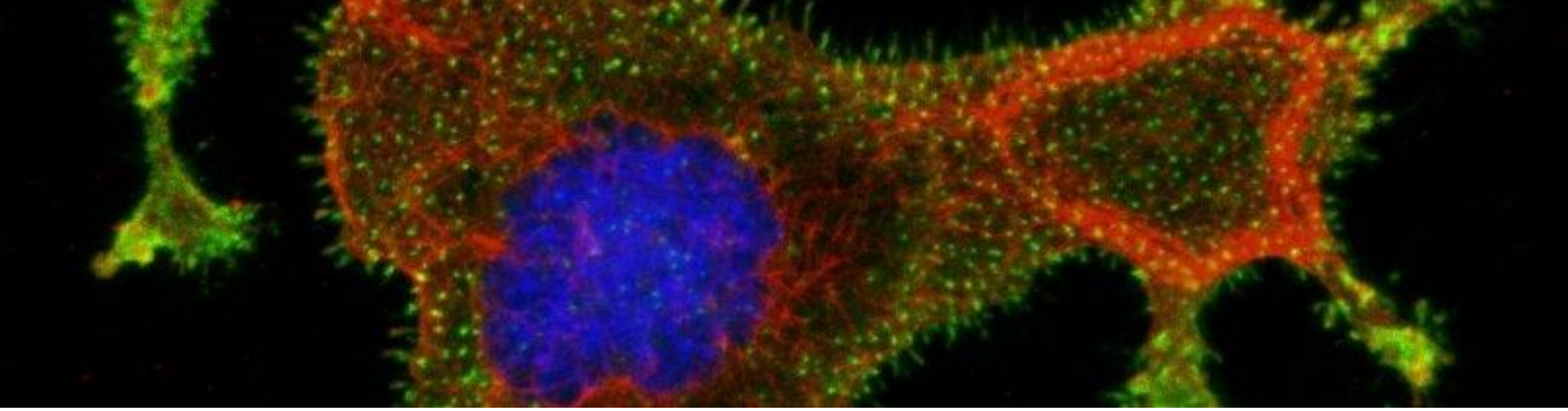


Intermediate Image Analysis

Jeremy Pike

Image analyst for COMPARE

Course website:
https://jeremypike.github.io/intermediate-image-analysis/

IN PARTNERSHIP:
The Universities of Birmingham and Nottingham

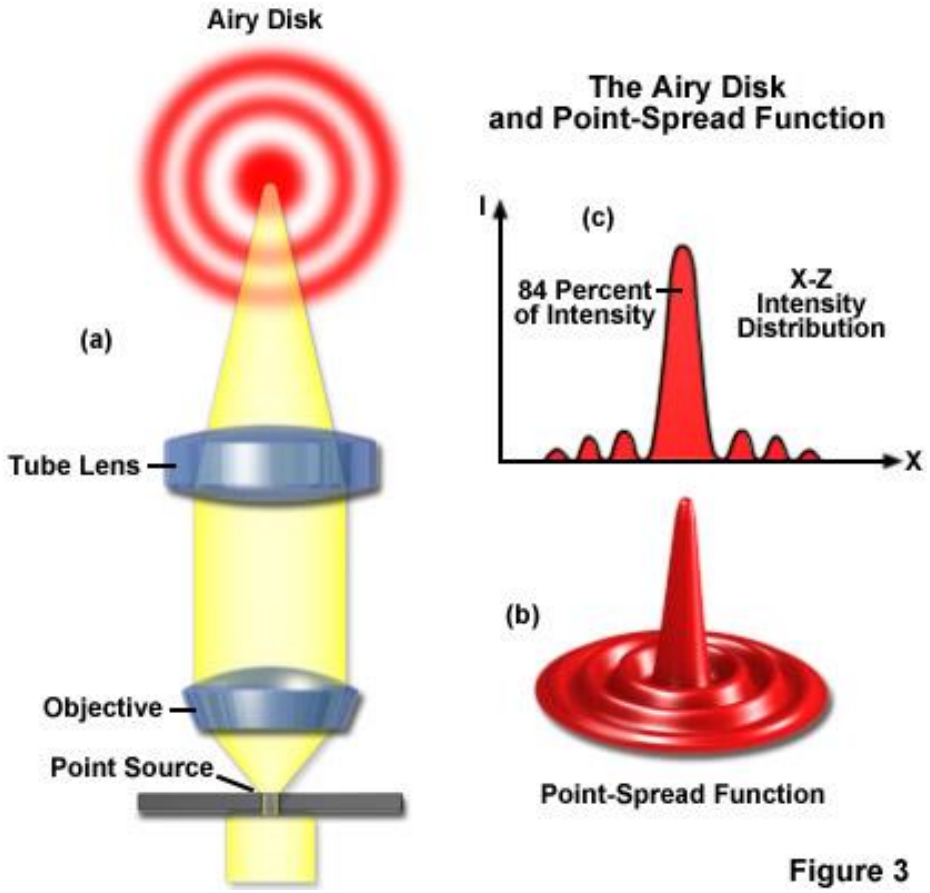
COMPARE
CENTRE OF MEMBRANE PROTEINS AND RECEPTORS

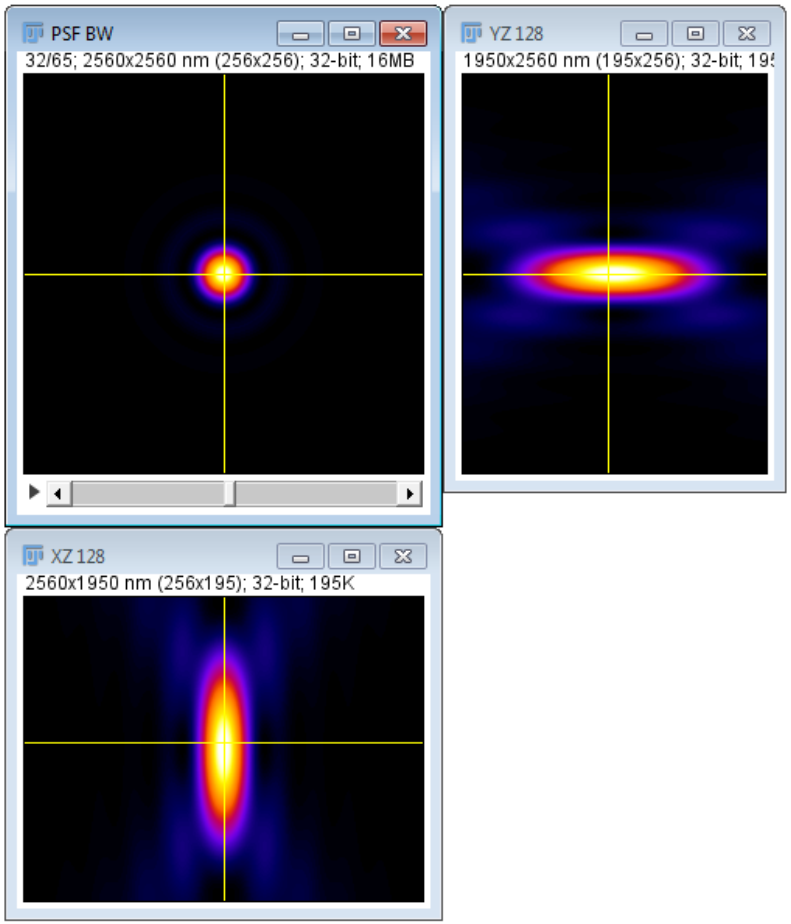
A brief tour of selected image processing and analysis tools

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

Section 1: Deconvolution

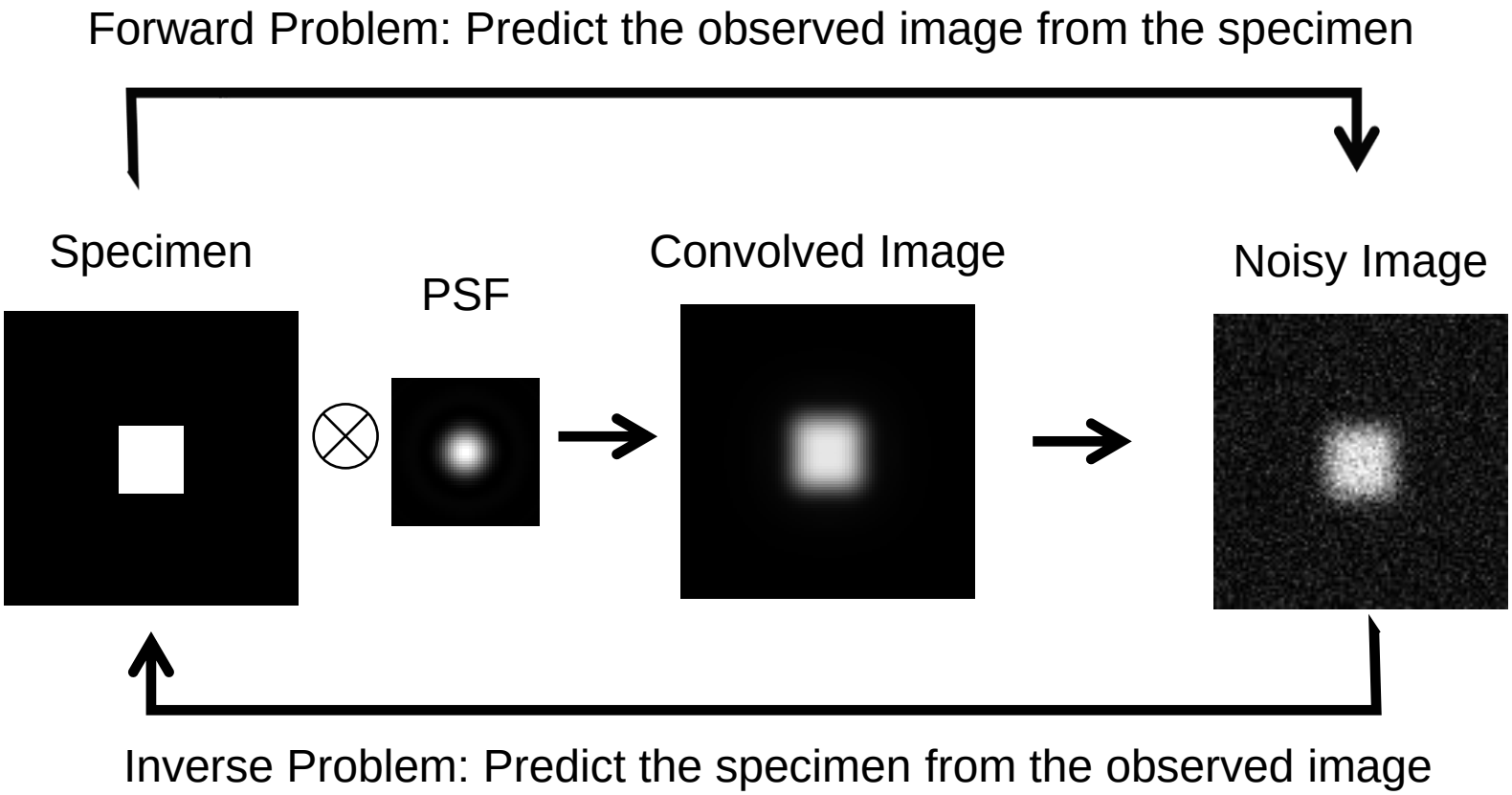
The Point-Spread Function (PSF)





http://zeiss-campus.magnet.fsu.edu/articles/basics/resolution.html

The Forward and Inverse problem in Fluorescence Microscopy

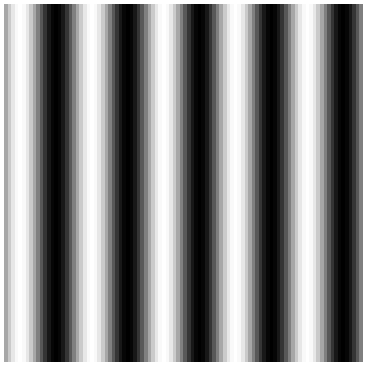


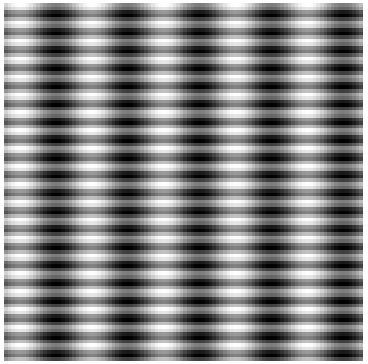
Sbalzarini, Ivo F. "Seeing Is Believing: Quantifying Is Convincing: Computational Image Analysis in Biology." Focus on Bio-Image Informatics. Springer International Publishing, 2016. 1-39.

The Fourier Transform

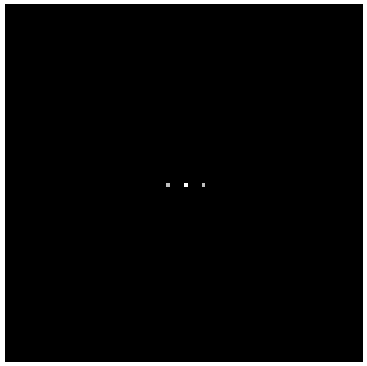
- An arbitrary signal can be expressed as the sum of sine waves
  http://www.falstad.com/fourier/
- Each sine wave has a amplitude and phase
- For images a 2D discrete transform is used

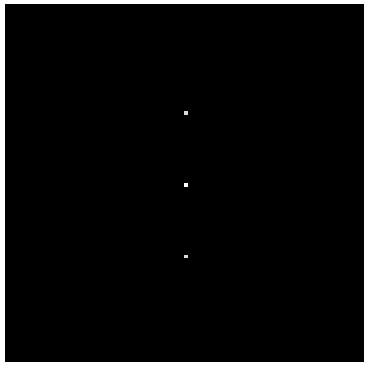
Image

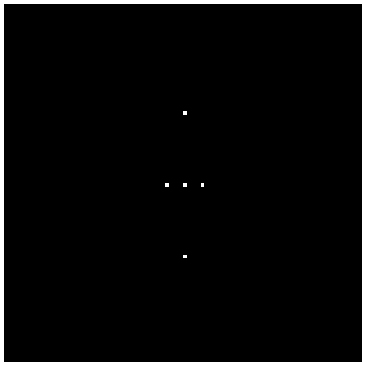




Fourier Transform







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:

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

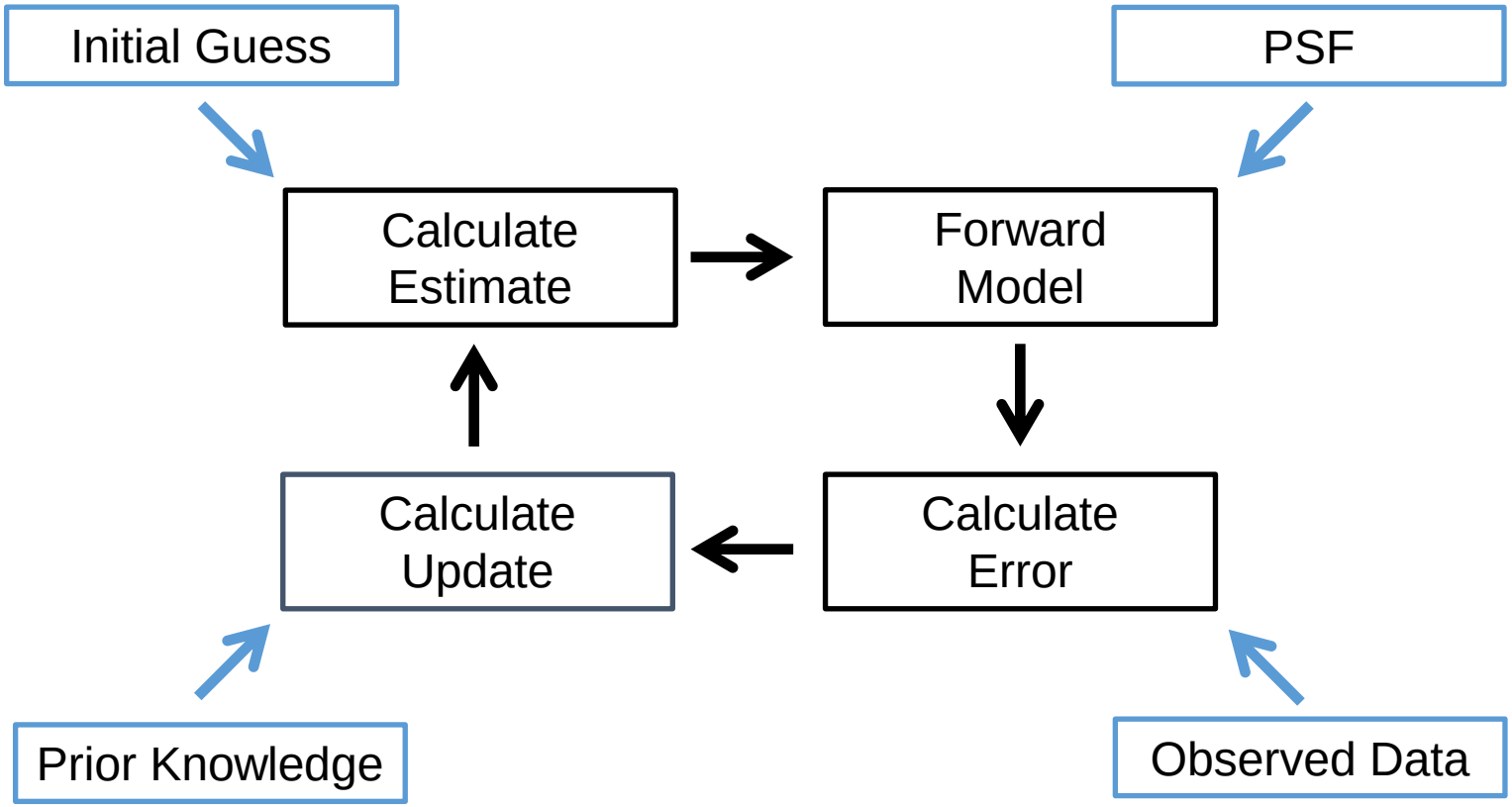
- But what about where $FT(PSF) \approx 0$?

- 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.

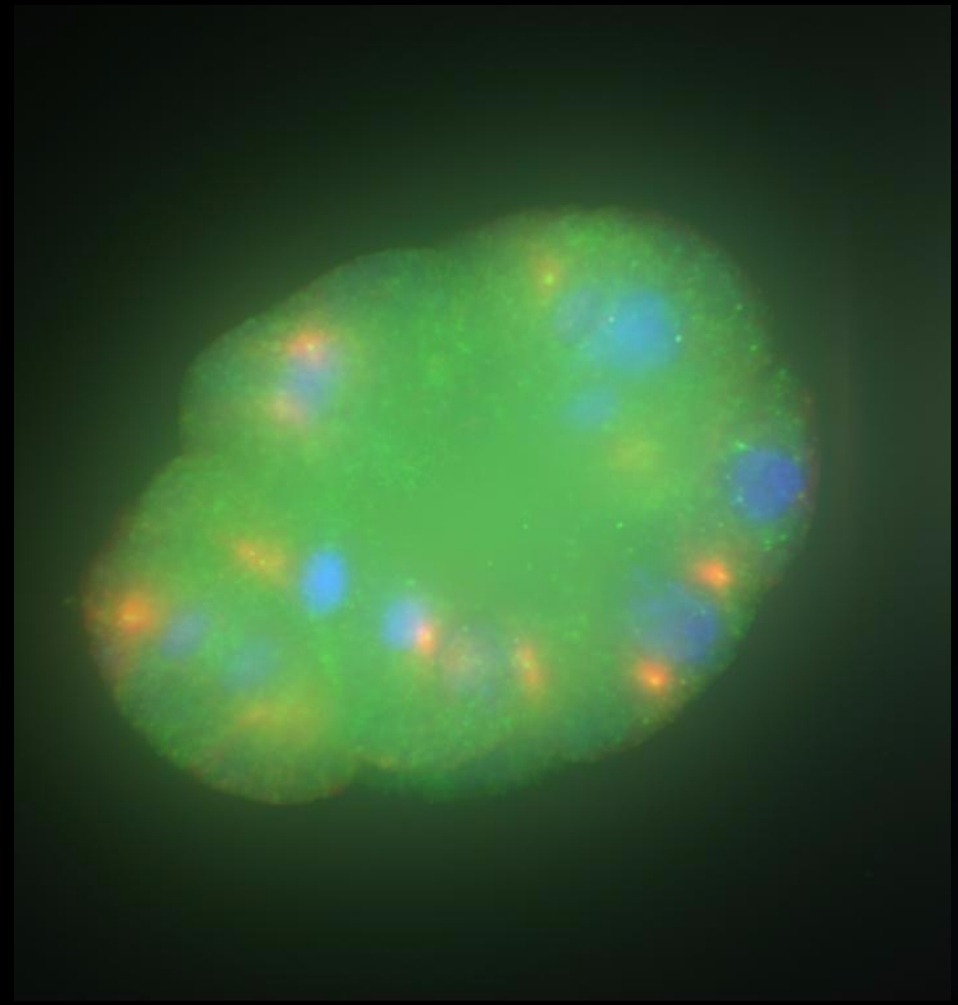
A. Griffa, N. Garin and D. Sage, Comparison of Deconvolution Software in 3D Microscopy. A User Point of View, Part I and Part II, G.I.T. Imaging & Microscopy, vol 1, pp. 43-45, 2010.

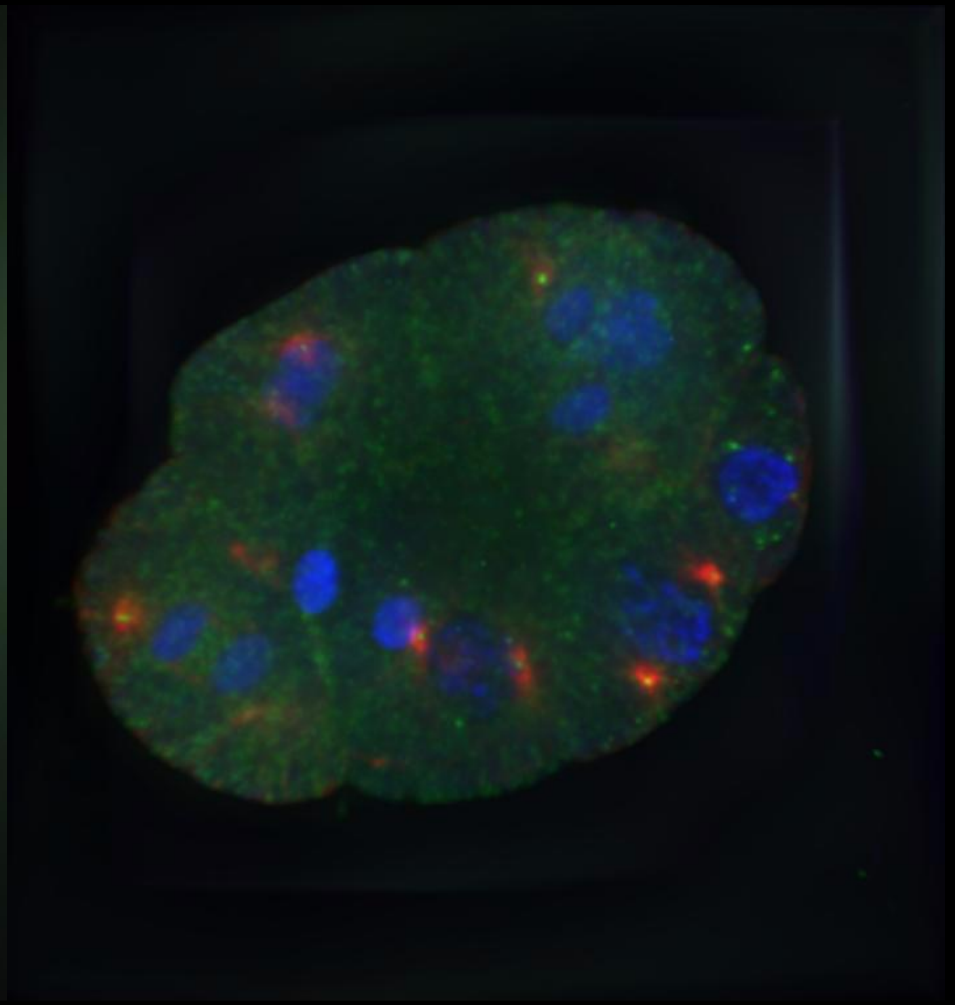
Huygens (Scientific Volume Imaging)

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

https://svi.nl/HuygensProfessional/

Richardson-Lucy Deconvolution using DeconvolutionLab

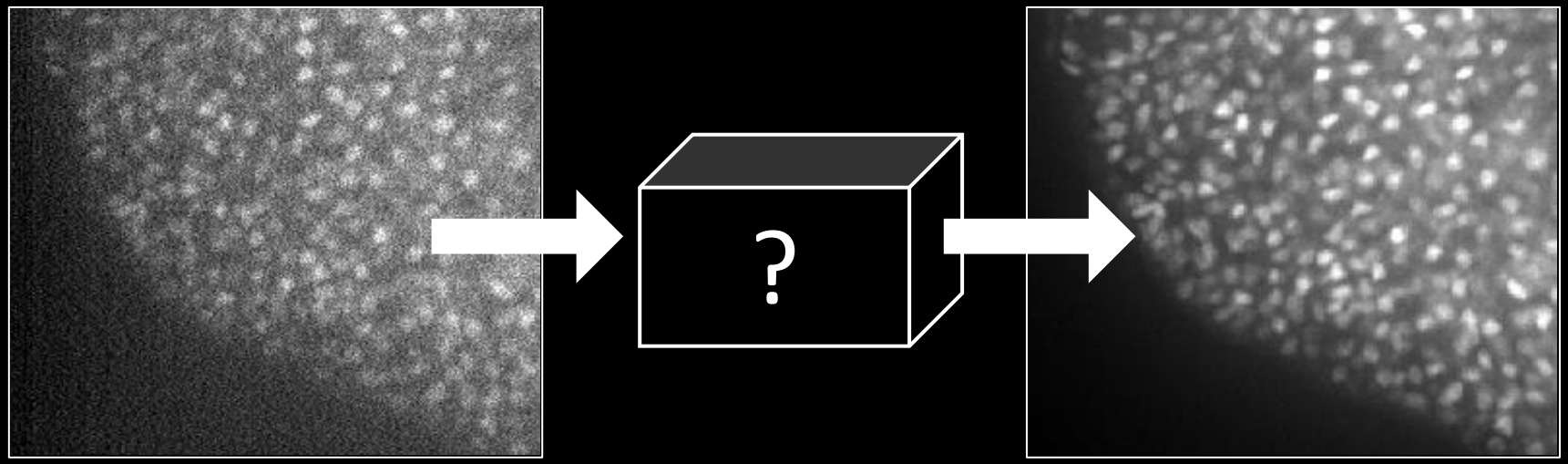




Section 3: Deep learning-based denoising

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

The Problem of Noise



Low exposure:

- Gentle ☺
- Noisy ☹

High exposure:

- Damaging ☹
- Clean ☺

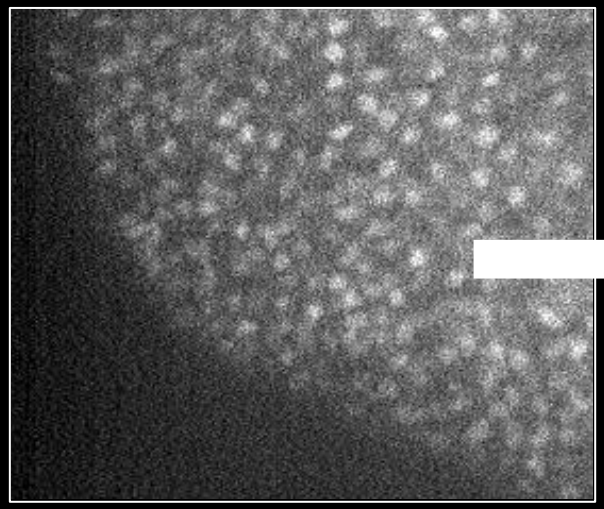
Slide by Alexander Krull

Traditional Supervised Training

You need clean data.

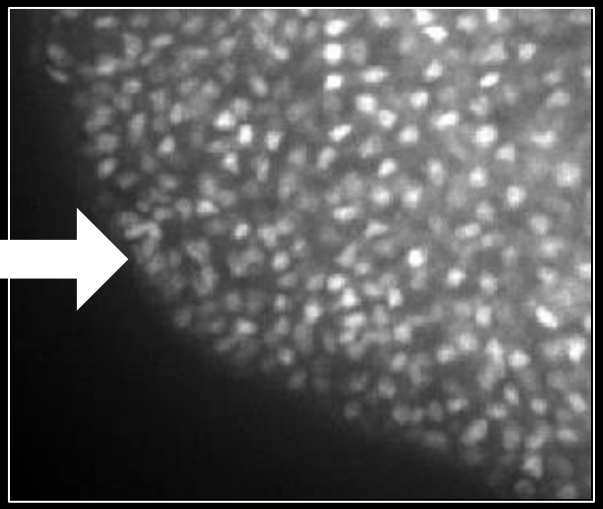
Slide by Alexander Krull

Deep Learning for Denoising



Convolutional Neural Network (CNN)

- 100k – 1M parameters
- Have to be trained



Low exposure:

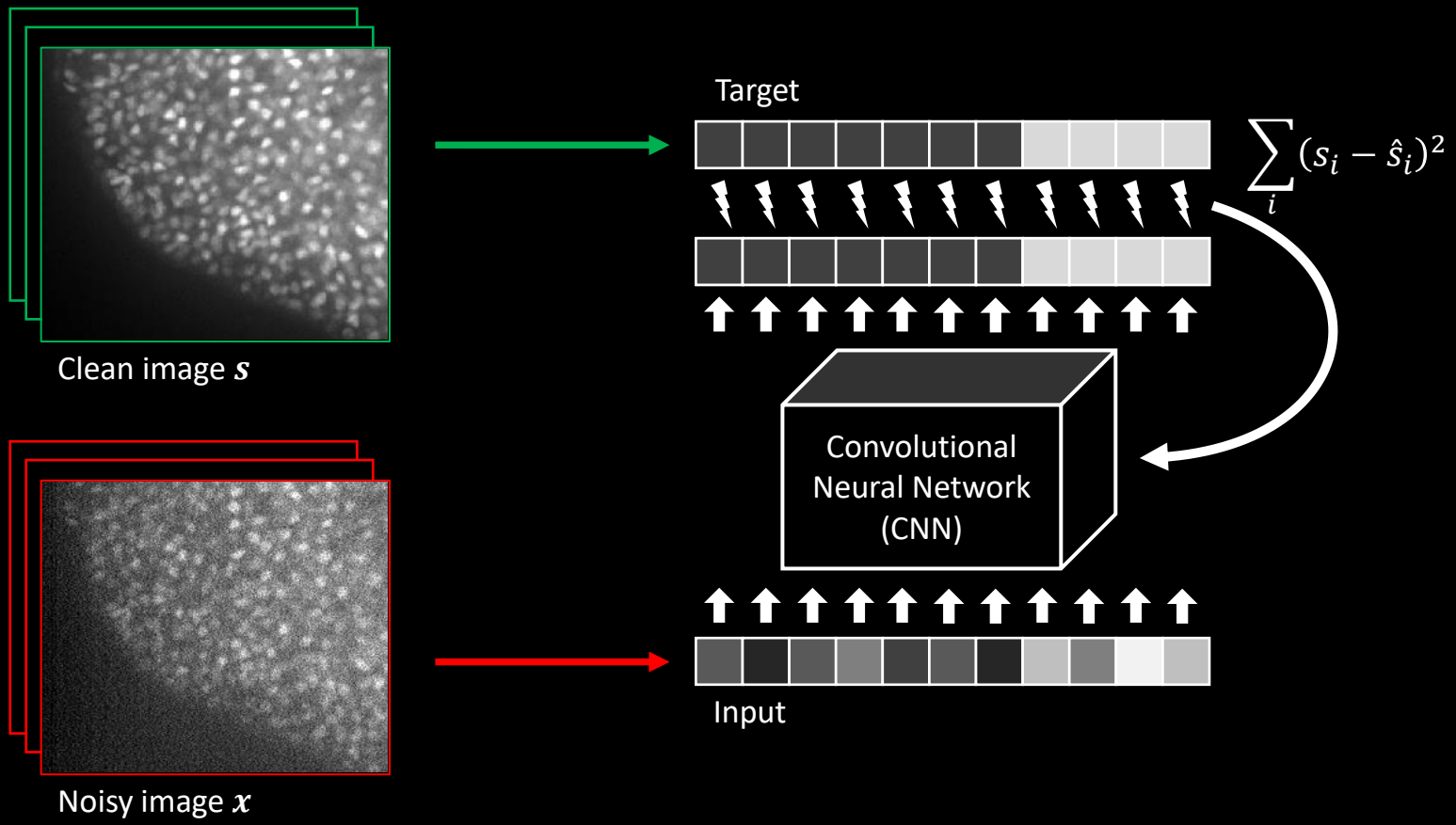
- Low photo toxicity ☺
- Low bleaching ☺
- Noisy ☹

High exposure:

- Strong photo toxicity ☹
- Strong bleaching ☹
- Less noise ☺

Slide by Alexander Krull

CARE – Traditional Supervised Training



Slide by Alexander Krull

Weigert, M., Schmidt, U., Boothe, T. et al. Content-aware image restoration: pushing the limits of fluorescence microscopy. Nat Methods 15, 1090–1097 (2018). https://doi.org/10.1038/s41592-018-0216-7

Noise2Noise

You only need noisy data!

Lehtinen et al. 2018

Slide by Alexander Krull

Noise2Noise

Noise $\boldsymbol{n} = (\boldsymbol{n}_1, \ldots, \boldsymbol{n}_m)$

=

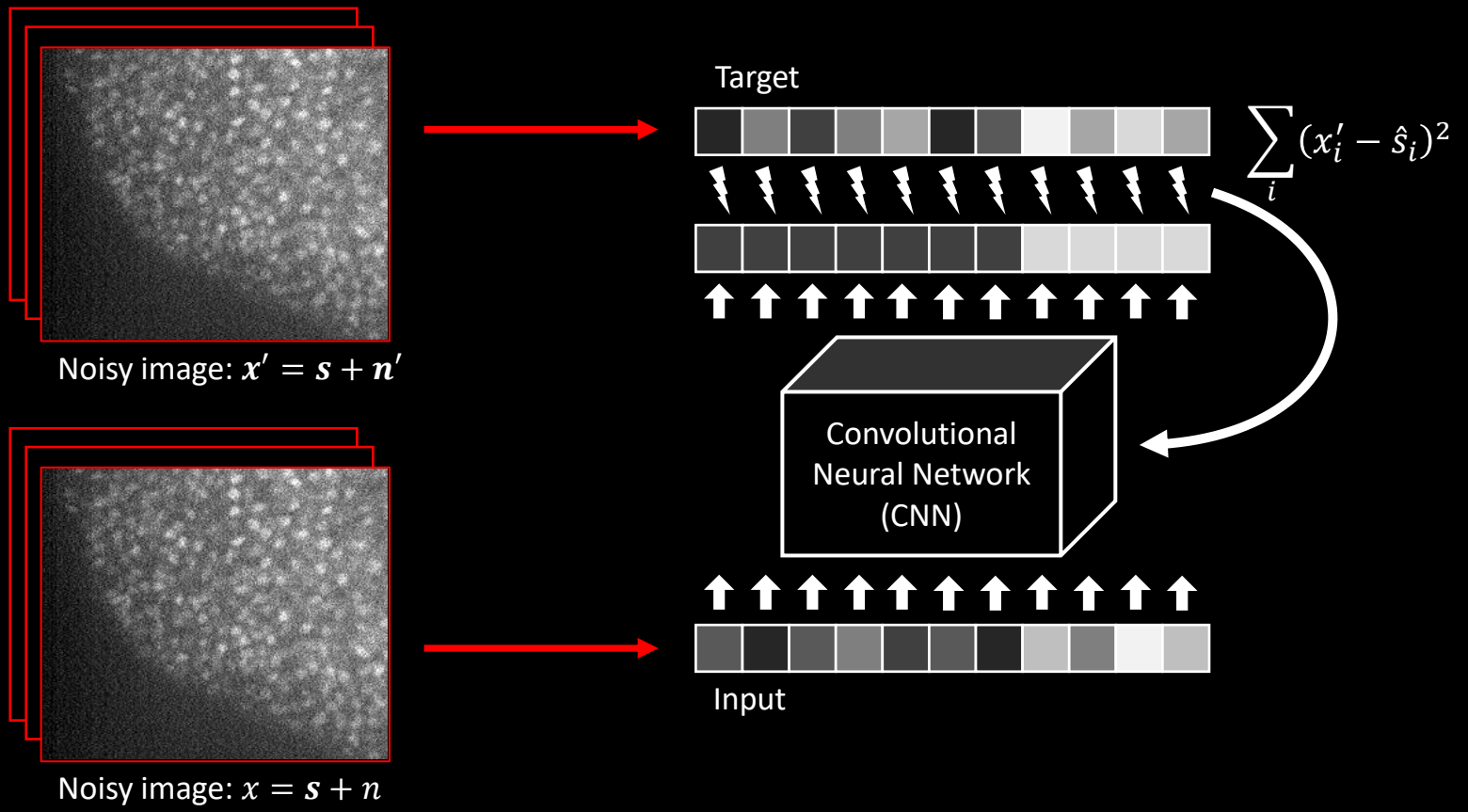
Noisy image $\boldsymbol{x} = (\boldsymbol{x}_1, \ldots, \boldsymbol{x}_m)$

−

Signal $\boldsymbol{s} = (\boldsymbol{s}_1, \ldots, \boldsymbol{s}_m)$

Slide by Alexander Krull

Noise2Noise training



Slide by Alexander Krull

Self-Supervised Denoising: Noise2Void

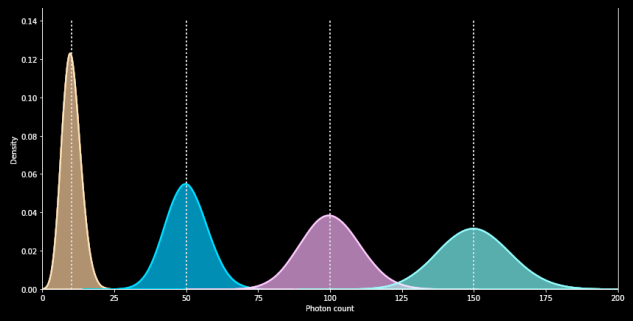
You only need individual noisy images!

Krull, Buchholz, and Jug 2019

Slide by Alexander Krull

Noise2Void – Assumptions

Noisy image ($\boldsymbol{x}$) aka *observation*



=

Signal $\boldsymbol{s} = (\boldsymbol{s}_1, \dots, \boldsymbol{s}_m)$

+

Noise $\boldsymbol{n} = (\boldsymbol{n}_1, \dots, \boldsymbol{n}_m)$

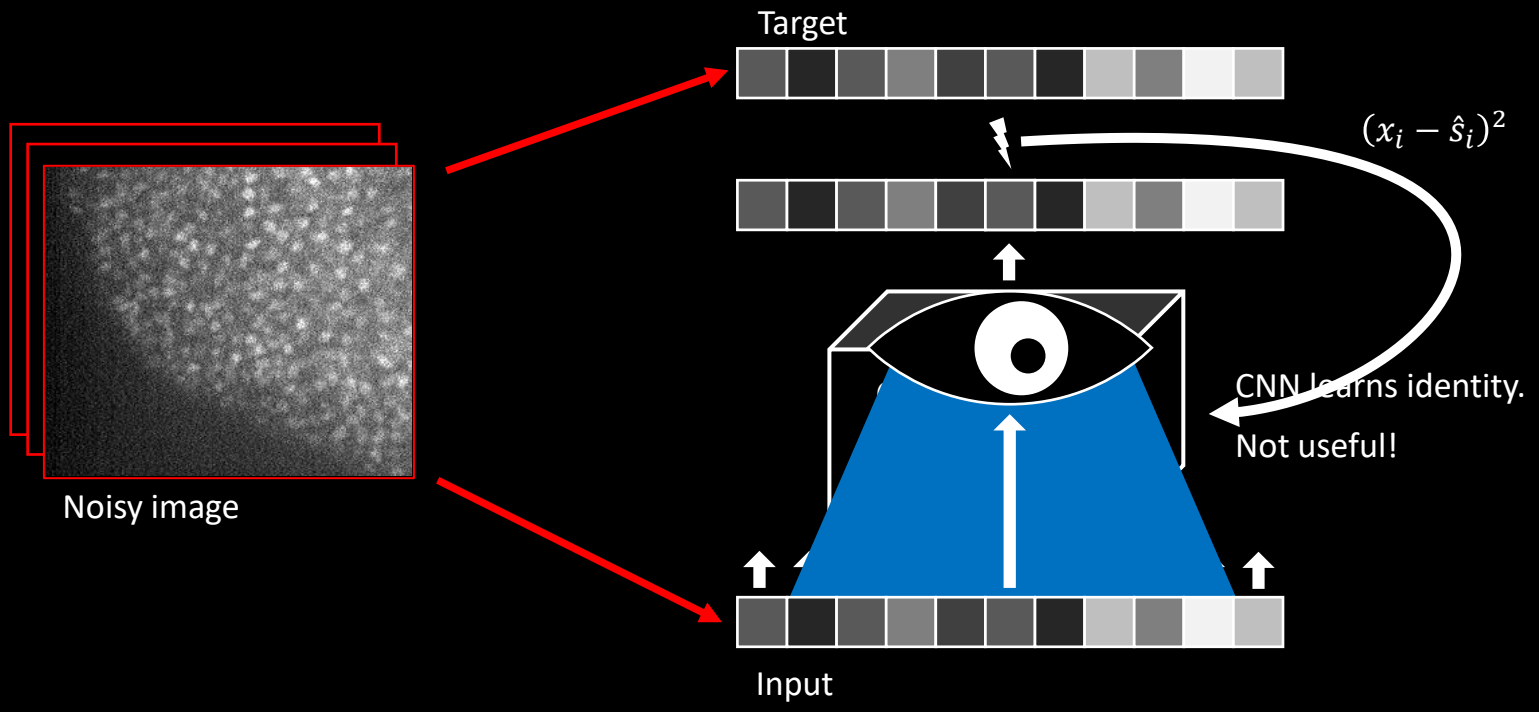
$$p(\boldsymbol{x}|\boldsymbol{s}) = \prod_{\boldsymbol{i}} p(x_i|s_i)$$

$$\mathbb{E}_{p(n_i|s_i)}[n_i] = 0$$

Zero centred noise

Slide by Alexander Krull

Noise2Void

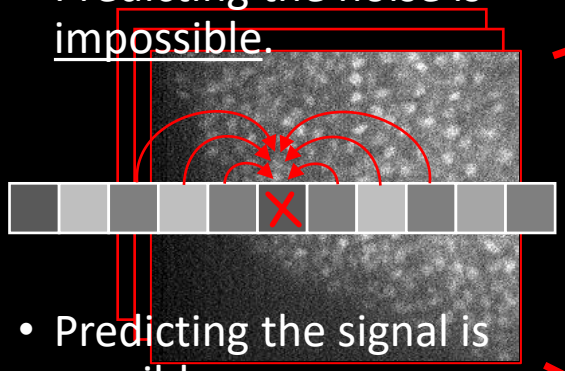


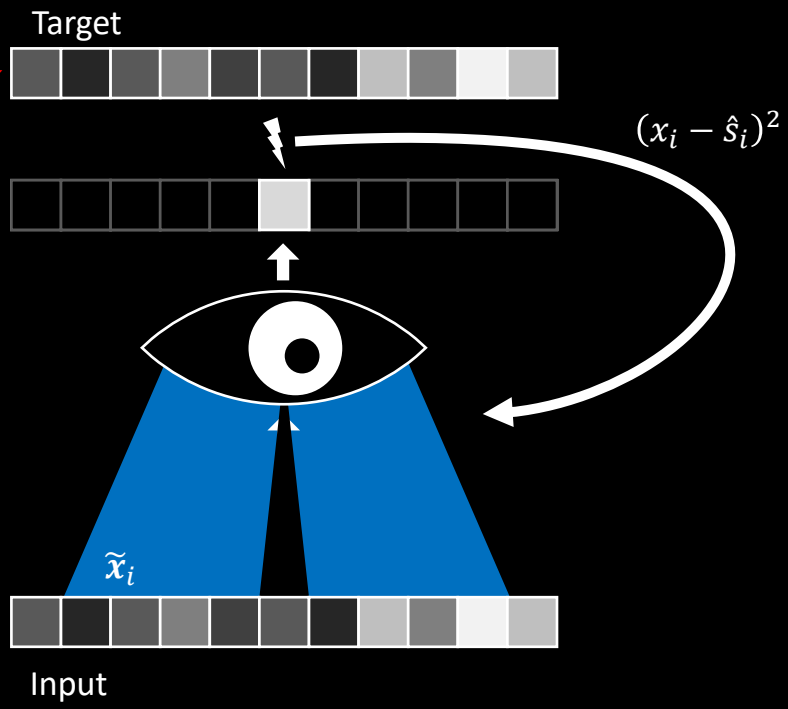
Slide by Alexander Krull

Noise2Void - Blind Spot Network

Why does it work?

- Predicting the noise is impossible.
- Predicting the signal is possible.

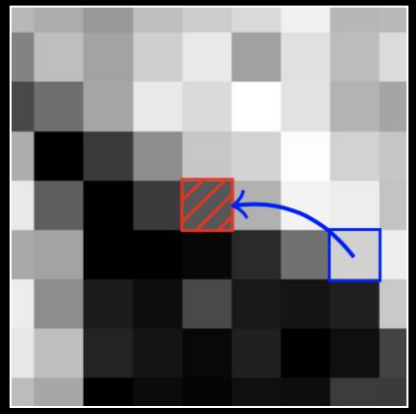




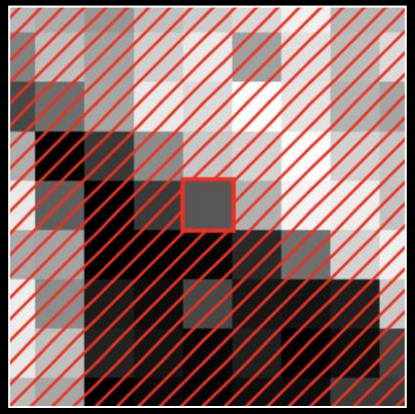
Slide by Alexander Krull

Noise2Void - Blind Spot Implementation

input

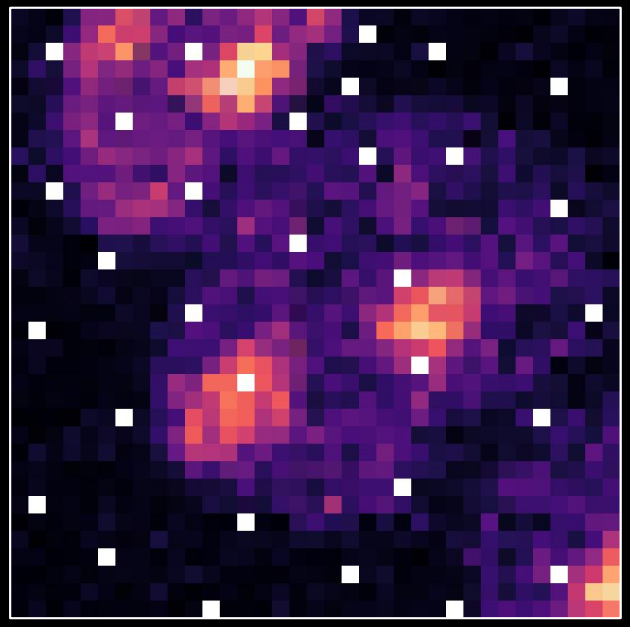


target

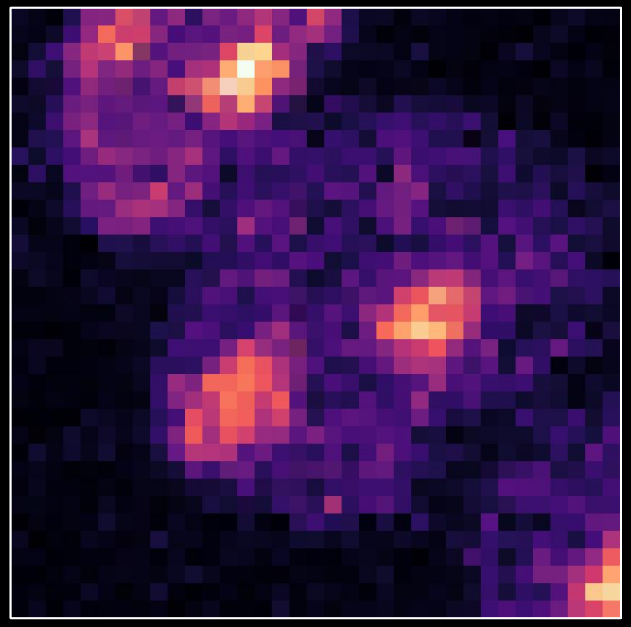


Slide by Alexander Krull

Noise2Void - Blind Spot Implementation



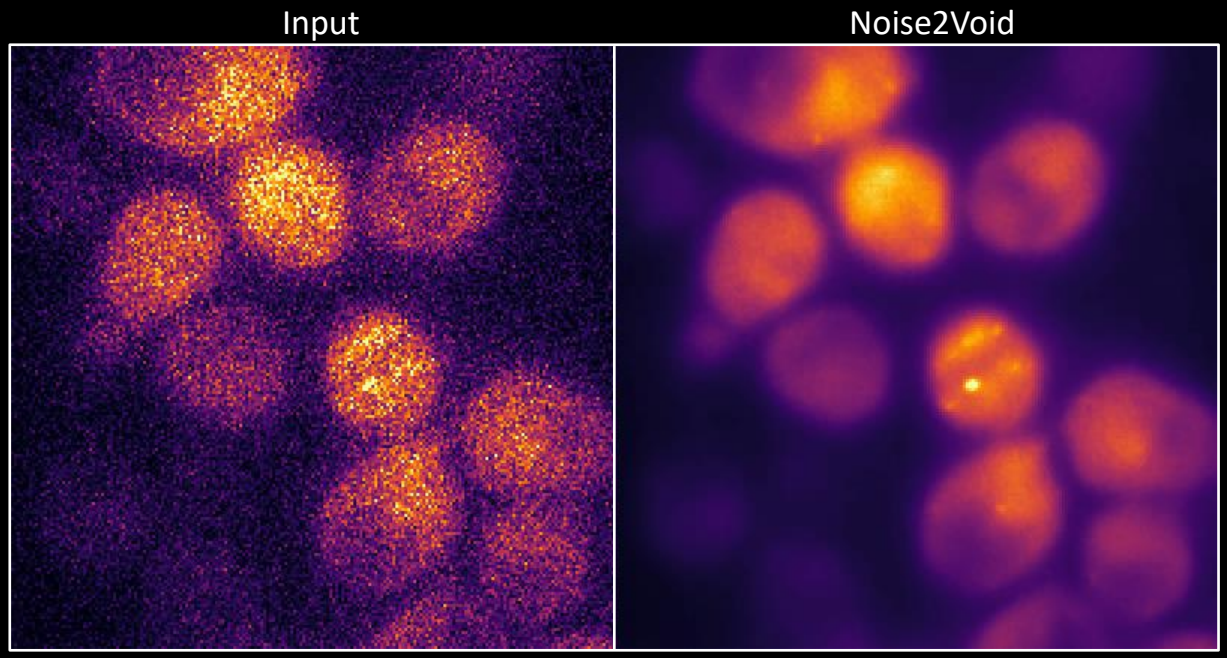
Input



Target

Slide by Alexander Krull

Noise2Void - Results

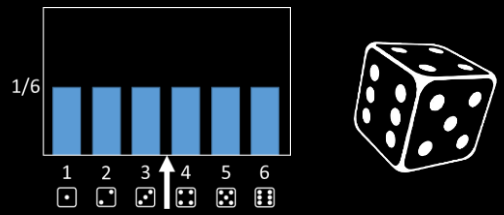


Data by Stephanie Heinrich

Slide by Alexander Krull

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

Slide by Alexander Krull

Section 3: Segmentation

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.

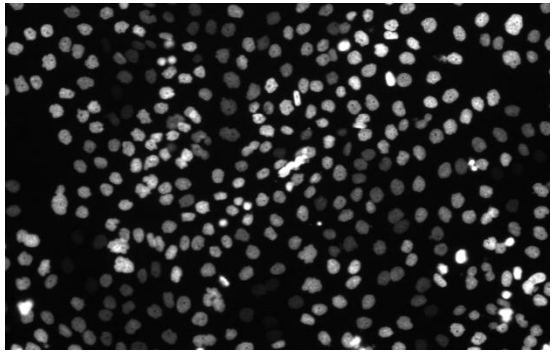
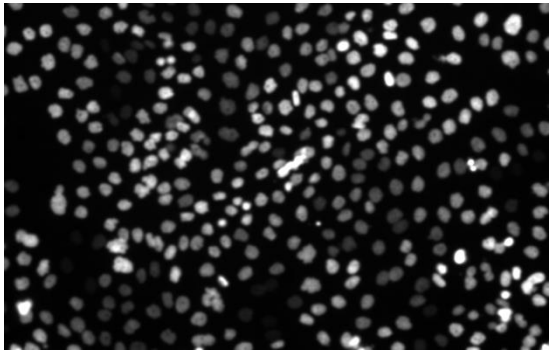
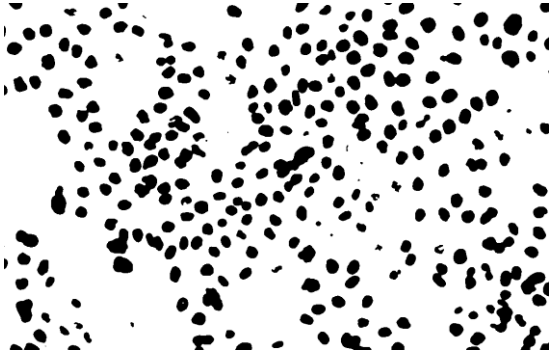




Image Segmentation:
The Model Based Approach

- How well does the segmentation explain the observed data given a specific model.

- Incorporate knowledge about the target(s) and/or the forward problem

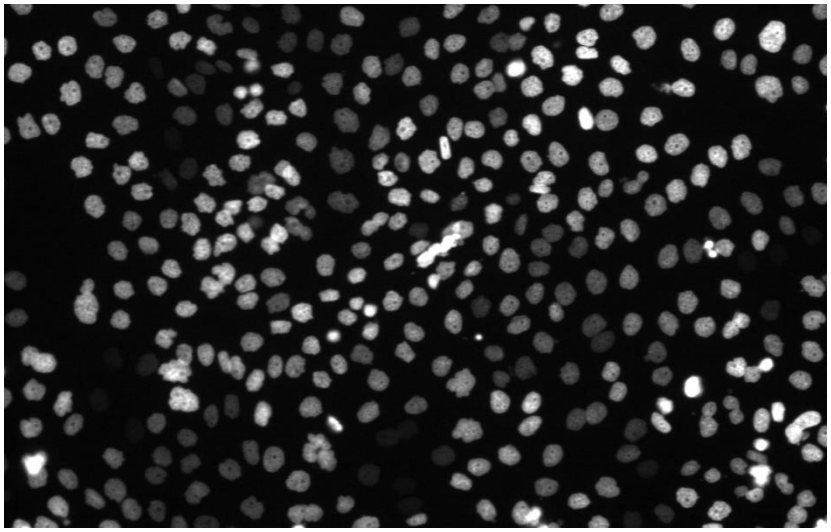
- Iiterately update segmentation until convergence

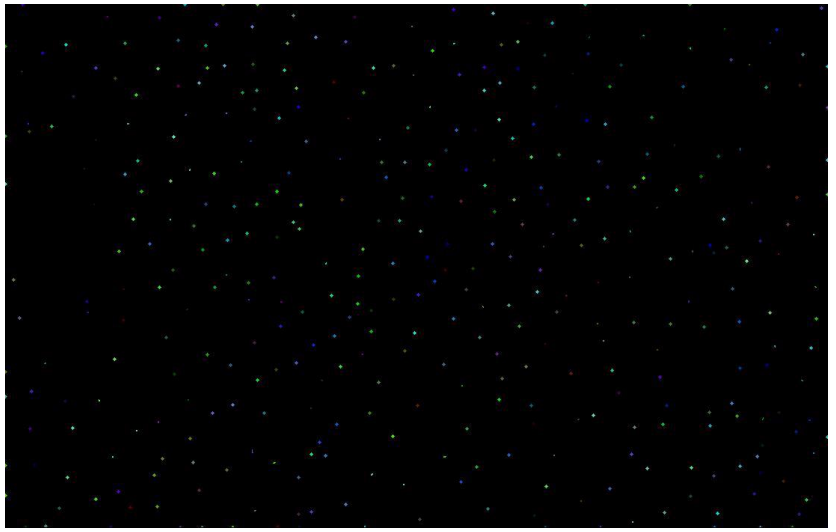
- Pros:
  - Can be flexible. Simply change the data model but not the algoithm!
  - Does not require training
  - Well reasoned

- 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/optimise

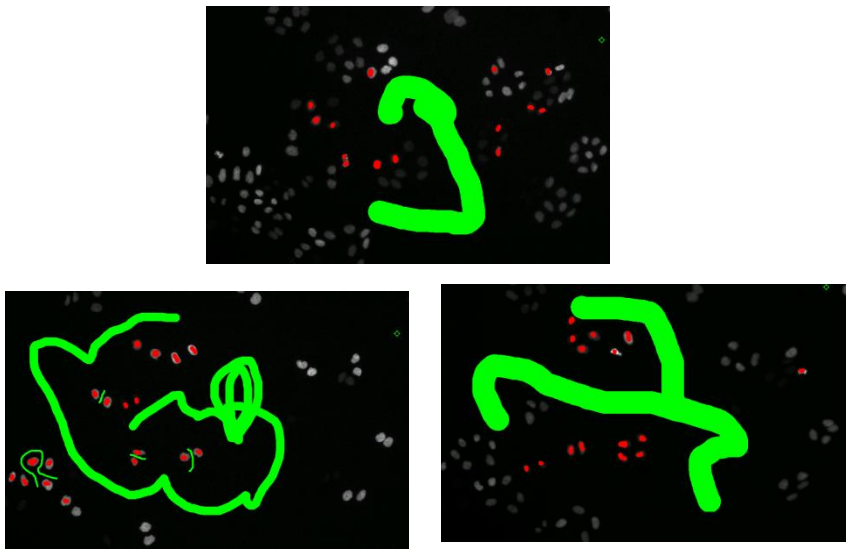
- Cons:
  - Training data and expert manually annotation required. Bias?
  - The classifier can be hard to understand and justify
  - Typically slower than a filter based approach

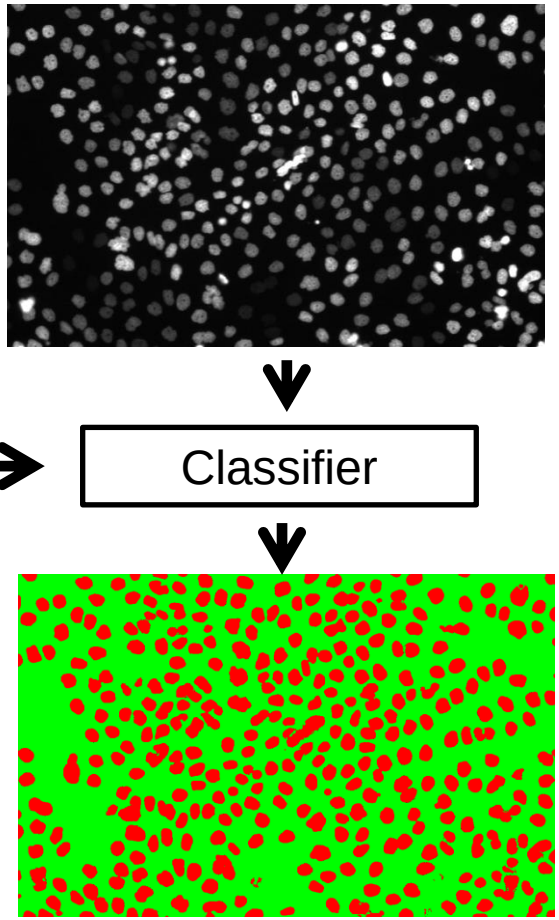
Sommer, Christoph, and Daniel W. Gerlich. "Machine learning in cell biology–teaching computers to recognize phenotypes." J Cell Sci 126.24 (2013): 5529-5539.

The Supervised Machine Learning Approach Example: Pixel Classification with ilastik

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

Training phase



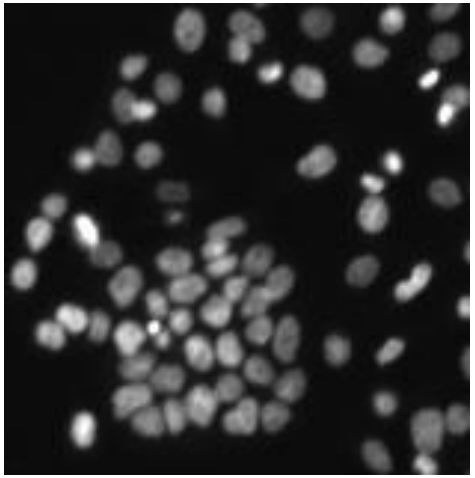


http://ilastik.org/index.html

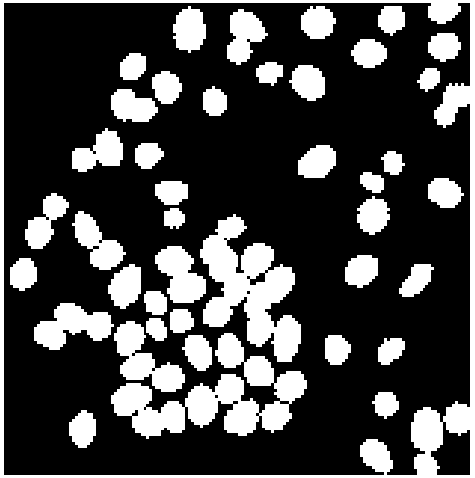
Sommer, Christoph, et al. "Ilastik: Interactive learning and segmentation toolkit." 2011 IEEE international symposium on biomedical imaging: From nano to macro. IEEE, 2011.

Instance segmentation

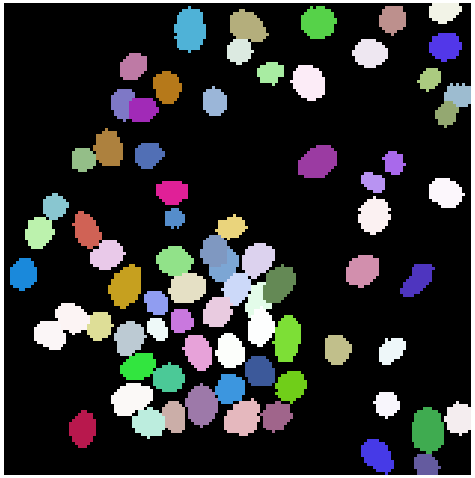
Data



Semantic segmentation
2 or more classes

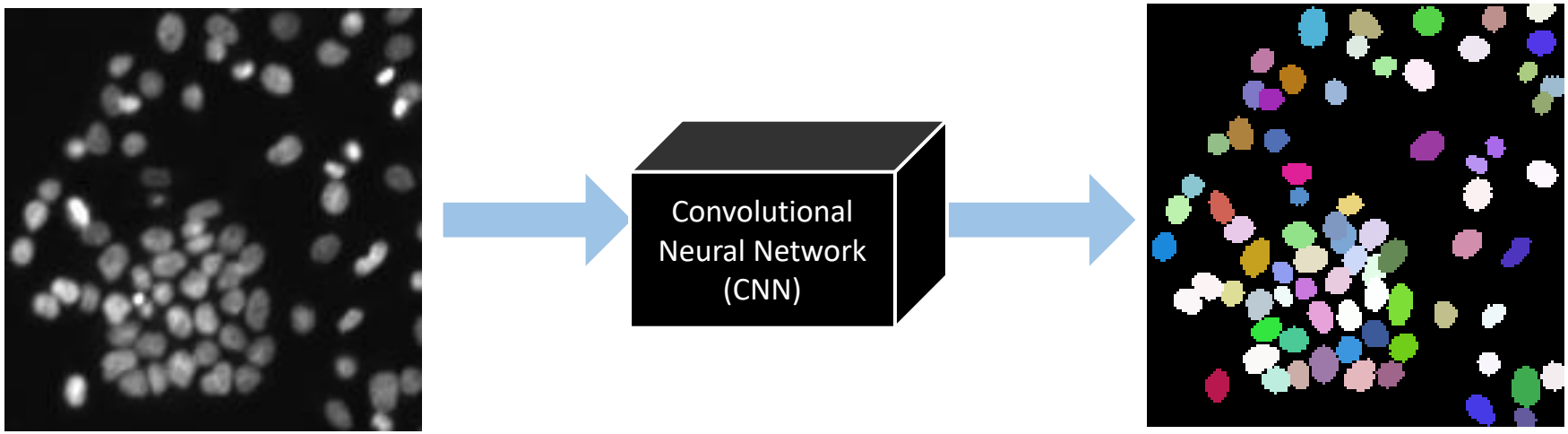


Instance segmentation
Individual objects



- 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



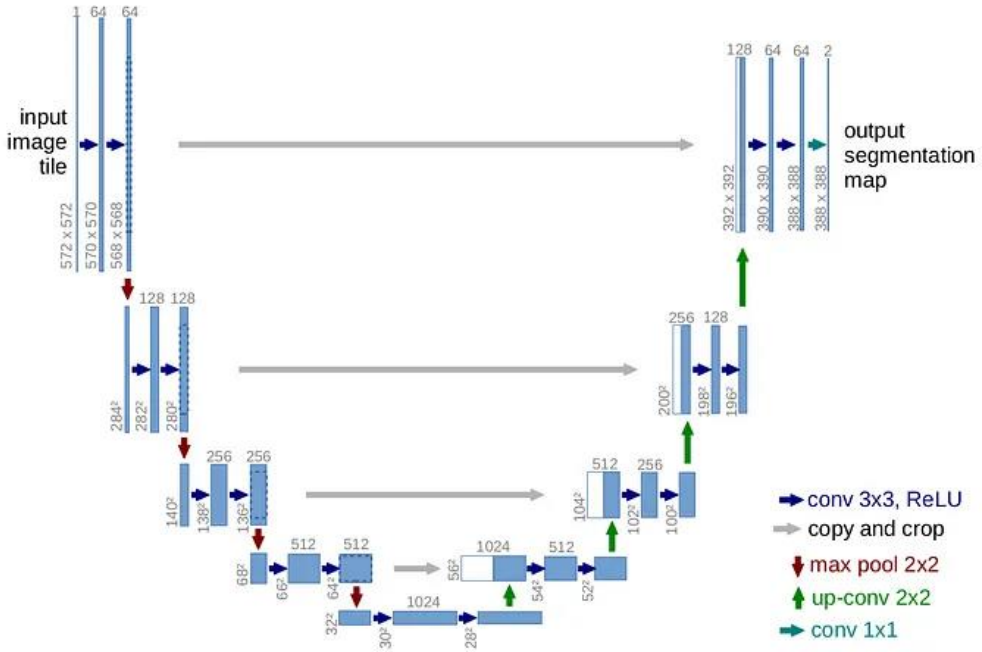
- Requires time consuming manual annotation of training data
- Pre-trained networks can be very useful with optional fine tuning on your own data

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)



Ronneberger, Olaf, Philipp Fischer, and Thomas Brox. "U-net: Convolutional networks for biomedical image segmentation." *Medical Image Computing and Computer-Assisted Intervention–MICCAI 2015: 18th International Conference, Munich, Germany, October 5-9, 2015, Proceedings, Part III 18*. Springer International Publishing, 2015.

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.

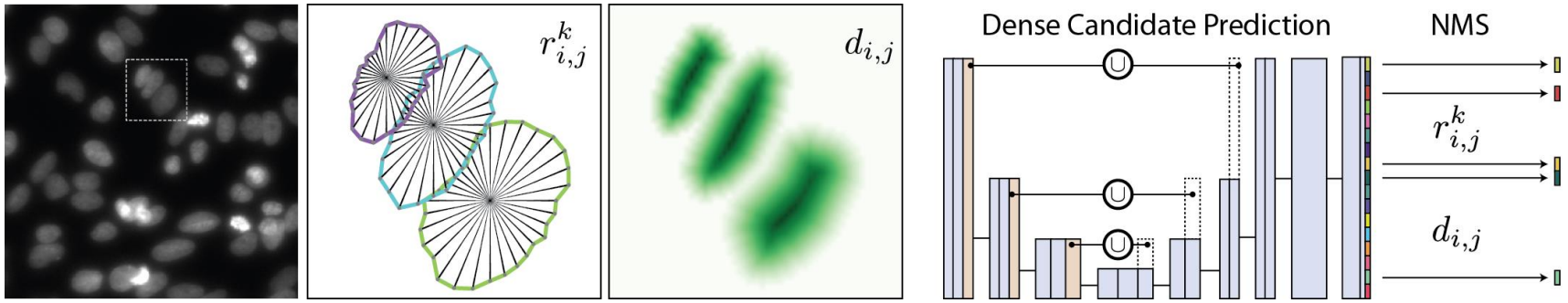


Image from https://github.com/stardist/stardist

- Awesome for nuclei segmentation in 2D and 3D.

- Not good for cell membrane boundaries wich often are not star-convex

- Great Fiji plugin for 2D with pretrained model for fluorescent nuclei and H&E staining

Schmidt, Uwe, et al. "Cell detection with star-convex polygons." *Medical Image Computing and Computer Assisted Intervention–MICCAI 2018: 21st International Conference, Granada, Spain, September 16-20, 2018, Proceedings, Part II 11*. Springer International Publishing, 2018.

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”

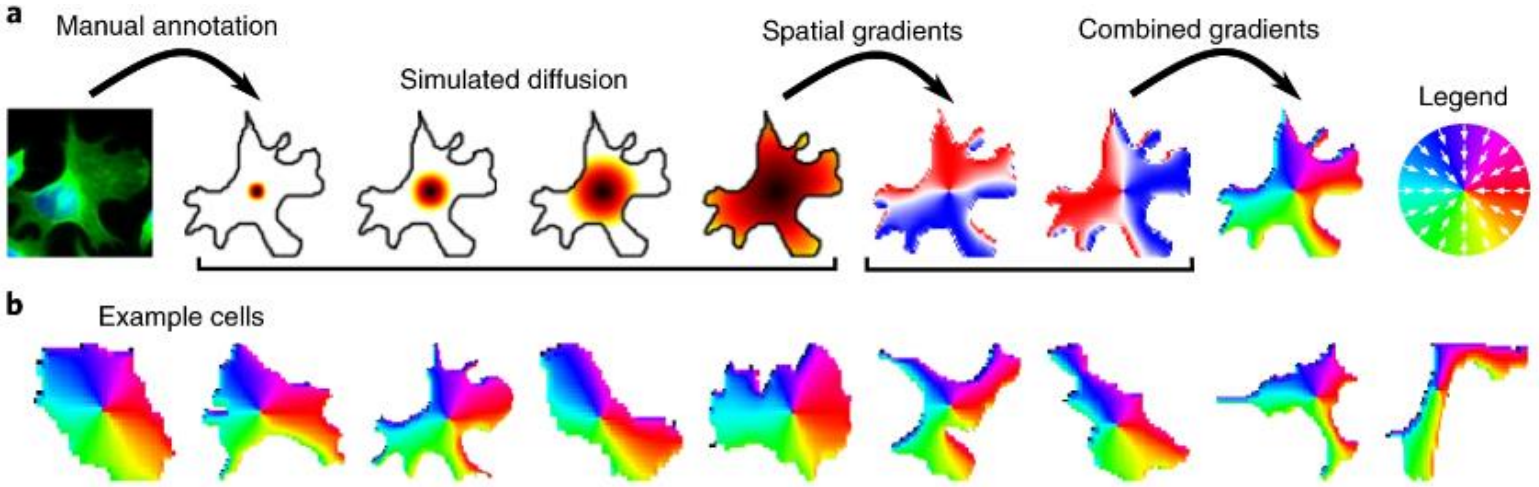


Image from Figure 1 of Stringer et al.

Stringer C, Wang T, Michaelos M, Pachitariu M. Cellpose: a generalist algorithm for cellular segmentation. Nature methods. 2021 Jan;18(1):100-6.

Cellpose
A generalist algorithm for cellular segmentation

- UNET trained with supervised training scheme

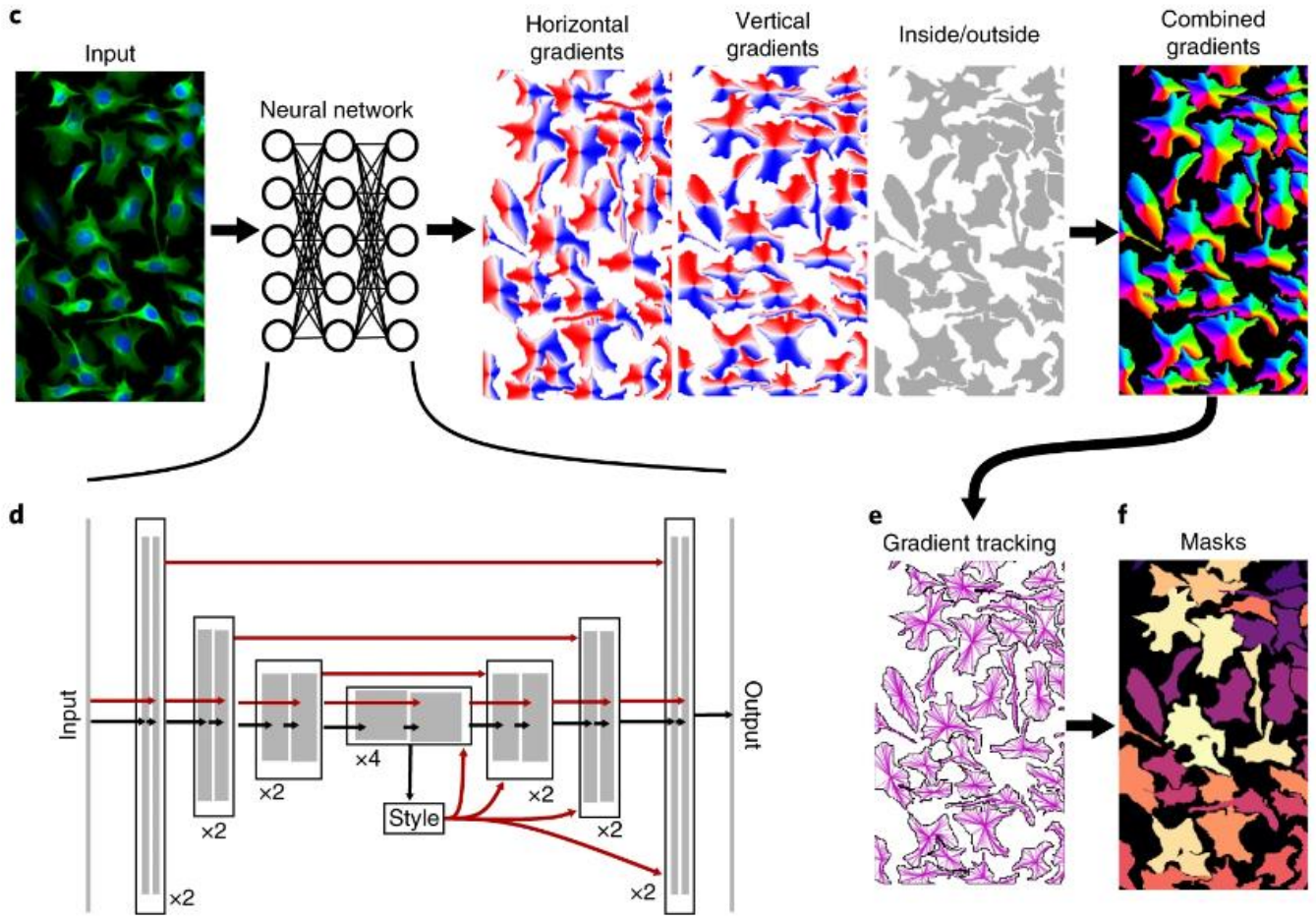


Image from Figure 1 of Stringer et al.

Stringer C, Wang T, Michaelos M, Pachitariu M. Cellpose: a generalist algorithm for cellular segmentation. Nature methods. 2021 Jan;18(1):100-6.

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

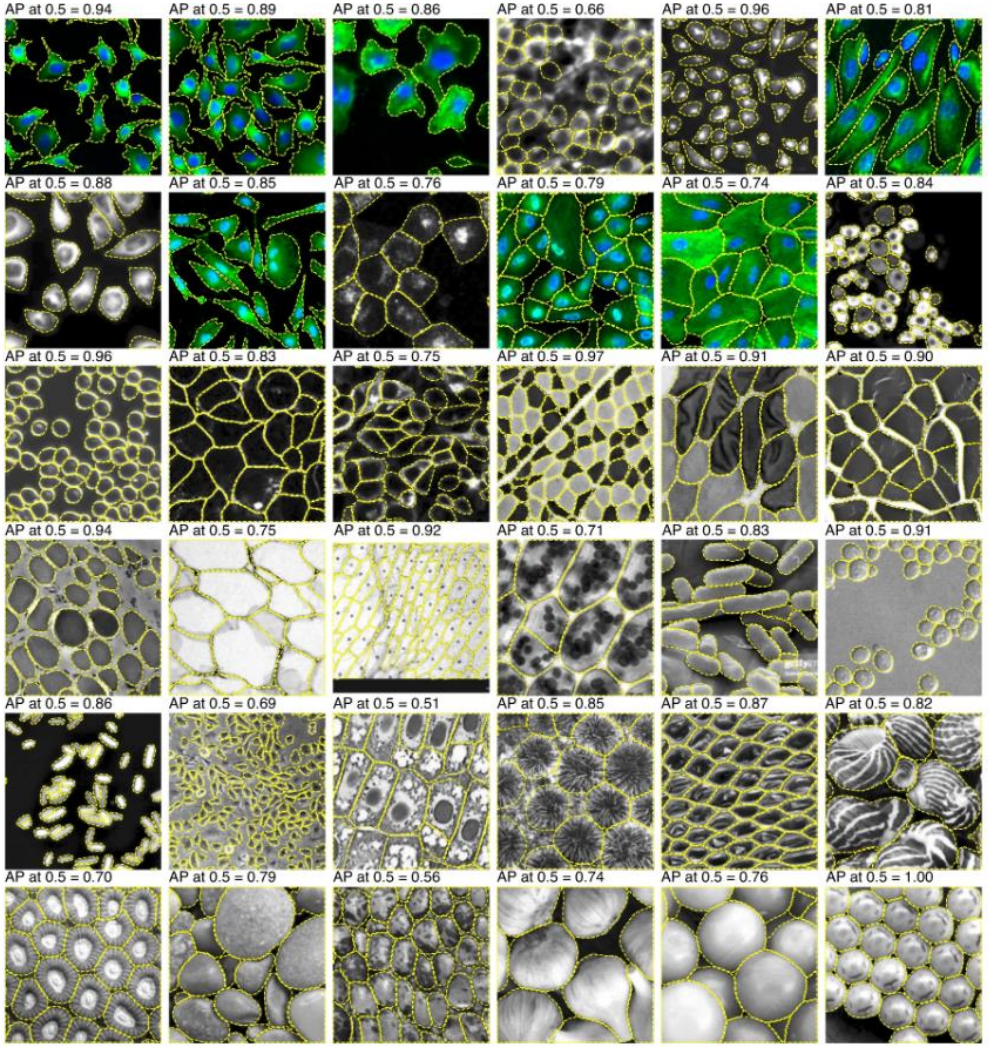


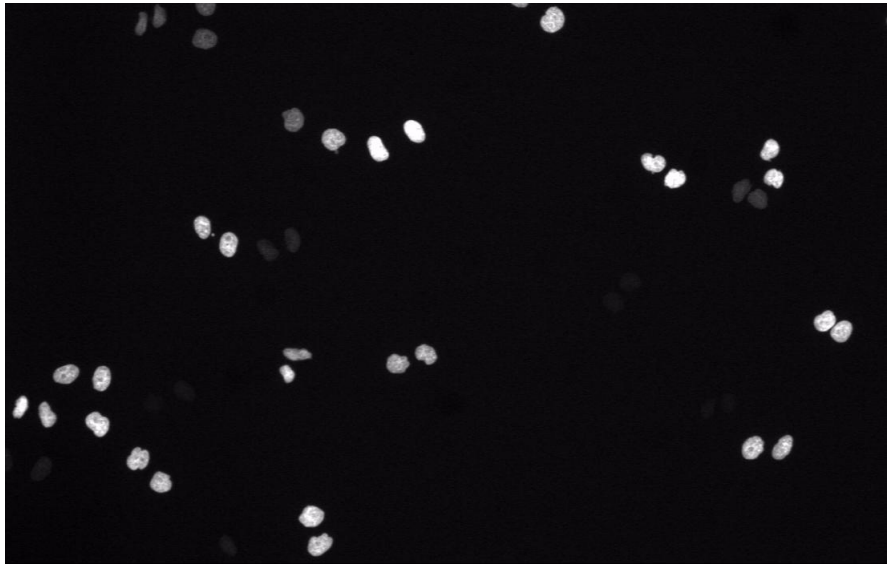
Image from Figure 3 of Stringer et al.

Stringer C, Wang T, Michaelos M, Pachitariu M. Cellpose: a generalist algorithm for cellular segmentation. Nature methods. 2021 Jan;18(1):100-6.
Pachitariu M, Stringer C. Cellpose 2.0: how to train your own model. Nature Methods. 2022 Nov 7:1-8.

Section 4: Single Particle Tracking

Single Particle Tracking

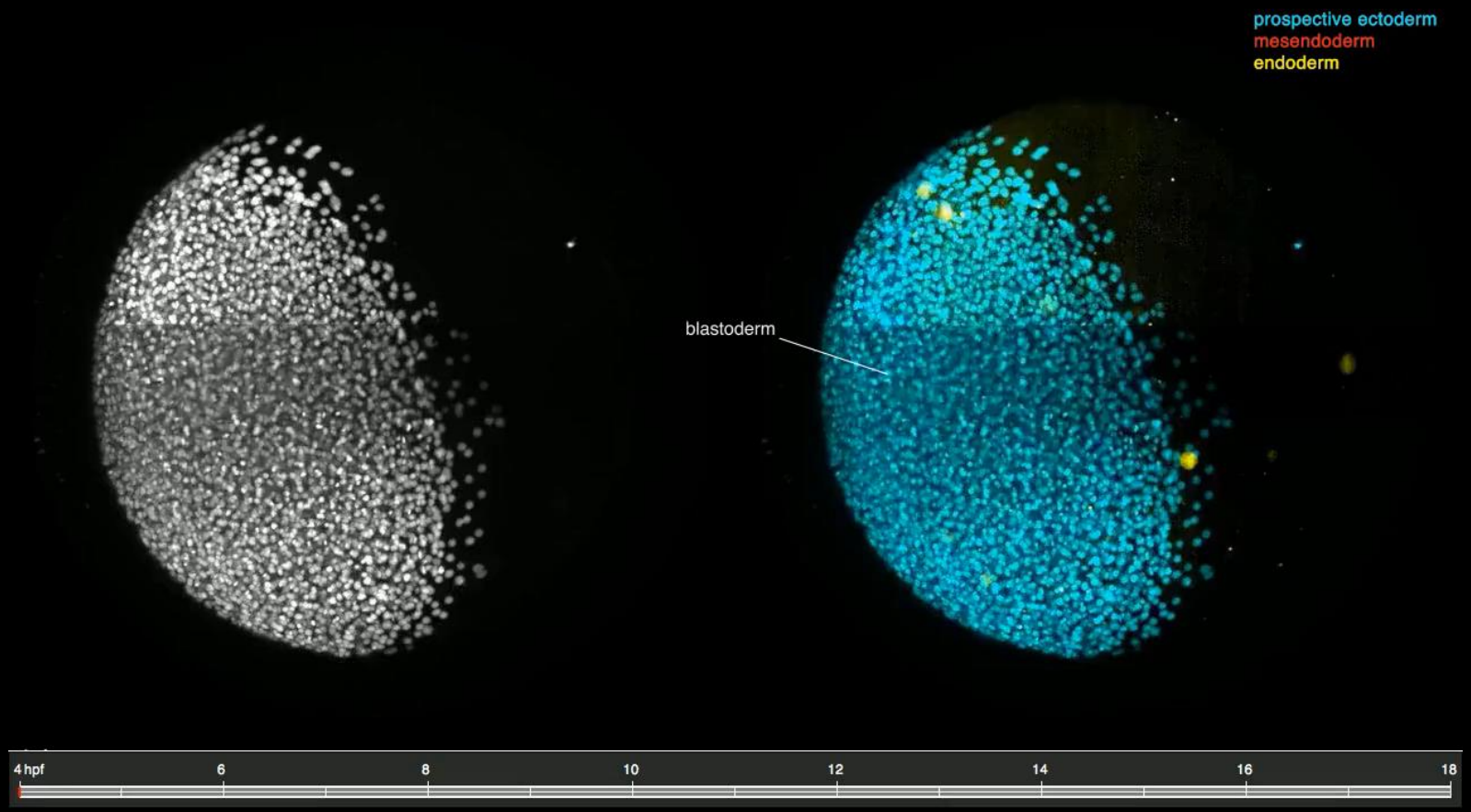
- A two step process
  1. Object detection
  2. Linking objects between frames (tracking)
- Complications include object merging and splitting
- If an object is missed in one (or more) frames then gap closing can be used to merge particle trajectories



http://www.codesolorzano.com/celltrackingchallenge/Cell_Tracking_Challenge/Datasets.html

Maška, Martin, et al. "A benchmark for comparison of cell tracking algorithms." Bioinformatics 30.11 (2014): 1609-1617.

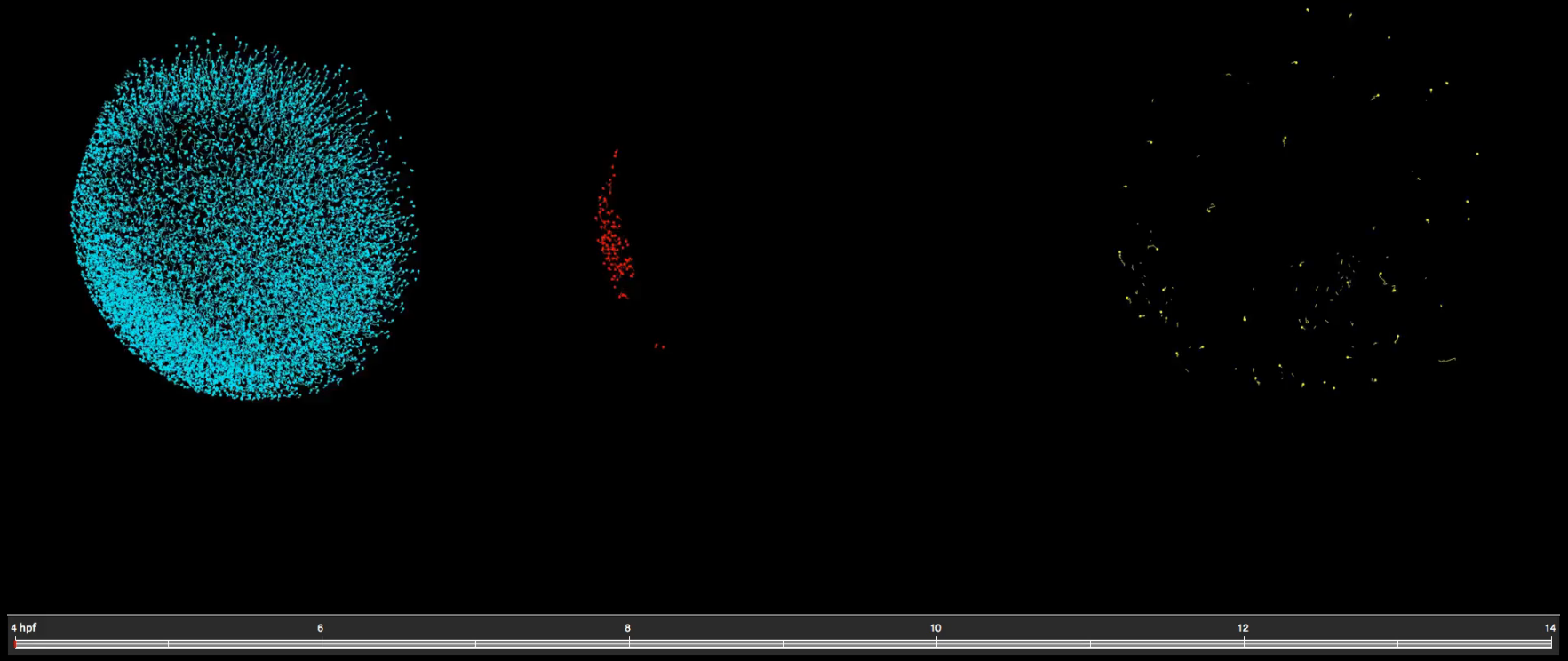
Application 1: Tracking cells in developing embryos



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).

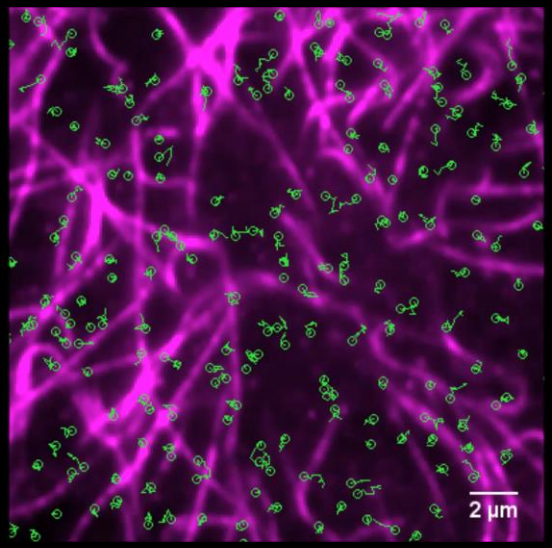
Application 1: Tracking cells in developing embryos



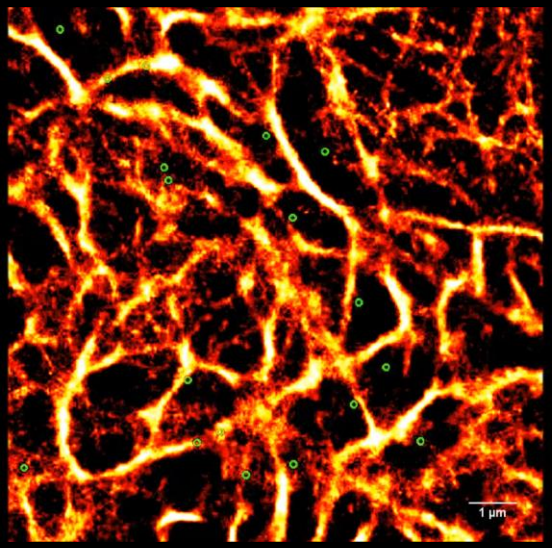
Shah, Gopi, et al. "Pan-embryo cell dynamics of germlayer formation in zebrafish." bioRxiv (2017): 173583.

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

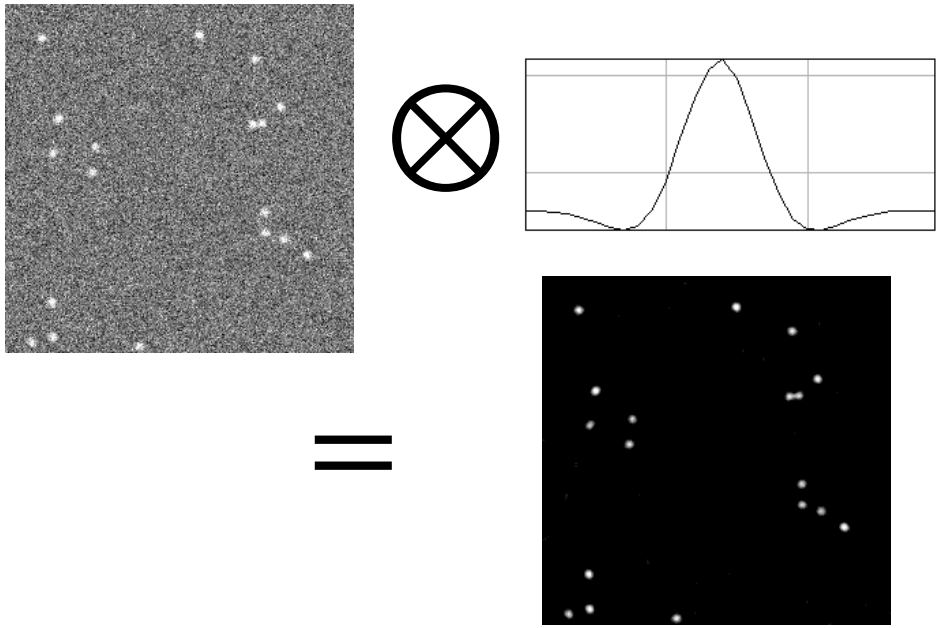


G-proteins over MTs

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-bulit difference of Gaussian schemes

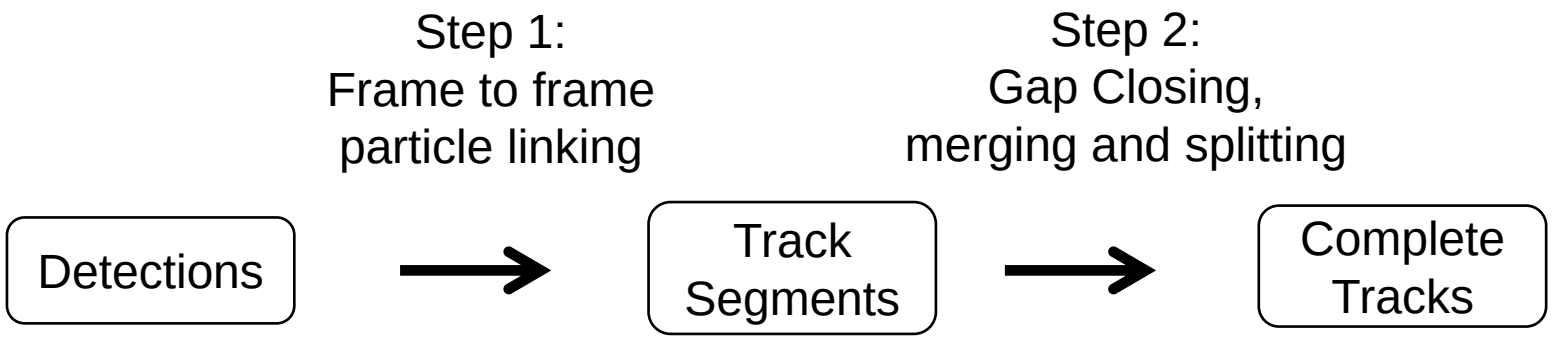


- More sophisticated algorithms such as wavelet based detection can be employed. For example the Spot Detector plugin in Icy.

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.

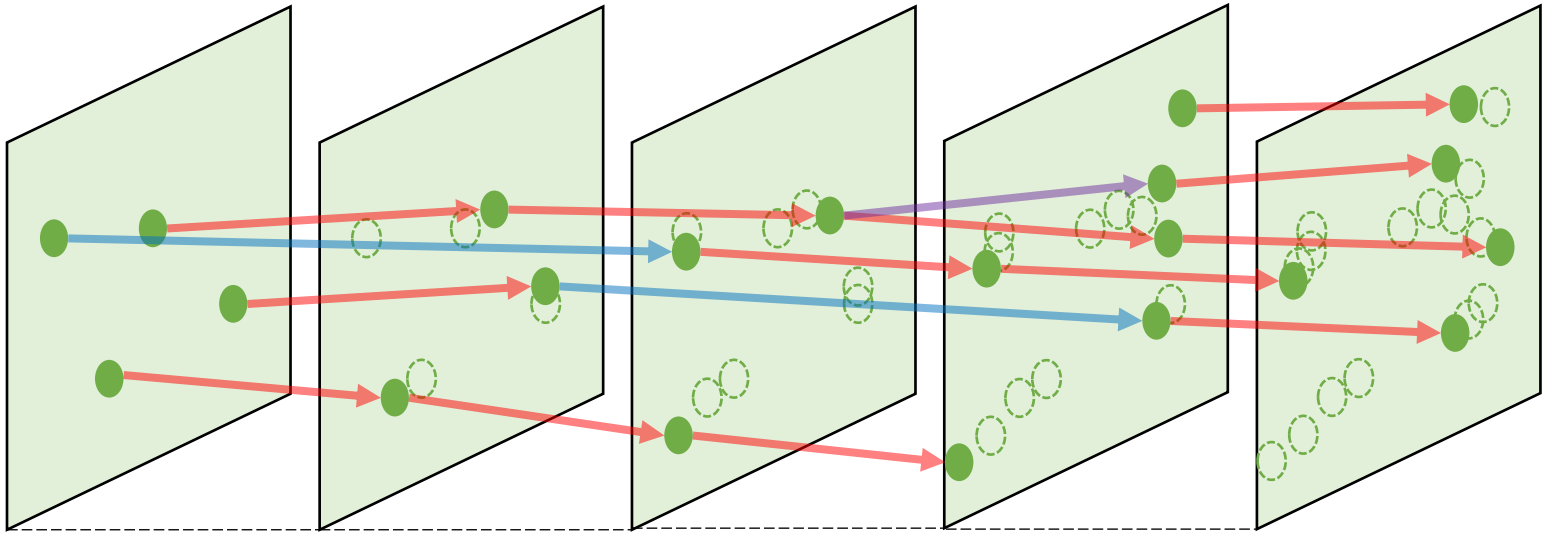
Linear Assignment Problem (LAP) trackers



- 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

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

The Linear Assignment Problem

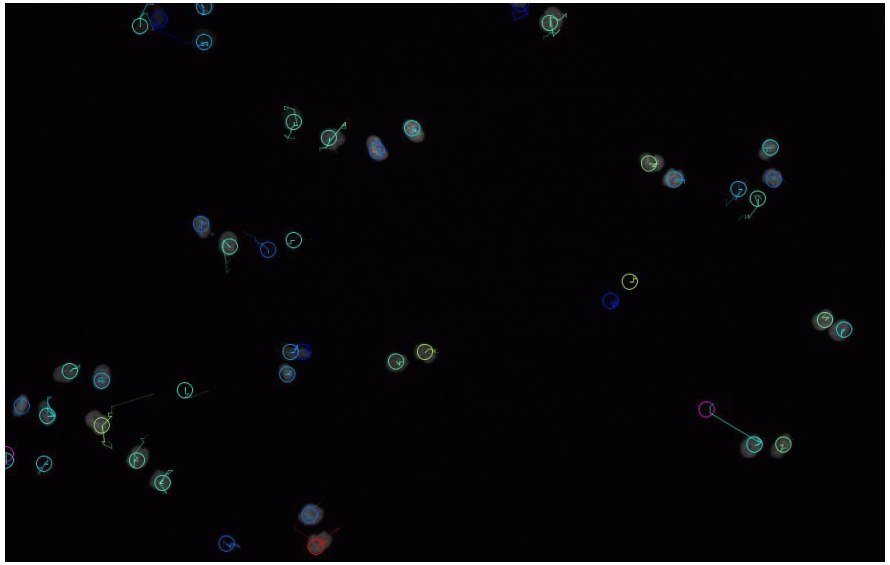


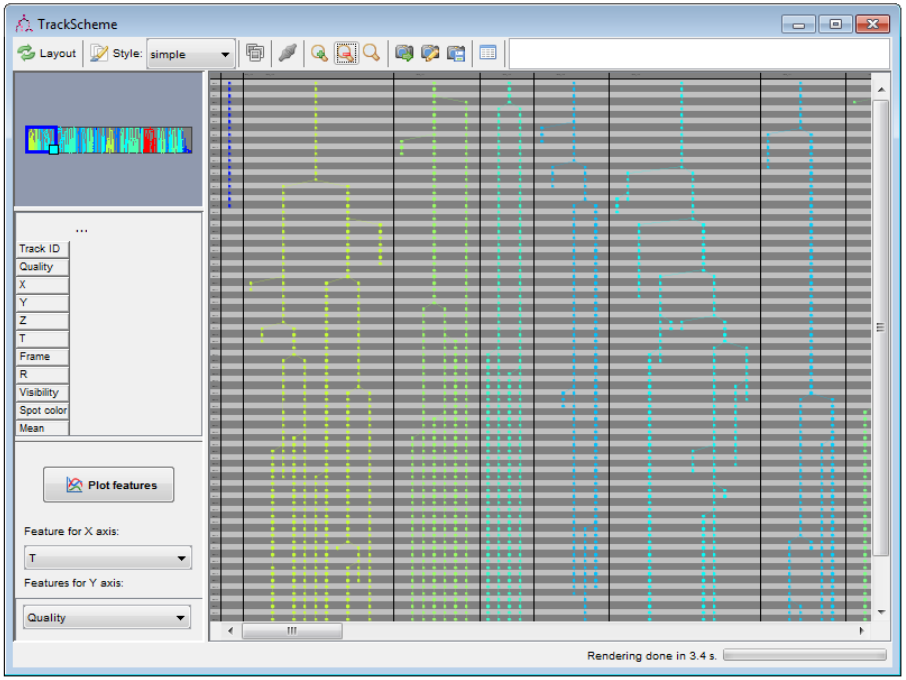
Frame-to-frame particle linking

Track segment linking – gap closing and splitting

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

Cell tracking with TrackMate using a LAP Tracker



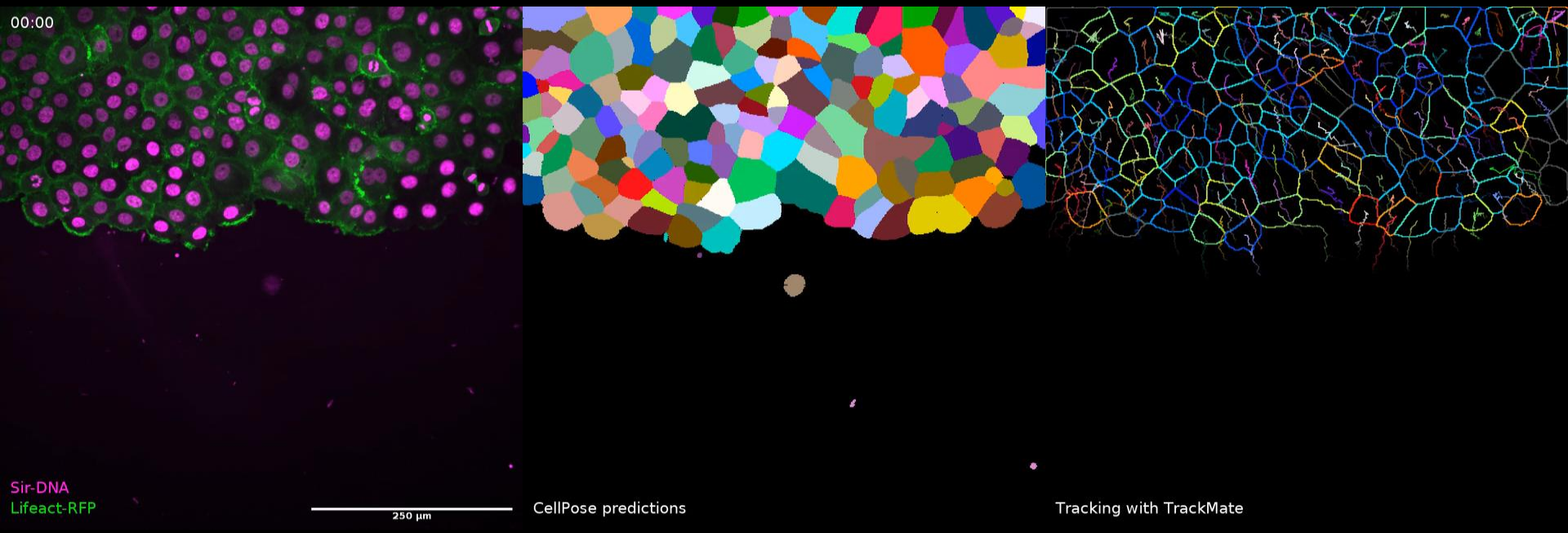


Tinevez J-Y, Perry N, Schindelin J, Hoopes GM, Reynolds GD, Laplantine E, et al. TrackMate: An open and extensible platform for single-particle tracking. Methods 2016.

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

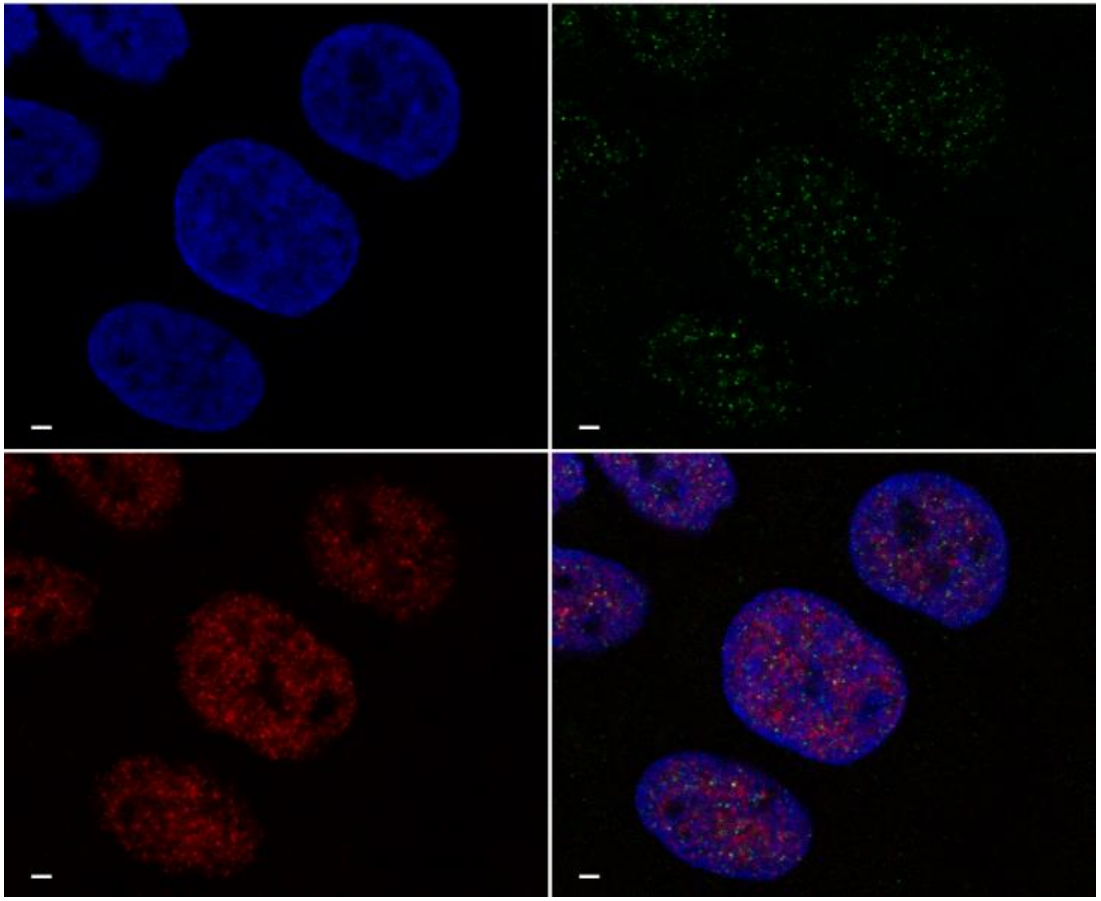
- Extensions which allow integration with Cellpose, Stardist, ilastik etc



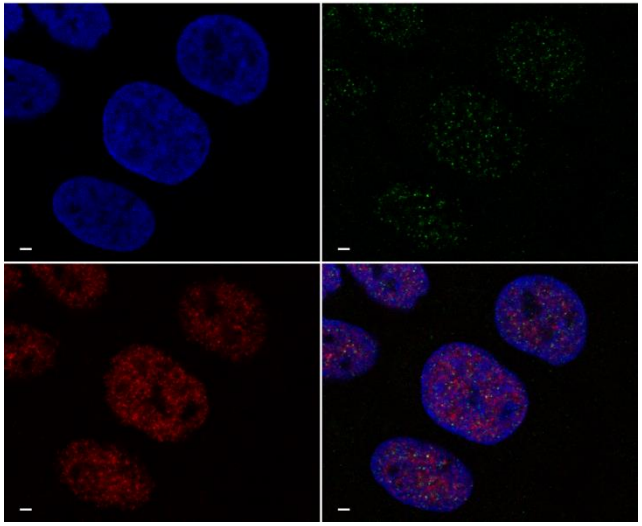
Video from https://imagej.net/plugins/trackmate/

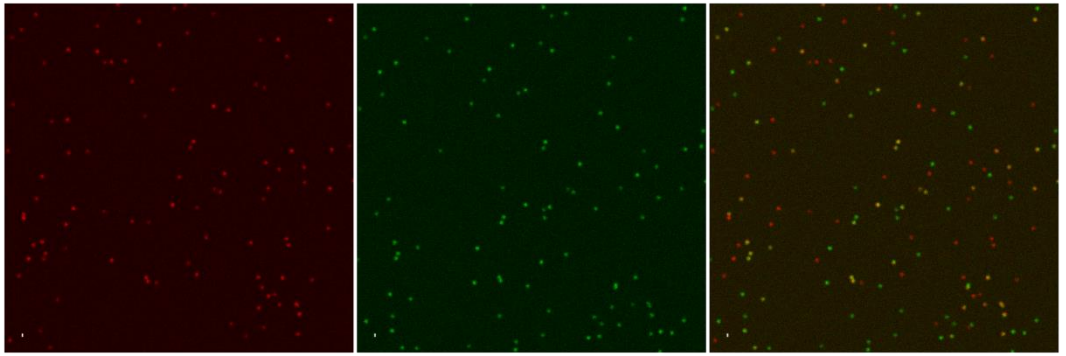
Ershov, Dmitry, et al. "TrackMate 7: integrating state-of-the-art segmentation algorithms into tracking pipelines." Nature methods 19.7 (2022): 829-832.

Section 5: Pixel Based Colocalization Analysis



Visualising Colocalization with Colour Overlays

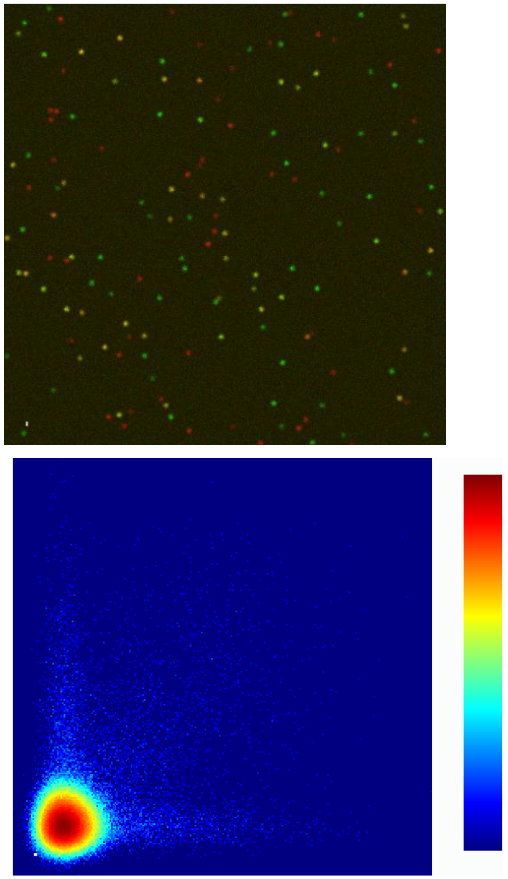




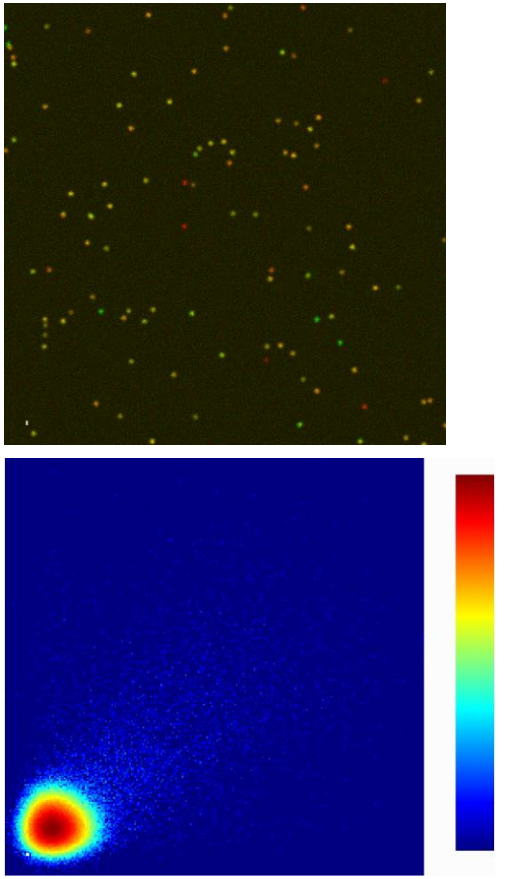
- Not very useful and cannot make any convincing conclusions…
- Visual interpretation is very sensitive to changes in display settings
- Some LUTs are better than others

Visualising Colocalization with Joint-Histograms

50% Spots Colocalized

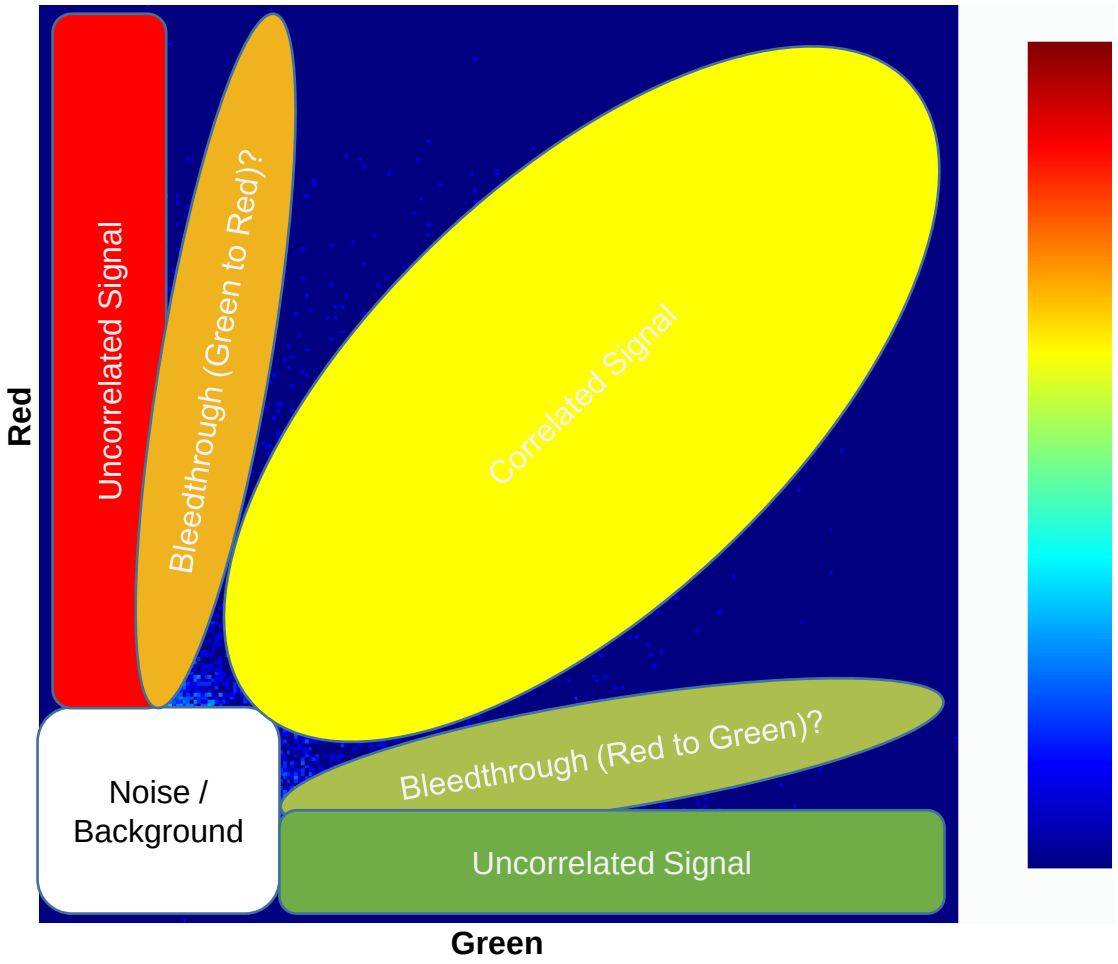


95% Spots Colocalized



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

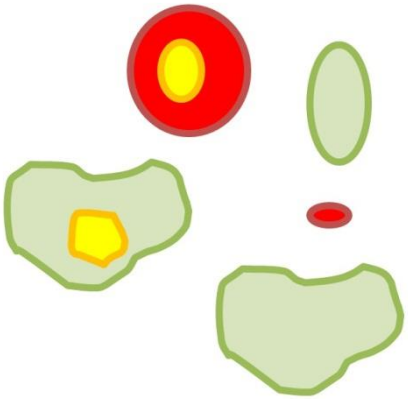
Visualising Colocalization with Joint-Histograms



Quantifying Colocalization

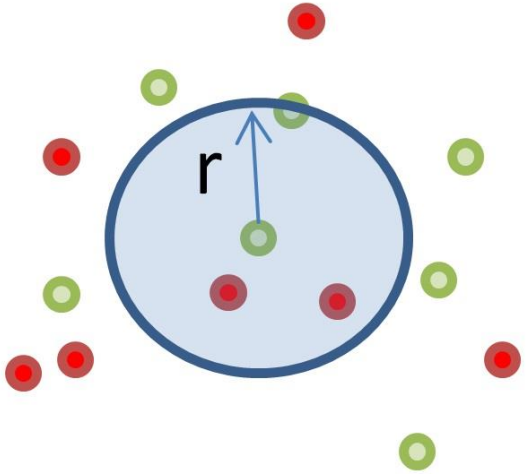
There are two approaches:

Pixel Based



Measures overlap and correlation of signal across individual pixels

Object Based



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

Lagache, Thibault, et al. "Statistical analysis of molecule colocalization in bioimaging." Cytometry Part A 87.6 (2015): 568-579.

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-occurence

The Manders coefficients:

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

- Together the Pearson and Manders coefficients measure and distinguish between correlation and co-occurence
- 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.

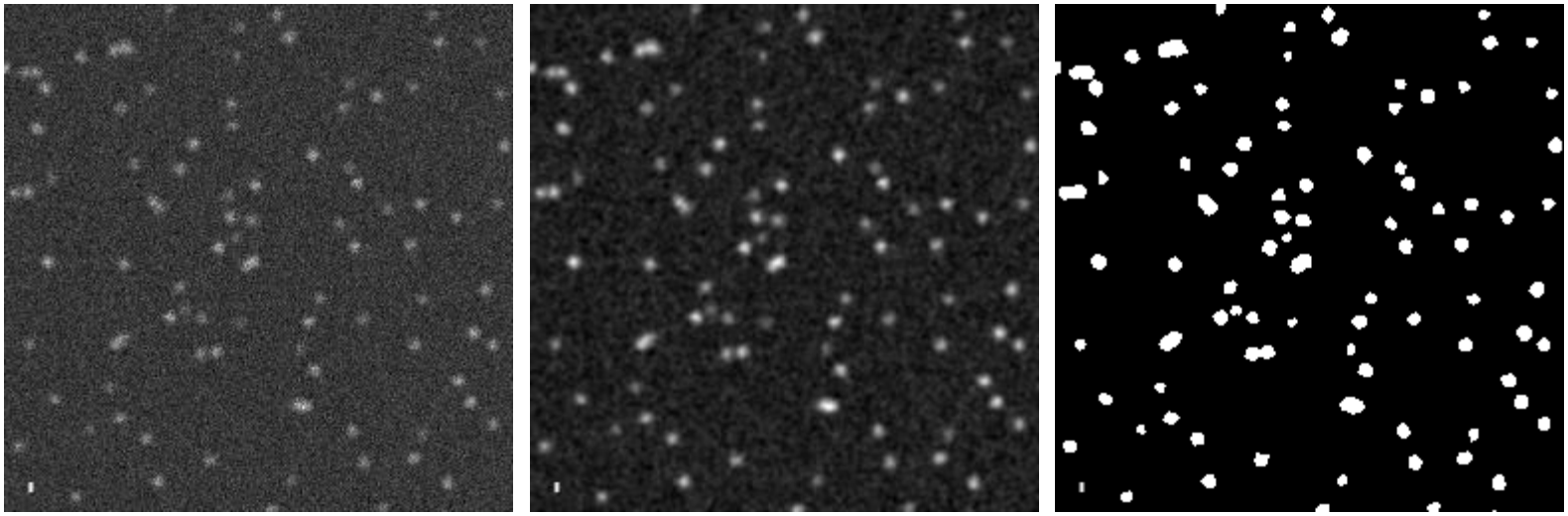
Image Acquisition and Pre-processing

- 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

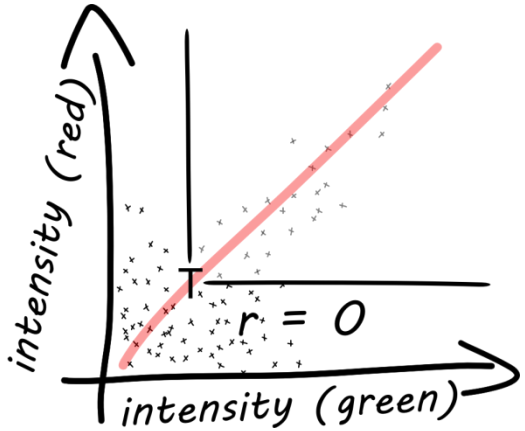
Signal Isolation

- Essential for calculation of the Manders coefficents 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.

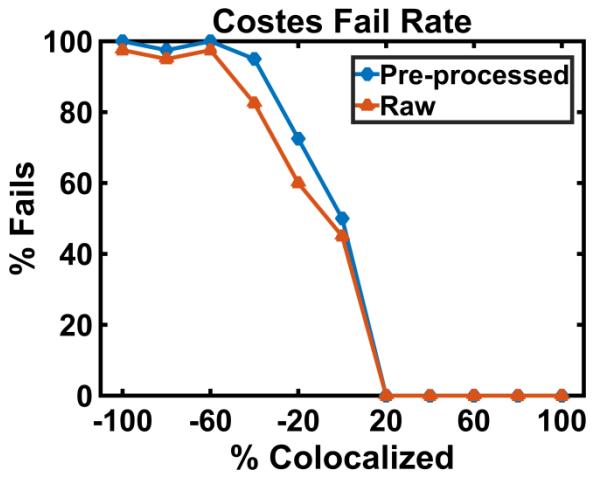
Raw → Gaussian Filter → Otsu Threshold



Costes' Thresholding



- Finds the point on the line of best bit bellow which the Person coefficent ≤ 0
- Sets threshold values for signal isolation at this point

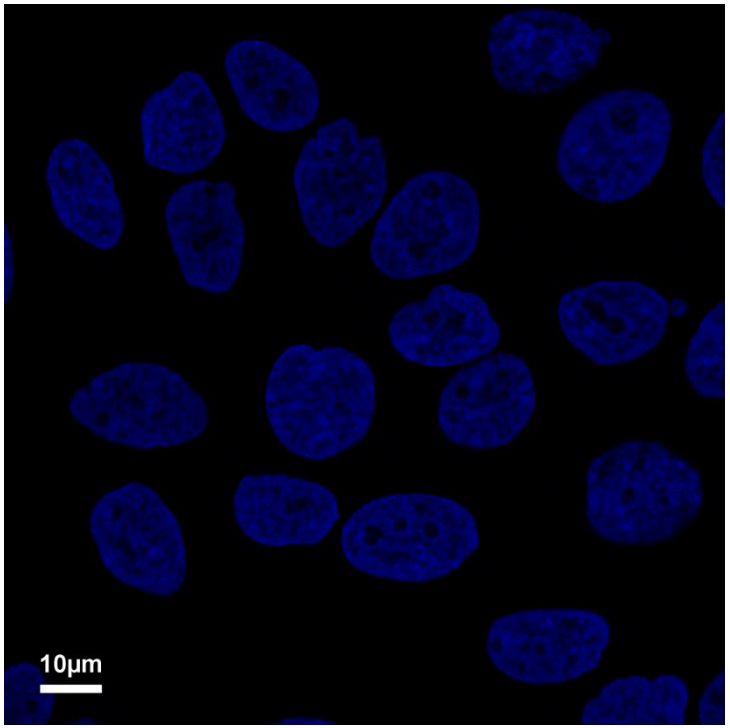


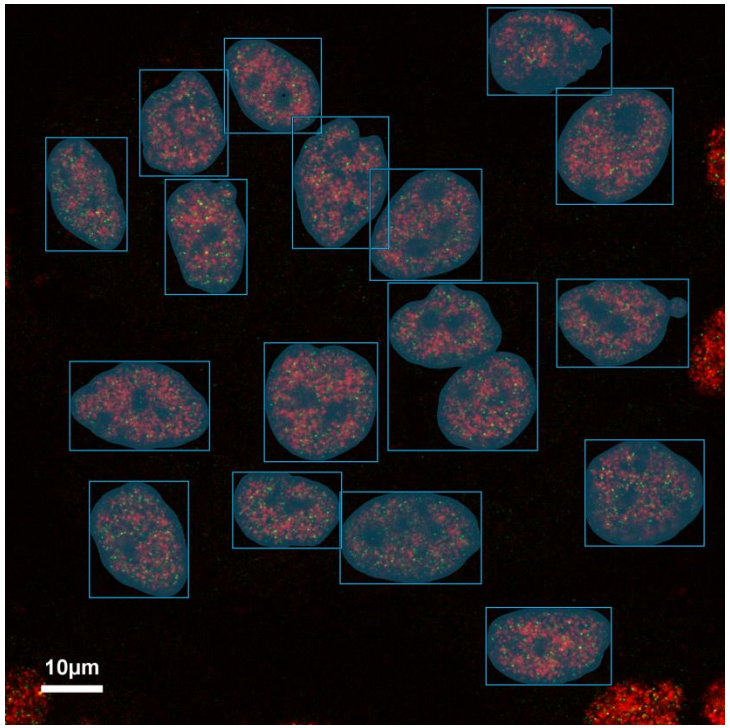
- Be careful, Costes' thresholding assumes a single linear correlation!
- Why use a test that assumes colocalization to test for colocalization?

Pike J., Styles I., Rappoport J.Z., Heath J."Quantifying Receptor Trafficking and Colocalization with Confocal Microscopy." Submitted to Methods 2016.

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!

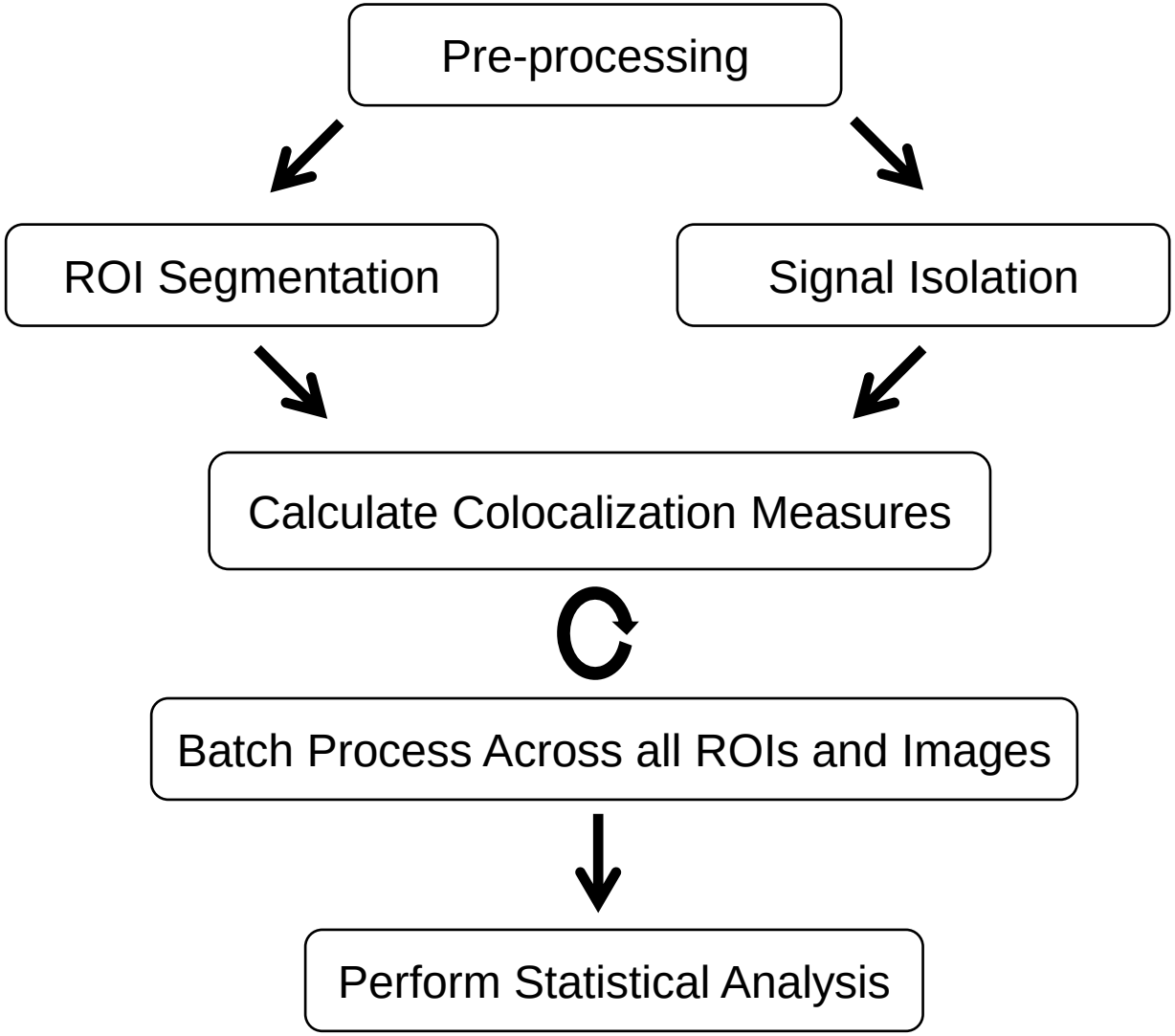
- Option 2: Perform standard statistical tests (eg t-tests) to compare colocalization across populations.

  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

Mcdonald, John H., and Kenneth W. Dunn. "Statistical tests for measures of colocalization in biological microscopy." Journal of microscopy 252.3 (2013): 295-302

Putting It All Together: A Colocalization Workflow



Acknowledgments

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

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Mark Dunning
Stefanie Reichelt