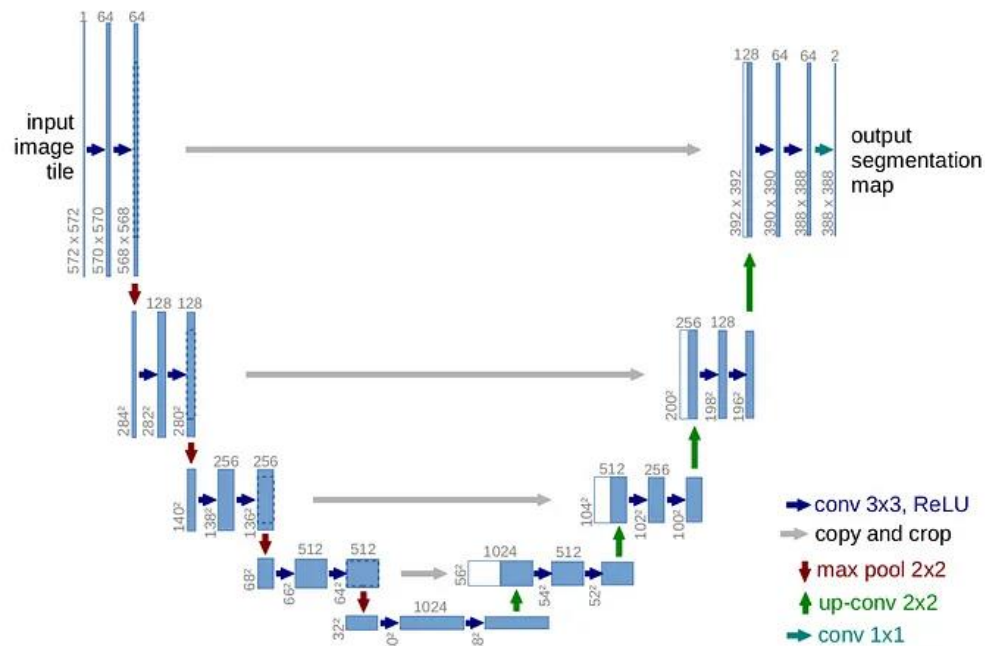


- Requires time consuming manual annotation of training data
- Pre-trained networks can be very useful with optional fine tuning on your own data

UNET

Convolutional Networks for Biomedical Image Segmentation

- Extremely popular fully convolutional neural network for segmentation (among other things including denoising; CARE, Noise2Void etc).
- Standard training scheme gives a semantic segmentation but can be adapted for instance segmentation (e.g StarDist / Cellpose)



StarDist

Object Detection with Star-convex Shapes

- Trains a CNN (UNET) to predict distances to object boundaries along fixed set of rays (r), and object probabilities (d).
- Together this gives candidate set of instances which are further processed by non maximal suppression (NMS) to give final result.

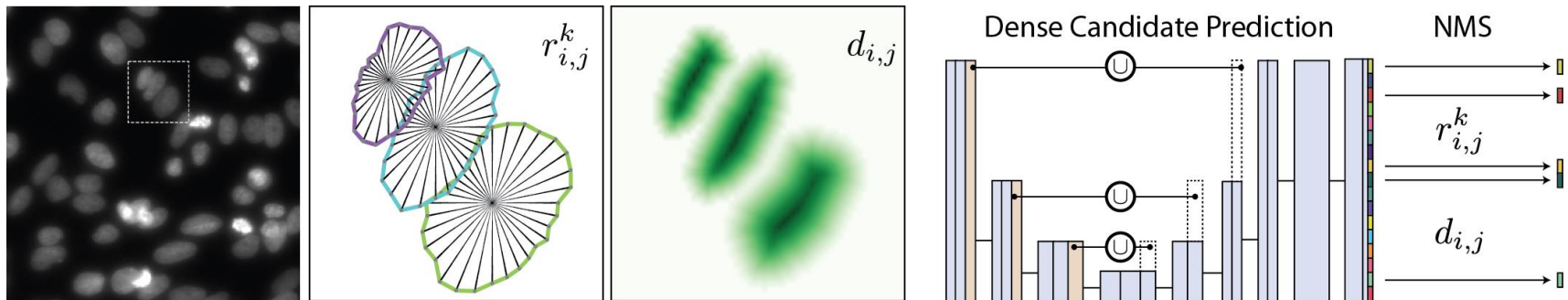


Image from <https://github.com/stardist/stardist>

- Awesome for nuclei segmentation in 2D and 3D.
- Not good for cell membrane boundaries which often are not star-convex
- Great Fiji plugin for 2D with pretrained model for fluorescent nuclei and H&E staining

Cellpose

A generalist algorithm for cellular segmentation

- Manual annotations transformed into vector flow representation which can be predicted by a CNN (e.g UNET)
- This representation is great because it allows for non star convex objects like complex cellular boundaries.
- Gradients point toward centre of objects but not necessarily directly to move around “corners”

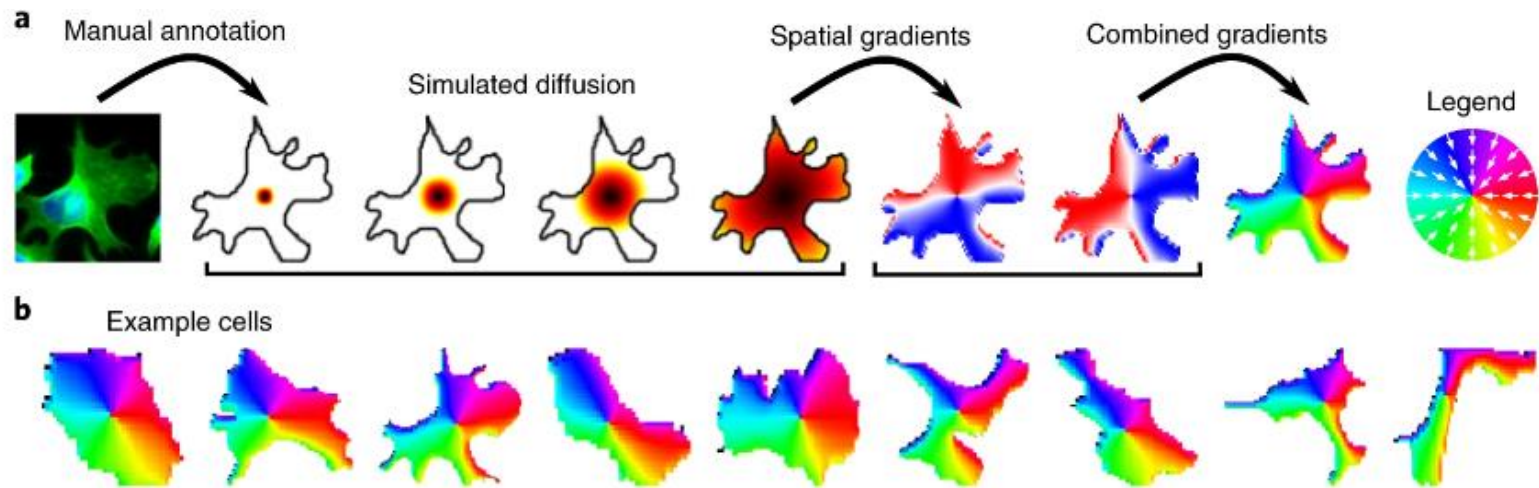


Image from Figure 1 of Stringer et al.

Cellpose

A generalist algorithm for cellular segmentation

- UNET trained with supervised training scheme

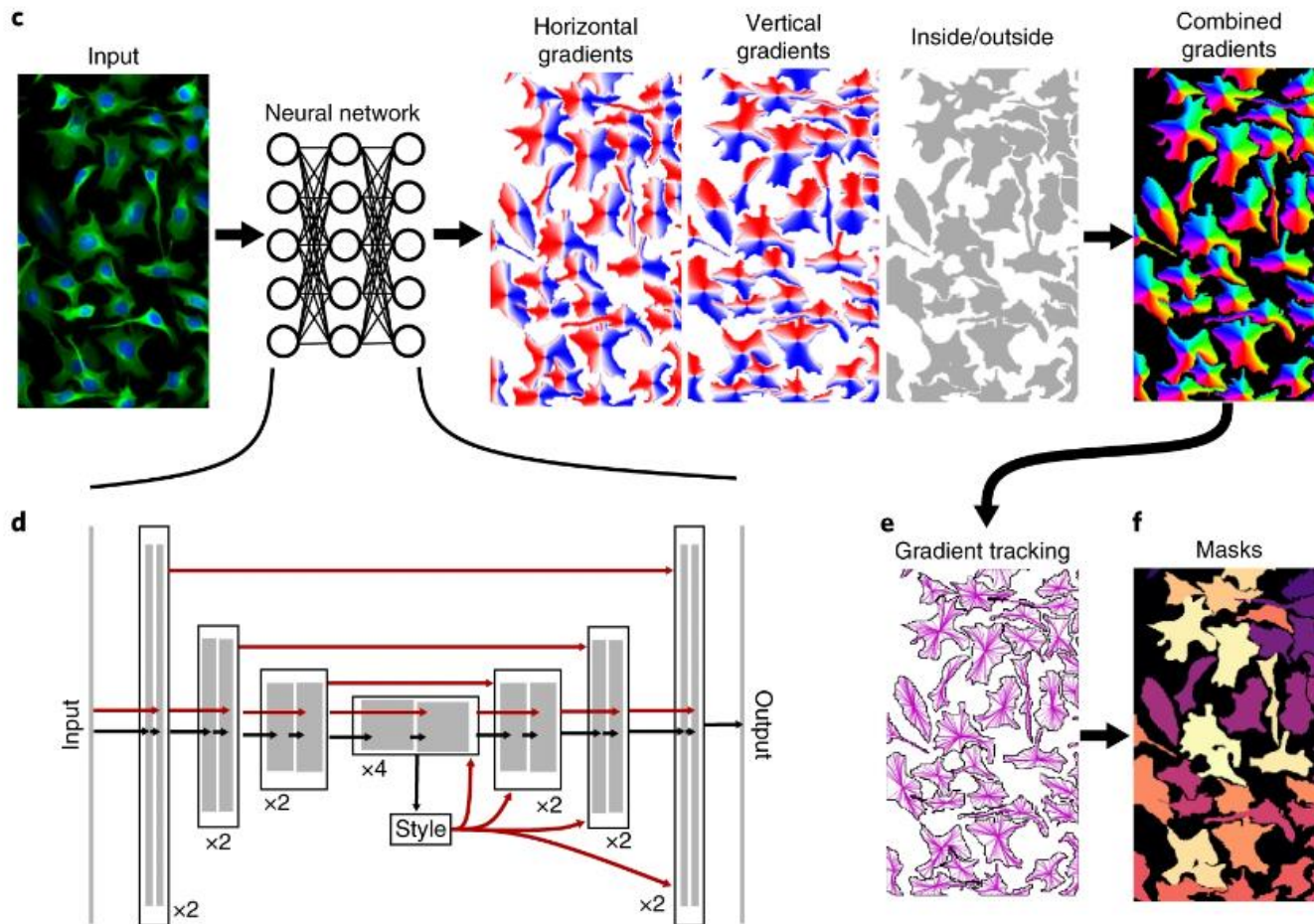


Image from Figure 1 of Stringer et al.

Cellpose

A generalist algorithm for cellular segmentation

- Varied training dataset gives high performance on many problems with pretrained models
- 2D but postprocessing gives 3D segmentations
- Variety of options to use:
 - Web app (single image)
 - GUI
 - Python using notebooks or scripts
 - Command line
 - Fiji plugin....
- Cellpose 2.0 introduces interactive fine tuning of pretrained networks on your own data using provided GUI

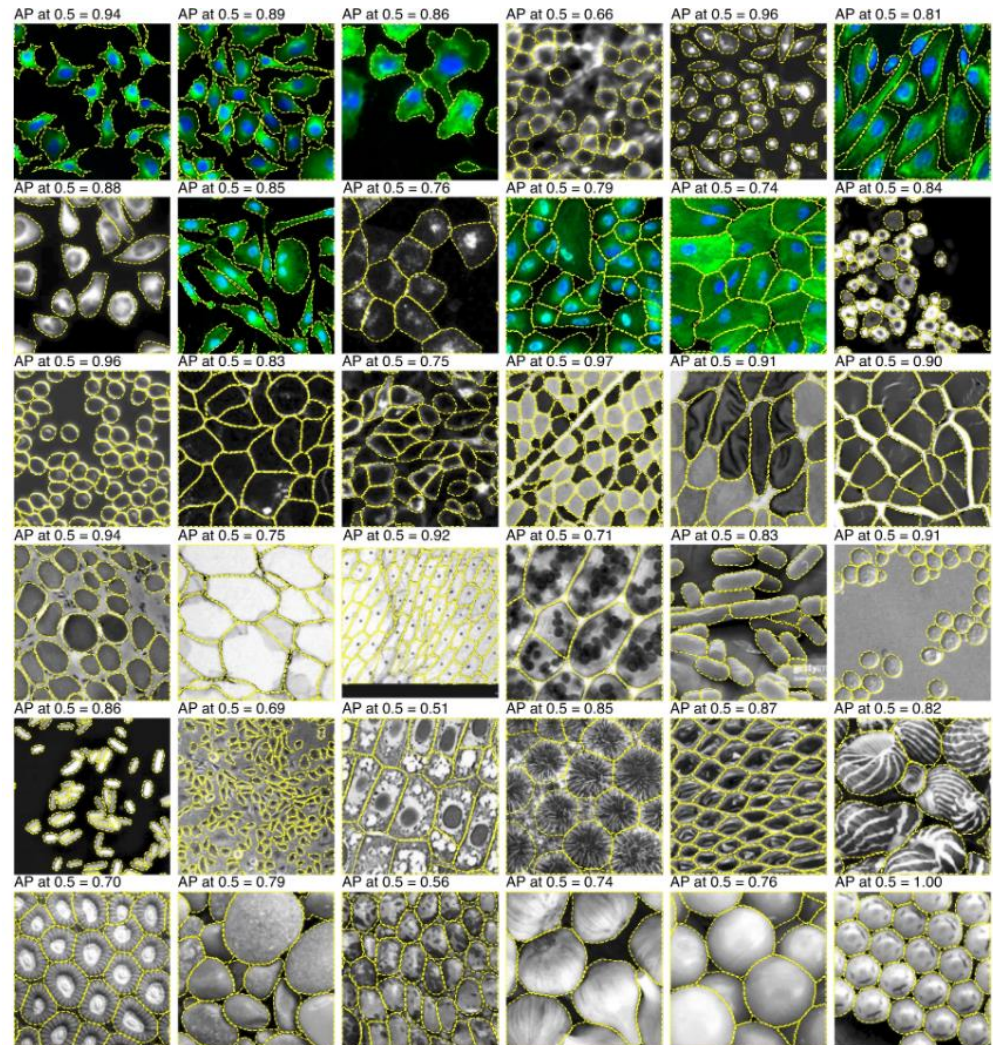
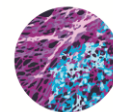


Image from Figure 3 of Stringer et al.

Section 4: Single Particle Tracking

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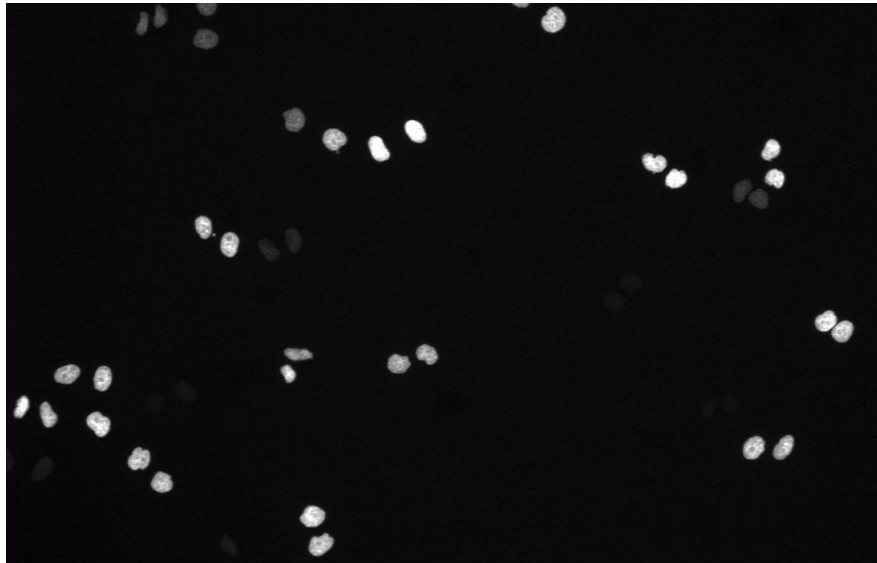
The Universities of Birmingham and Nottingham



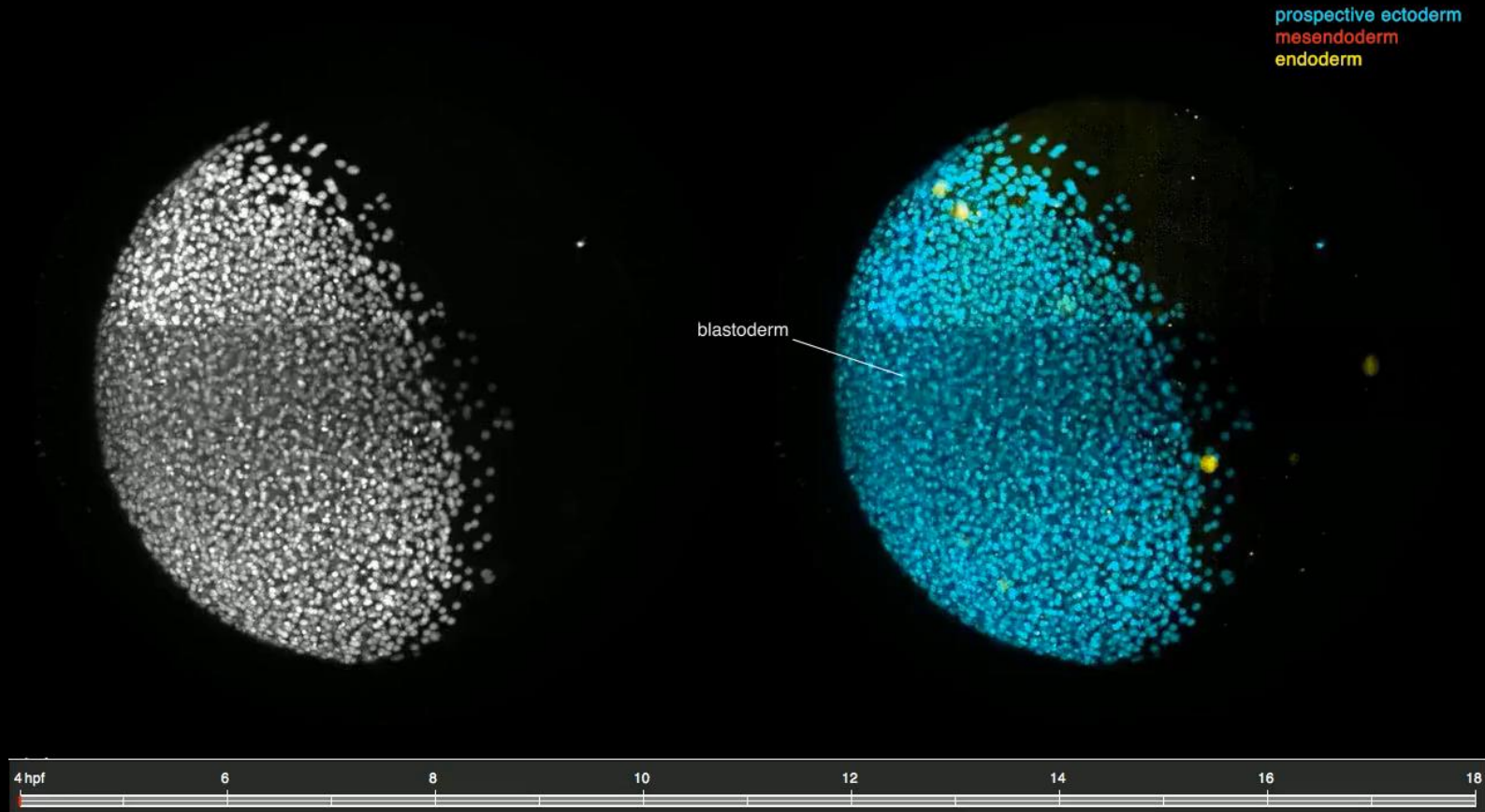
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Single Particle Tracking

- A two step process
 1. Object detection
 2. Linking objects between frames (tracking)
- Complications include object merging and splitting
- If an object is missed in one (or more) frames then gap closing can be used to merge particle trajectories



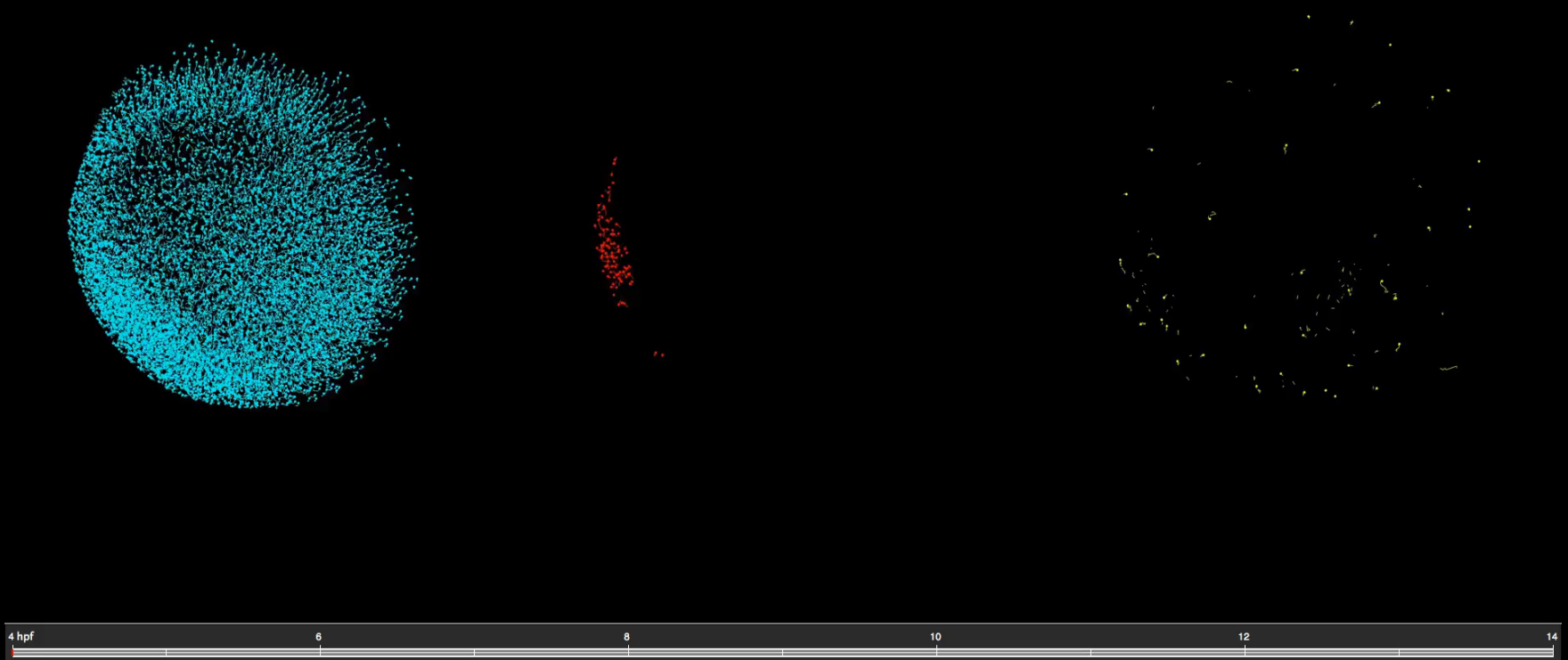
Application 1: Tracking cells in developing embryos



Shah, Gopi, et al. "Pan-embryo cell dynamics of germlayer formation in zebrafish." *bioRxiv* (2017): 173583.

Amat, Fernando, et al. "Fast, accurate reconstruction of cell lineages from large-scale fluorescence microscopy data." *Nature methods* (2014).

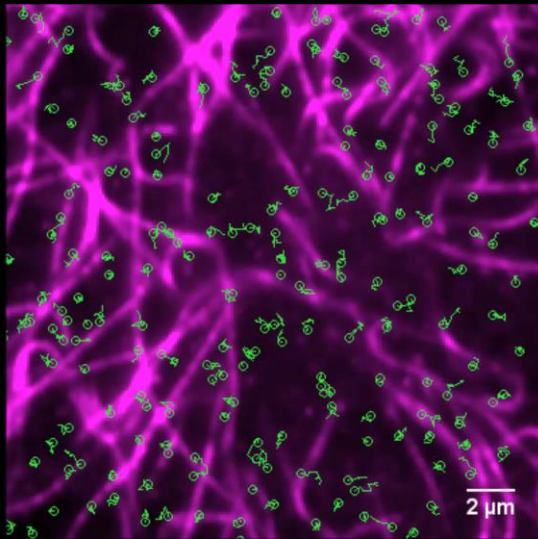
Application 1: Tracking cells in developing embryos



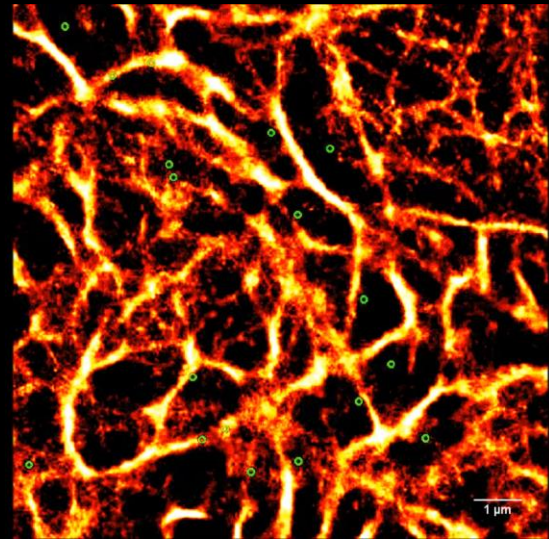
Shah, Gopi, et al. "Pan-embryo cell dynamics of germlayer formation in zebrafish." *bioRxiv* (2017): 173583.

Amat, Fernando, et al. "Fast, accurate reconstruction of cell lineages from large-scale fluorescence microscopy data." *Nature methods* (2014).

Application 2: Tracking individual receptors at the plasma membrane



G-proteins over actin

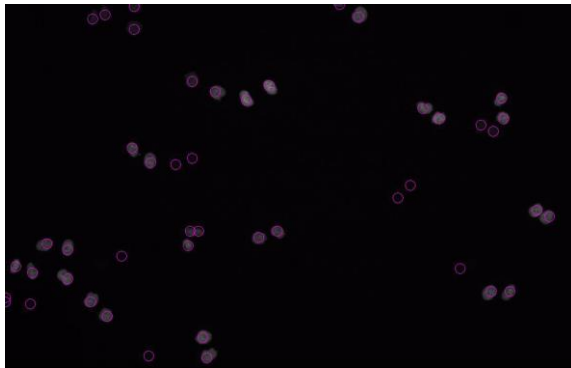
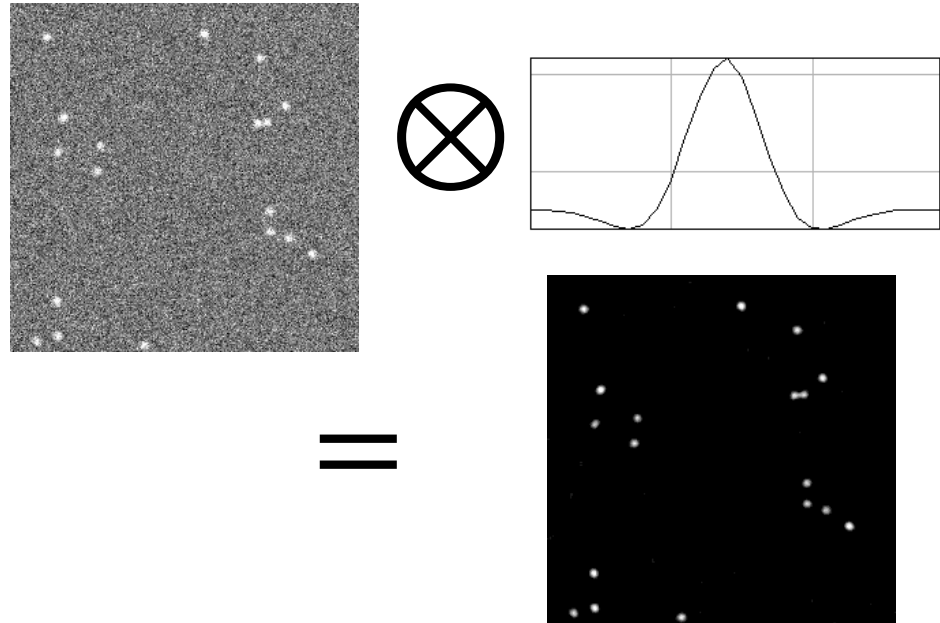


G-proteins over MTs

Sungkaworn, Titiwat, et al. "Single-molecule imaging reveals receptor–G protein interactions at cell surface hot spots." *Nature* 550.7677 (2017): 543-547.

Object Detection

- Typically a “spot detection” protocol is employed.
- TrackMate (Fiji) has simple in-built difference of Gaussian schemes

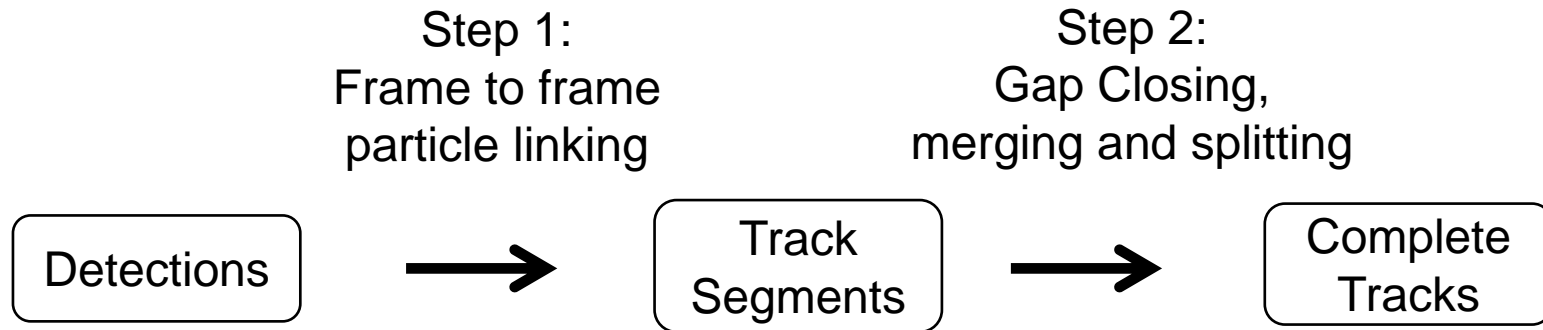


- More sophisticated algorithms such as wavelet based detection can be employed. For example the Spot Detector plugin in Icy.

Tinevez J-Y, Perry N, Schindelin J, Hoopes GM, Reynolds GD, Laplantine E, et al. TrackMate: An open and extensible platform for single-particle tracking. Methods 2016.

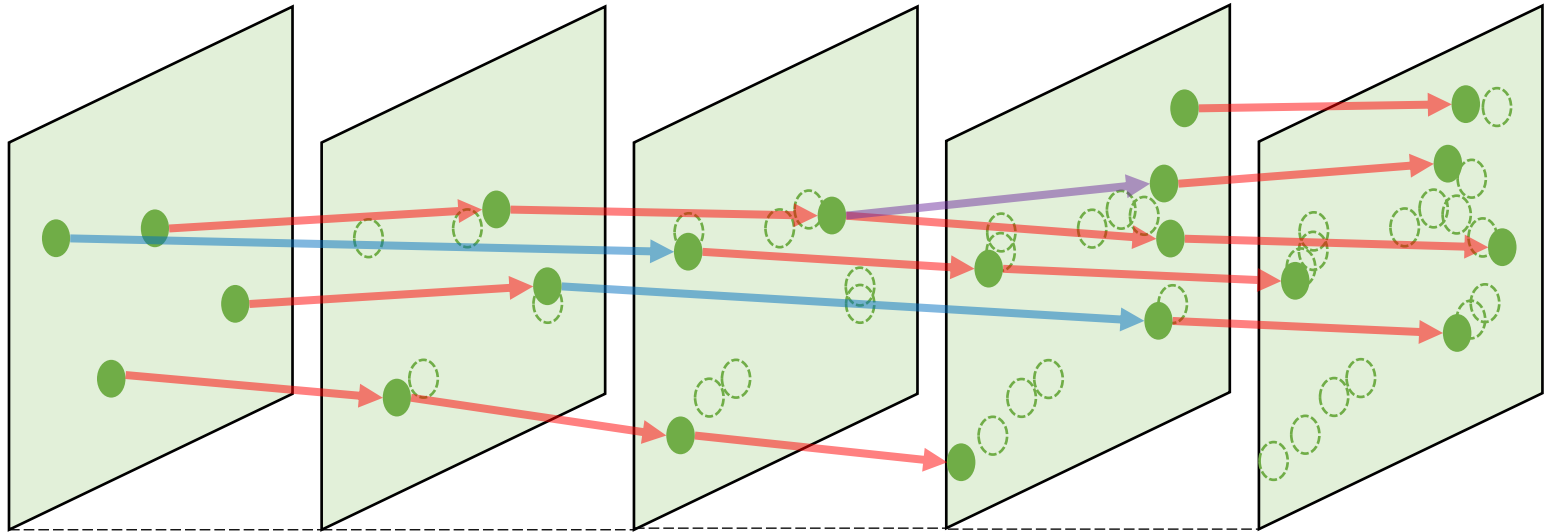
Olivo-Marin, Jean-Christophe. "Extraction of spots in biological images using multiscale products." Pattern recognition 35.9 (2002): 1989-1996.

Linear Assignment Problem (LAP) trackers



- For both steps cost matrices are constructed and minimised using a LAP framework
- Linking costs are based on distance between detections
- Well suited for particles undergoing Brownian motion
- TrackMate allows for costs to be weighted by detection or track segment properties

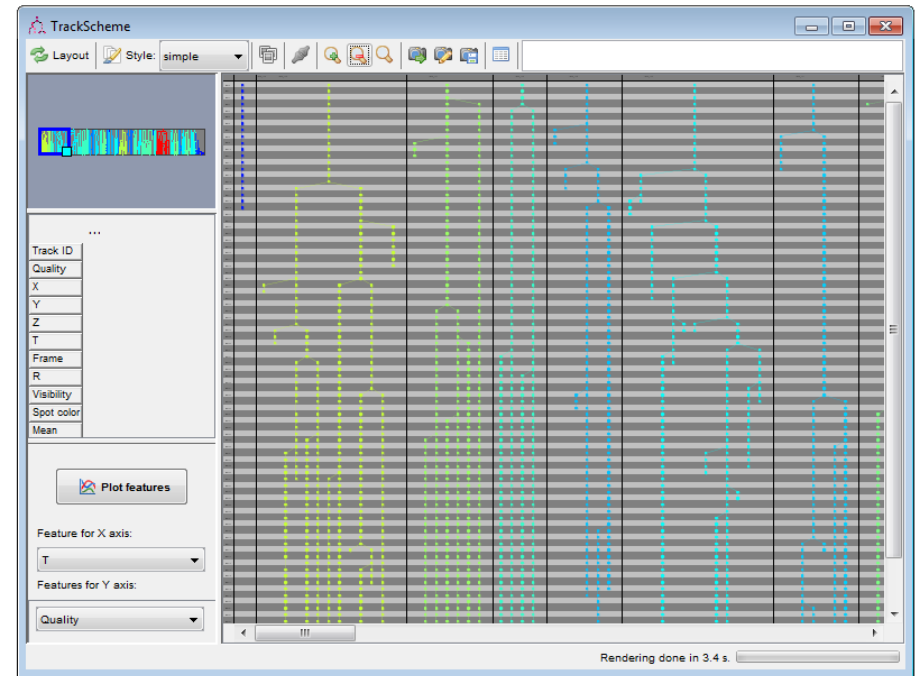
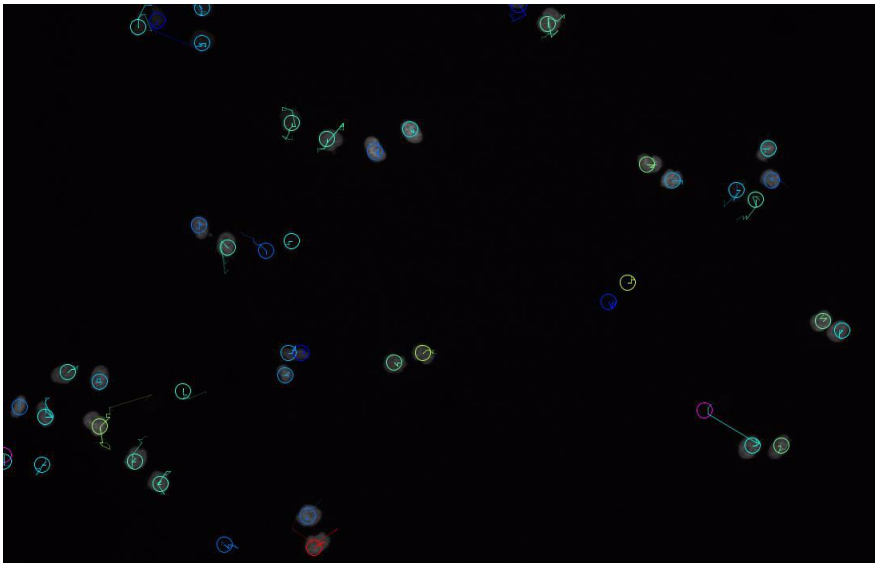
The Linear Assignment Problem



Frame-to-frame particle linking

Track segment linking – gap closing and splitting

Cell tracking with TrackMate using a LAP Tracker

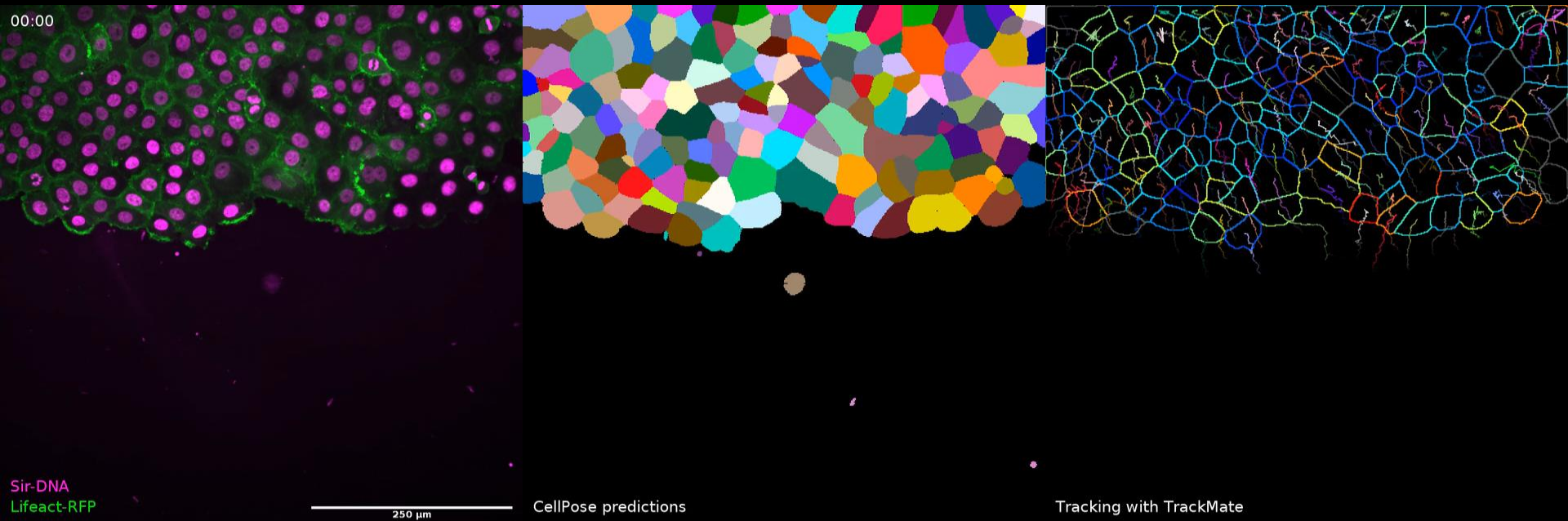


Tinevez J-Y, Perry N, Schindelin J, Hoopes GM, Reynolds GD, Laplantine E, et al. TrackMate: An open and extensible platform for single-particle tracking. *Methods* 2016.

Jaqaman, Khuloud, et al. "Robust single-particle tracking in live-cell time-lapse sequences." *Nature methods* 5.8 (2008): 695-702.

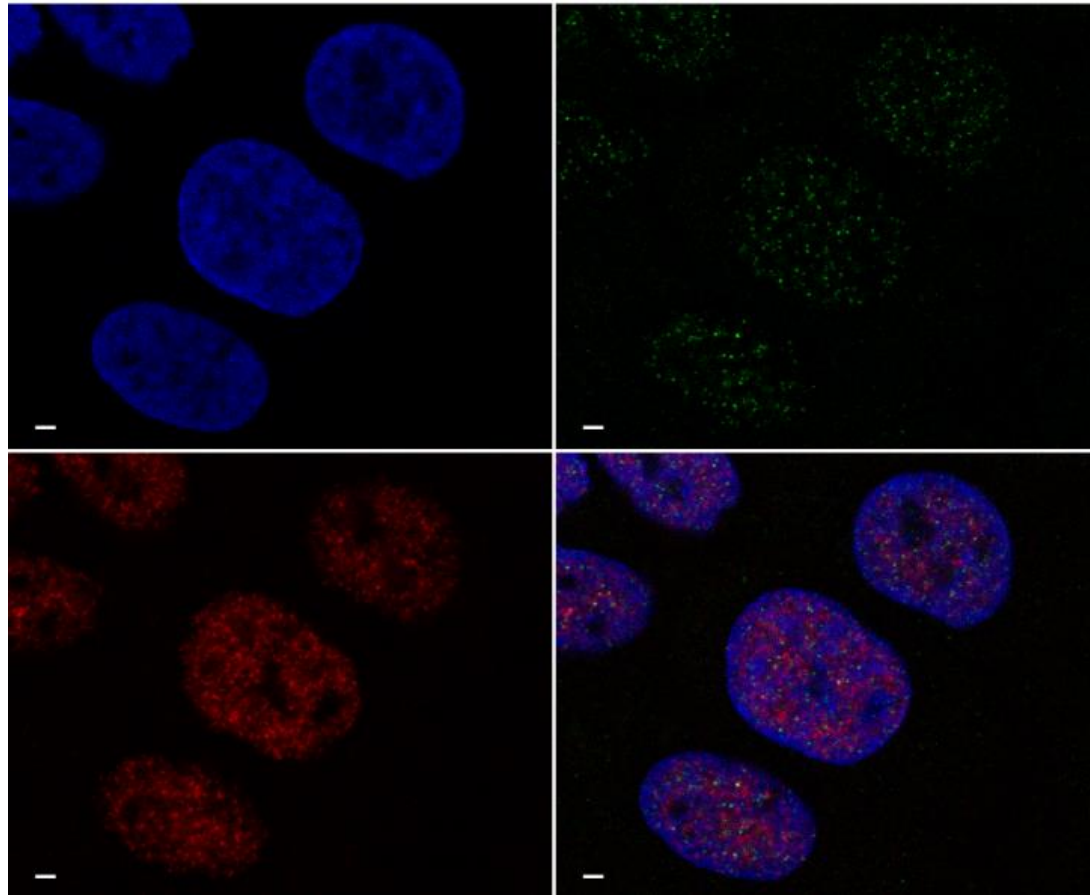
Combine TrackMate with advanced segmentation algorithms

- Extensions which allow integration with Cellpose, Stardist, ilastik etc

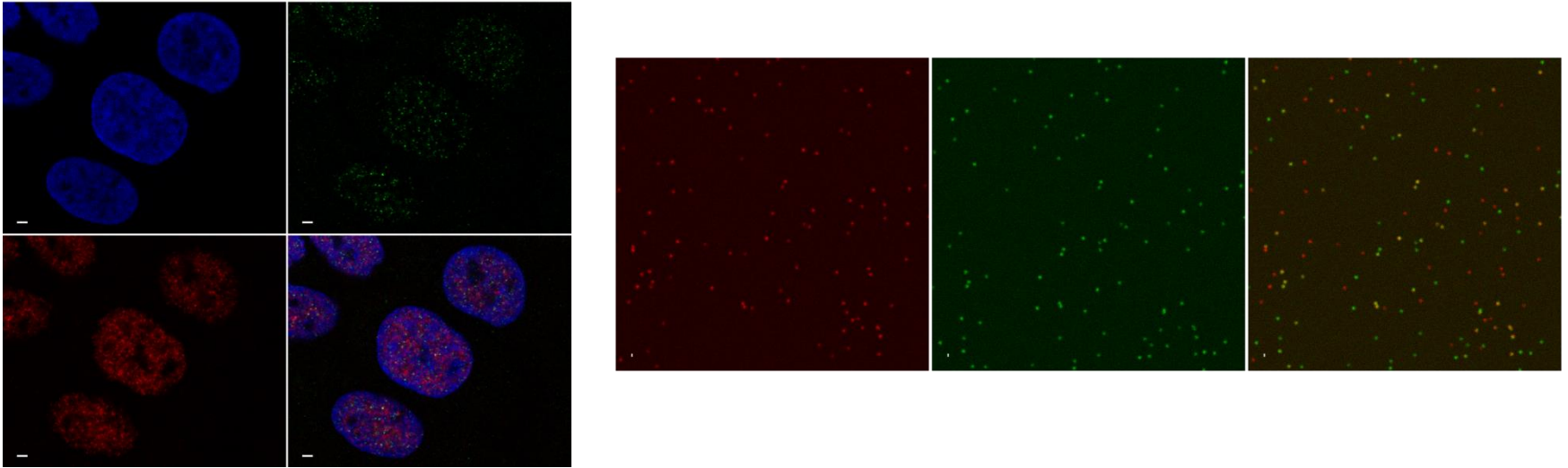


Video from <https://imagej.net/plugins/trackmate/>

Section 5: Pixel Based Colocalization Analysis



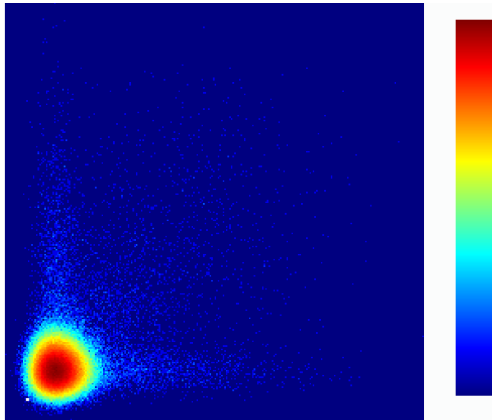
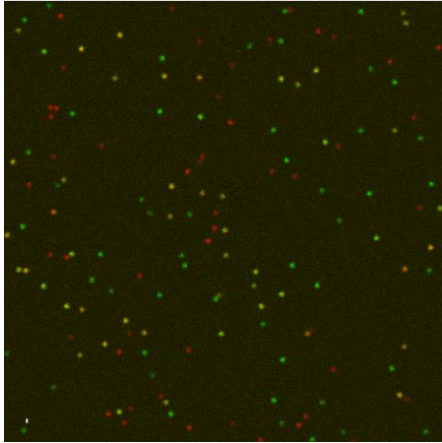
Visualising Colocalization with Colour Overlays



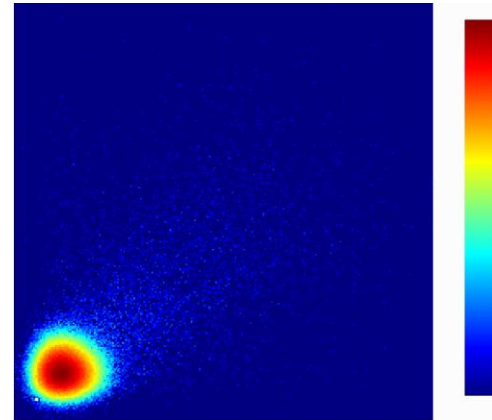
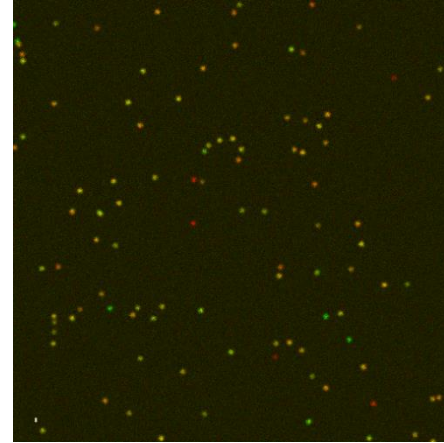
- Not very useful and cannot make any convincing conclusions...
- Visual interpretation is very sensitive to changes in display settings
- Some LUTs are better than others

Visualising Colocalization with Joint-Histograms

50% Spots Colocalized

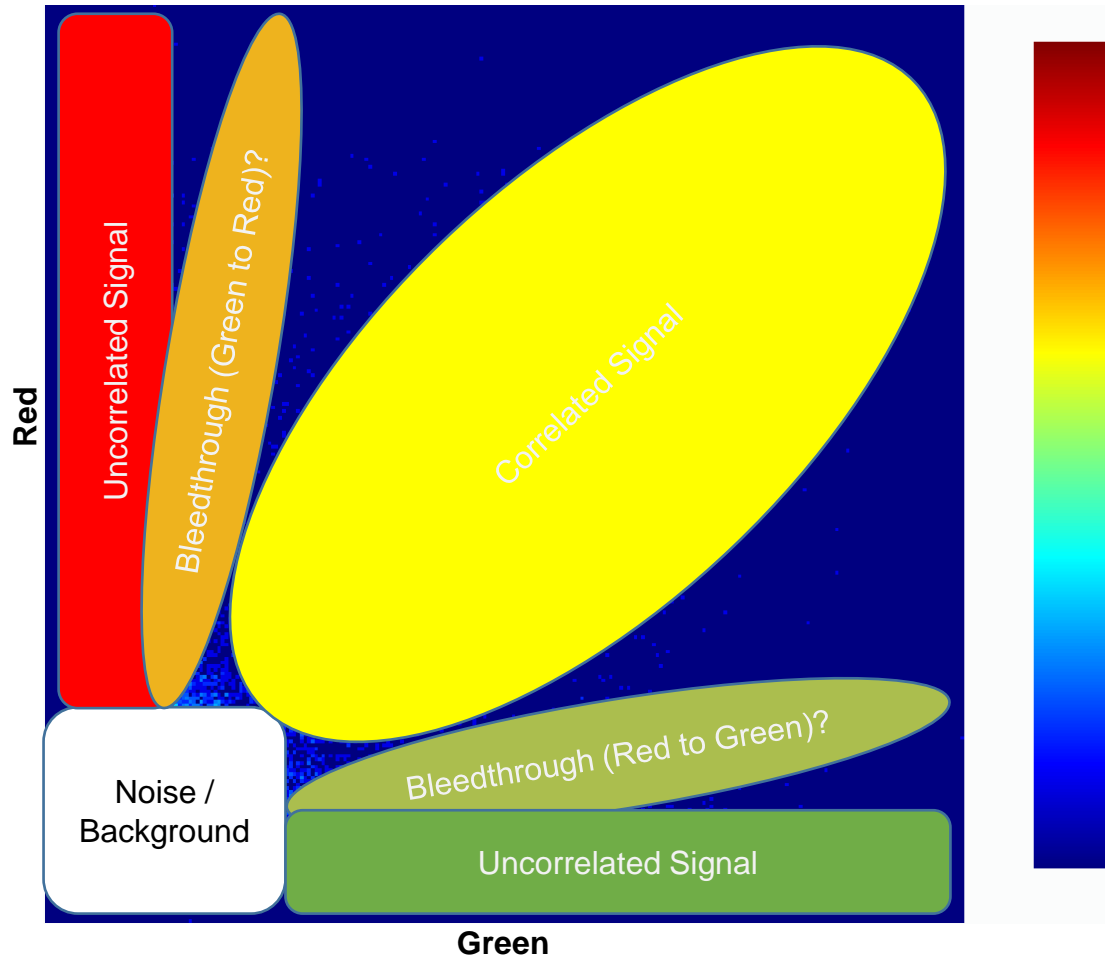


95% Spots Colocalized



- Allows for visual assessment of correlation
- Better than colour overlays but not a replacement for robust quantitative analysis

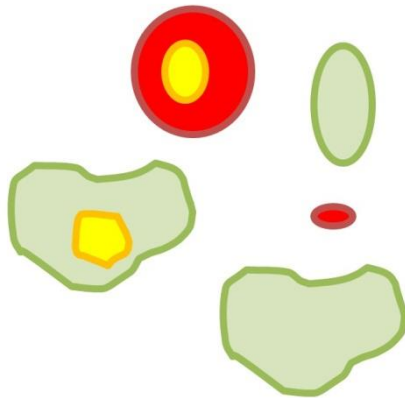
Visualising Colocalization with Joint-Histograms



Quantifying Colocalization

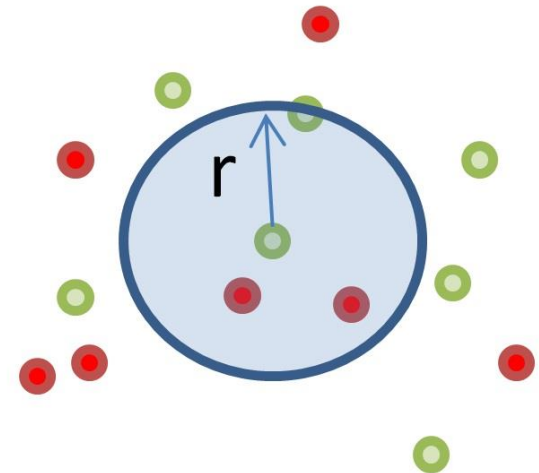
There are two approaches:

Pixel Based



Measures overlap and correlation of signal across individual pixels

Object Based



Spatial analysis using the center of mass (COM) of each detected object

Quantifying Colocalization: Pixel Based

Two types of measures:

Correlation

The Pearson coefficient:

$$R = \frac{\sum_i (C1_i - C1_{av}) \times (C2_i - C2_{av})}{\sqrt{\sum_i (C1_i - C1_{av})^2 \times \sum_i (C2_i - C2_{av})^2}}$$

Co-occurrence

The Manders coefficients:

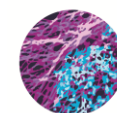
$$M1 = \sum_i \frac{C1_{i,coloc}}{C1_i} \quad M2 = \sum_i \frac{C2_{i,coloc}}{C2_i}$$

- Together the Pearson and Manders coefficients measure and distinguish between correlation and co-occurrence
- For example signal can have a high level of co-occurrence but be weakly correlated
- There are many other pixel based colocalization measures but why?

Adler, Jeremy, and Ingela Parmryd. "Quantifying colocalization by correlation: the Pearson correlation coefficient is superior to the Mander's overlap coefficient." Cytometry Part A 77.8 (2010): 733-742.

Image Acquisition and Pre-processing

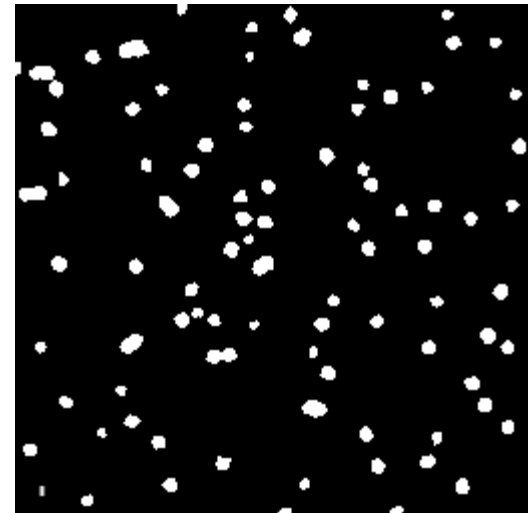
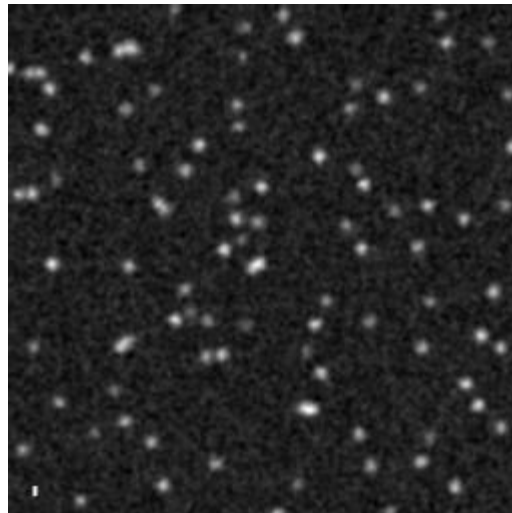
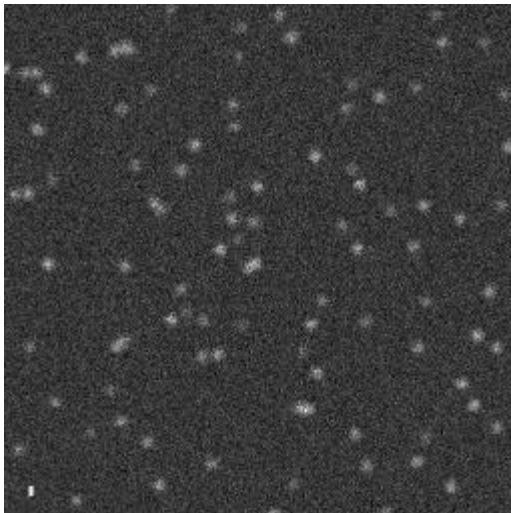
- Care should be taken to avoid cross-talk and bleed-through. Use of single labelled controls is a good idea!
- Watch out for chromatic aberrations
- Pre-processing is important and should not be ignored in colocalization analysis
- Application specific deconvolution, denoising and/or background subtraction steps should be used



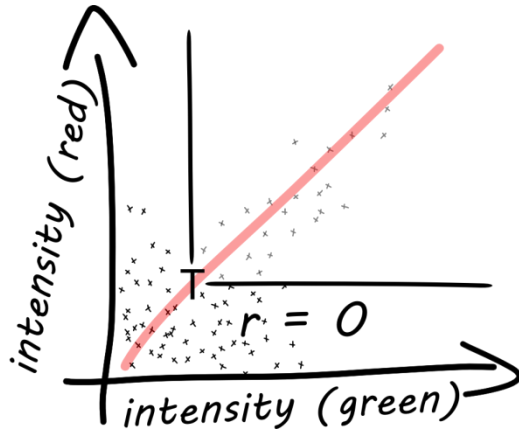
Signal Isolation

- Essential for calculation of the Manders coefficients and best practice for the Pearson coefficient.
- Aim is to segment the regions in both channels containing biologically relevant signal.
- Needs to be automated!
- There is no “one size fits all” strategy. Need to develop an approach that works reliably for your data.

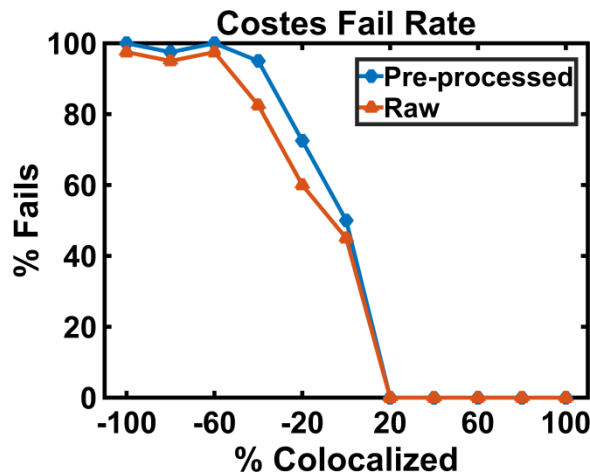
Raw → Gaussian Filter → Otsu Threshold



Costes' Thresholding



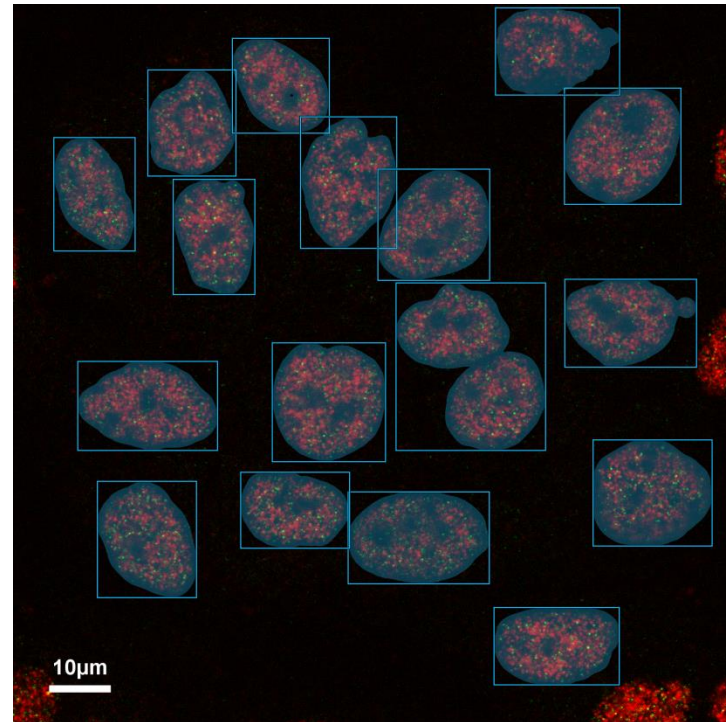
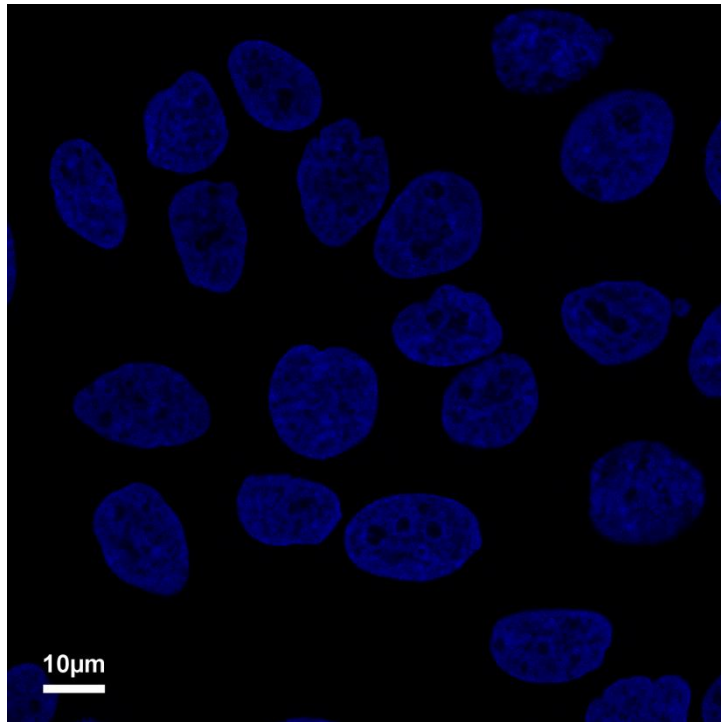
- Finds the point on the line of best fit below which the Pearson coefficient ≤ 0
- Sets threshold values for signal isolation at this point



- Be careful, Costes' thresholding assumes a single linear correlation!
- Why use a test that assumes colocalization to test for colocalization?

Regions of Interest (ROIs)

- Often appropriate to restrict (or perform separate) colocalization analysis using ROIs
- This is typically individual cells or nuclei



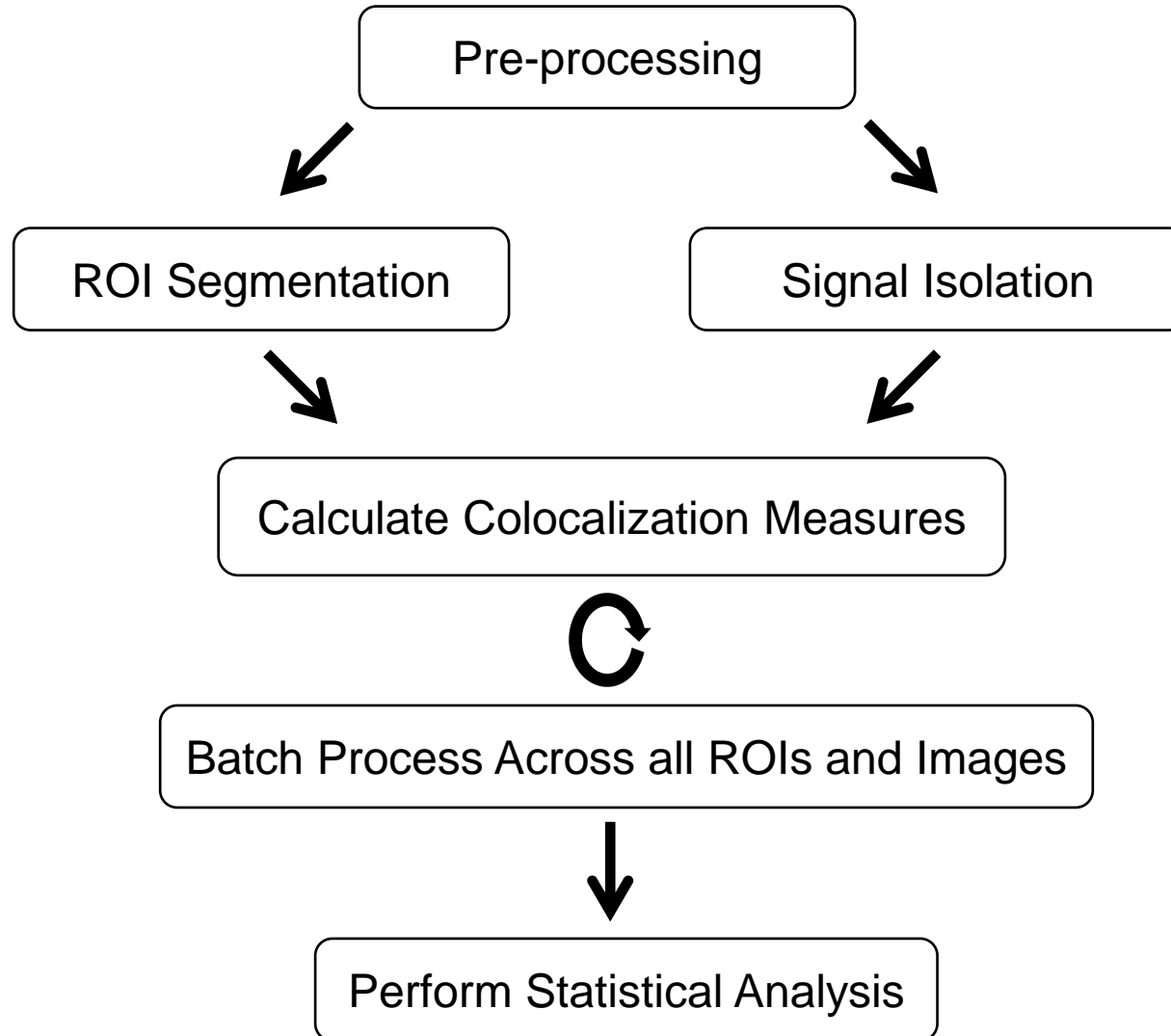
Statistical Testing

- Formulate a null hypotheses. Typically either:
 1. The signal from both channels is randomly distributed within the ROI
 2. There is no difference in the level of colocalization between two populations
- Option 1: Perform a statistical analysis for each ROI using pixel scrambling or simulations methods
 - Hard to completely remove auto-correlation effects
 - Individual ROIs are typically not very relevant, populations are!
- Option 2: Perform standard statistical tests (eg t-tests) to compare colocalization across populations.

Hypothesis 1: Subtract the expected value from each ROI measurement and compare the population to zero

Hypothesis 2: Compare distributions between replicates using two-sample statistical tests

Putting It All Together: A Colocalization Workflow



Acknowledgments

This course was organised and run by the Centre of Membrane Proteins and Receptors (COMPARE), a partnership between the Universities of Birmingham and Nottingham.

<http://www.birmingham-nottingham.ac.uk/compare/>

The material was adapted from a course original run at the University of Cambridge:

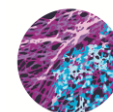
Cancer Research UK Cambridge Institute:

Mark Dunning

Stefanie Reichelt

IN PARTNERSHIP:

The Universities of Birmingham and Nottingham



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