Automated Spot Counting and Modeling to Estimate Microbial Concentration in Serial Dilution Experiments

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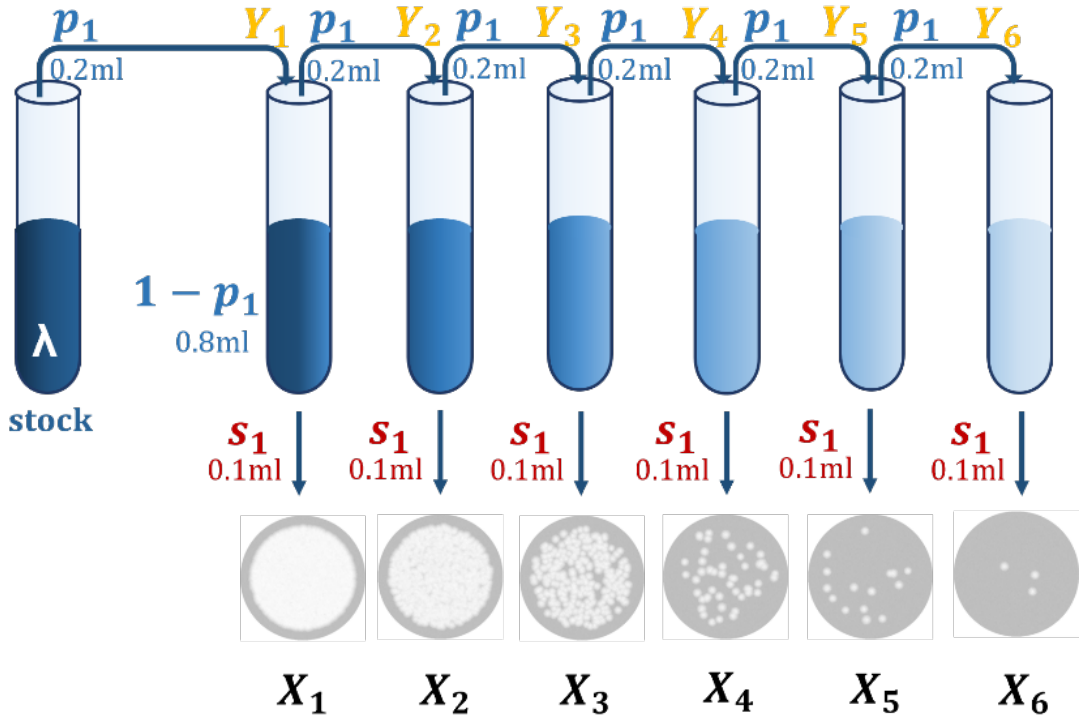
Agenda

1. **Motivation and experimental design**
2. **Automated PFU counting**
3. **Bias correction**
4. **Dilution series modeling**
5. **LoST R package and Shiny application**
6. **Q&A**

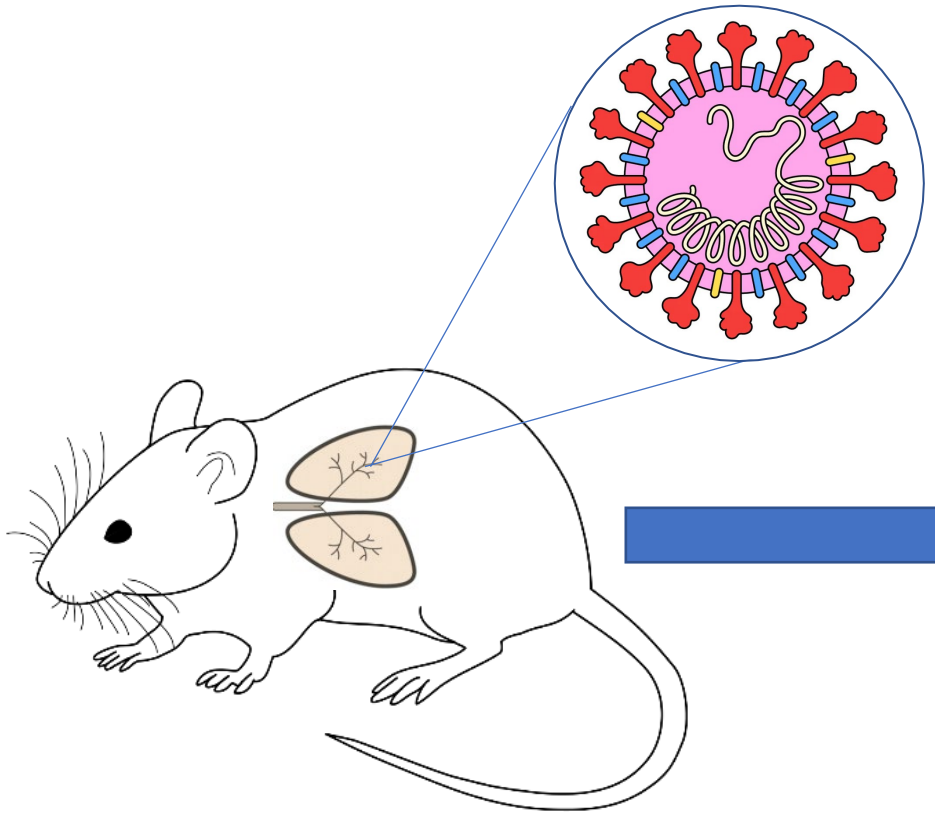
1. Motivation and experimental design

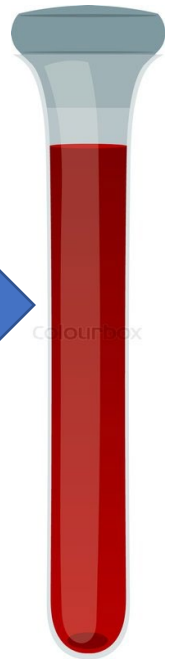
Motivation

- Dilution series common to quantification of organisms in a sample
  - Bacterial colony forming units (CFUs)
  - Phage Spot Forming Units (SFUs)
- Assumes each spot starts as a single organism that divides exponentially and consistently in all directions
  - Resulting spot should be a circle/sphere



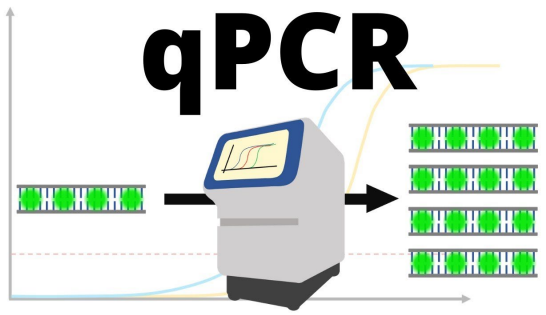
Viral Sample Collection and Quantification



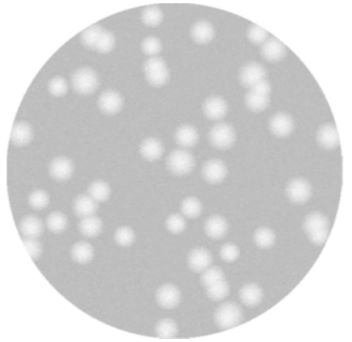


OR

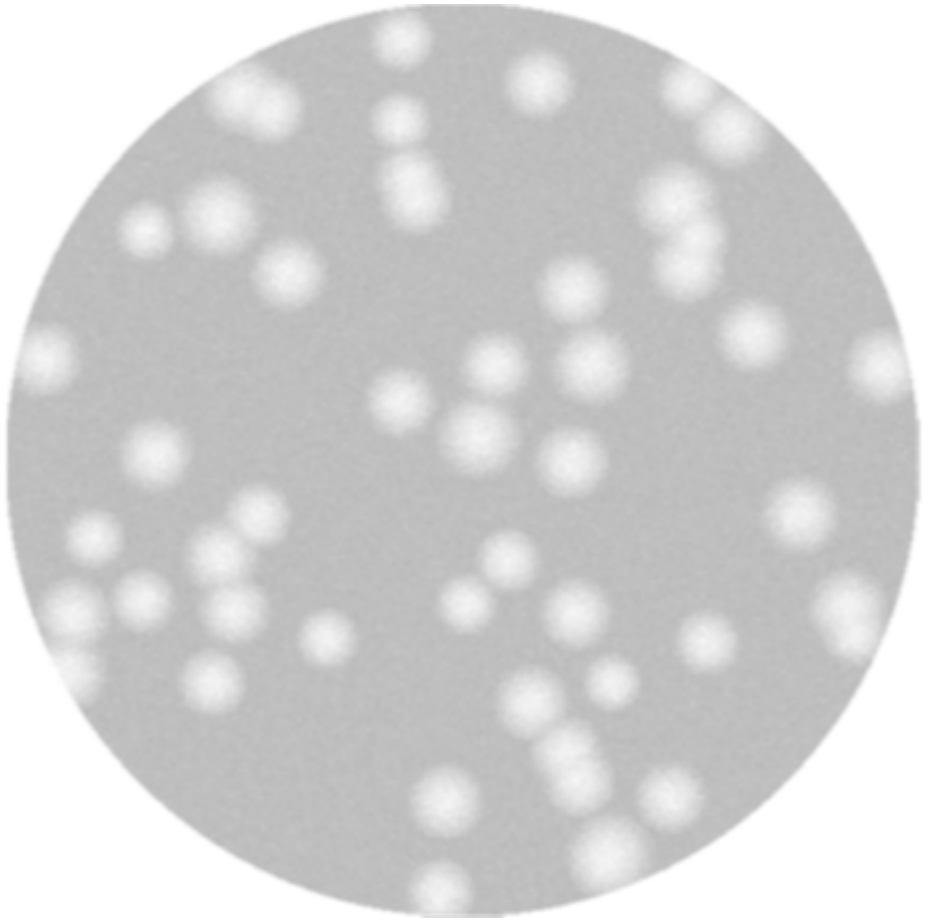
All viral particle quantification



Active viral particle quantification



Viral Sample Collection and Quantification



- Samples are loaded onto established cultures – a lawn of bacteria or a confluent monolayer of mammalian cells
- Active viral particles begin infecting and lysing host cells, and propagate
- “Clearance zones” are forming, i.e., regions that do not contain host cells
- Each clearance zone forms a circular spot putatively initiated by the propagation of a single, actively replicating infectious agent

Challenge 1

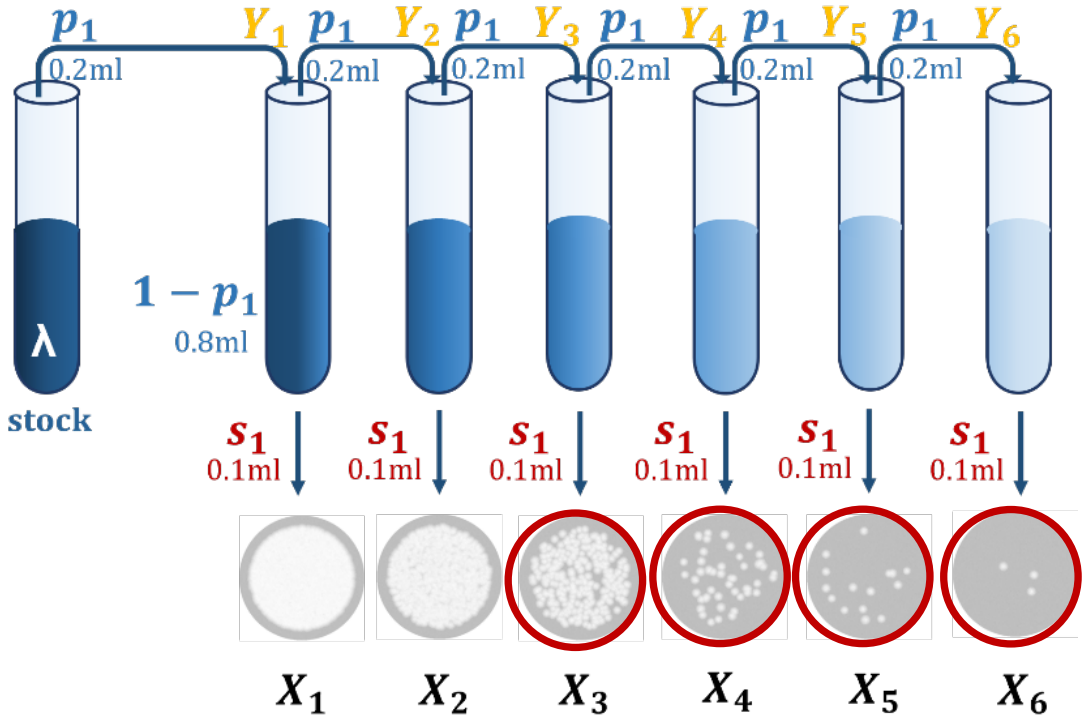
Counting: Scientists carrying out the spot counting

- Humans are fallible
- Intra-rater reliability is not assured
- Time limitations – impossible in high-throughput situations

Challenge 2

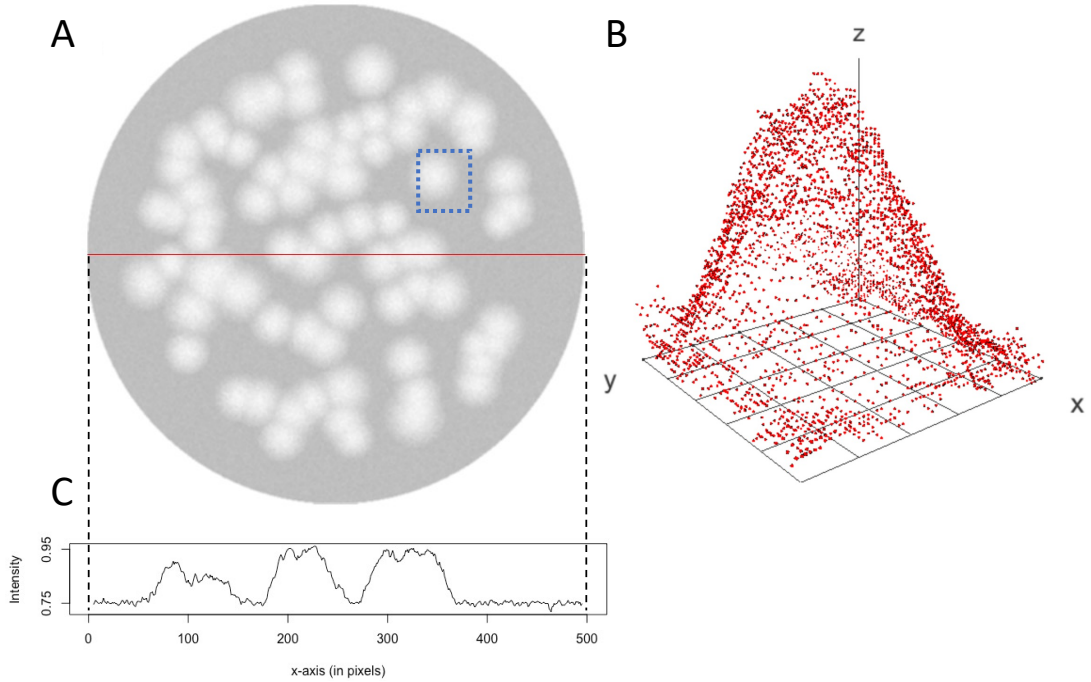
Modeling: Usually not done

- FDA Bacterial Analysis Manual (BAM) recommends counting one plate with 25-250 CFUs, then multiplying by the dilution to get the original concentration
- FDA makes no recommendation if more than one plate meets that rule
- ASTM recommends averaging if more than on plate meets the rule



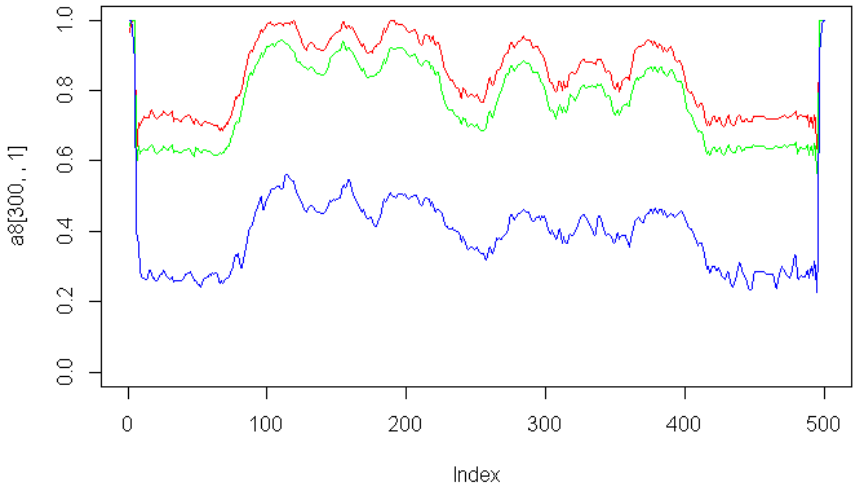
2. Automated PFU Counting

PFU Counting From Images

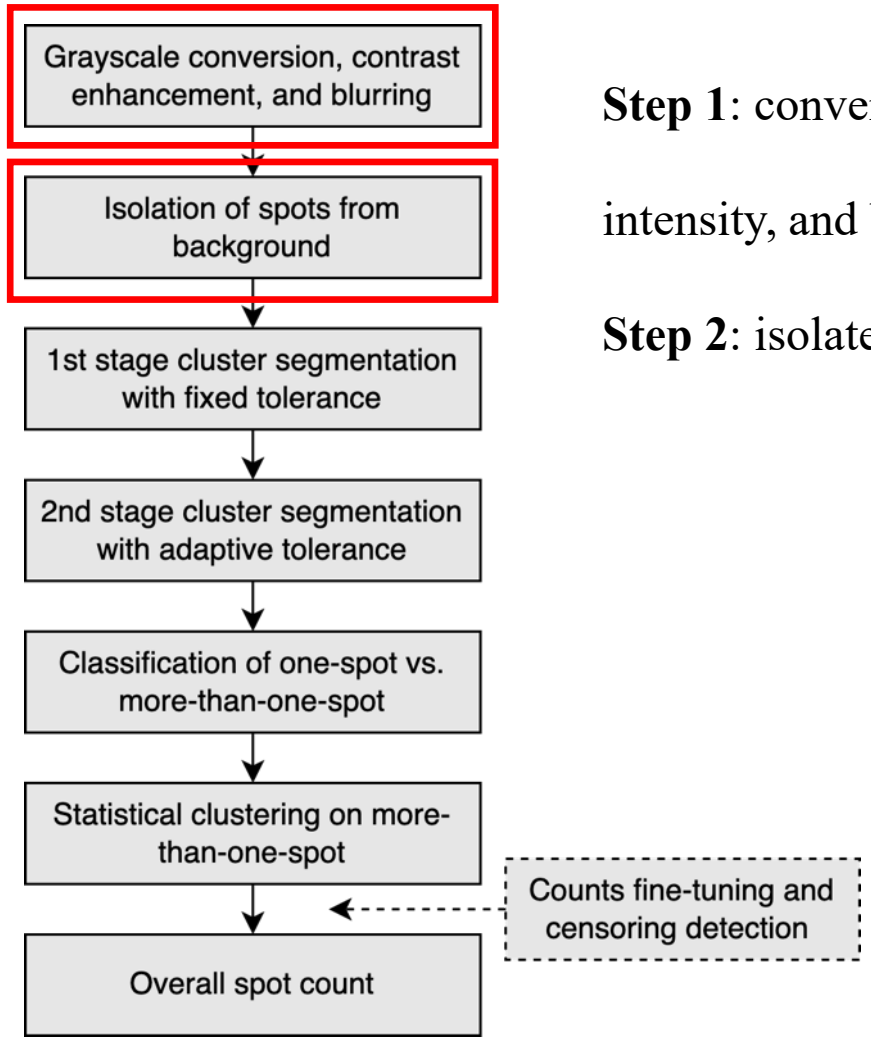


- PNG or JPEG images of the wells are used to count PFUs manually
- The images can be processed automatically, and several such algorithms already exist
- The existing tools struggle identifying individual spots if they overlap

- Images received for this project were 3-channel JPEG, 500x500 pixels light intensity (between 0 and 1)
- Hence, the data was 500x500 matrices, and each spot could be plotted as a 3-dimensional “mound”

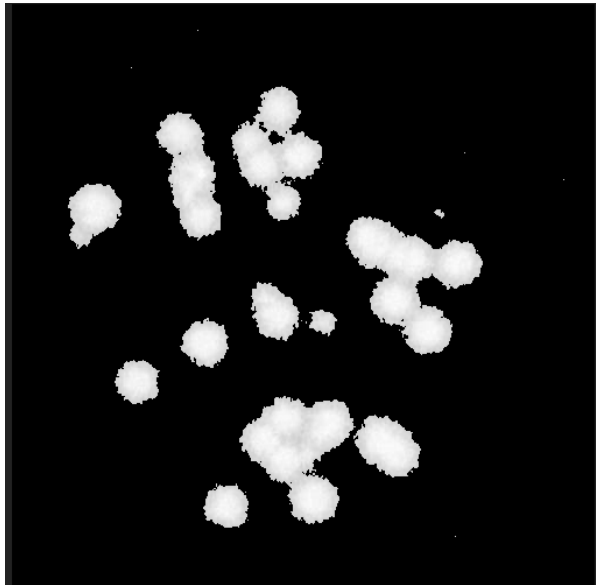


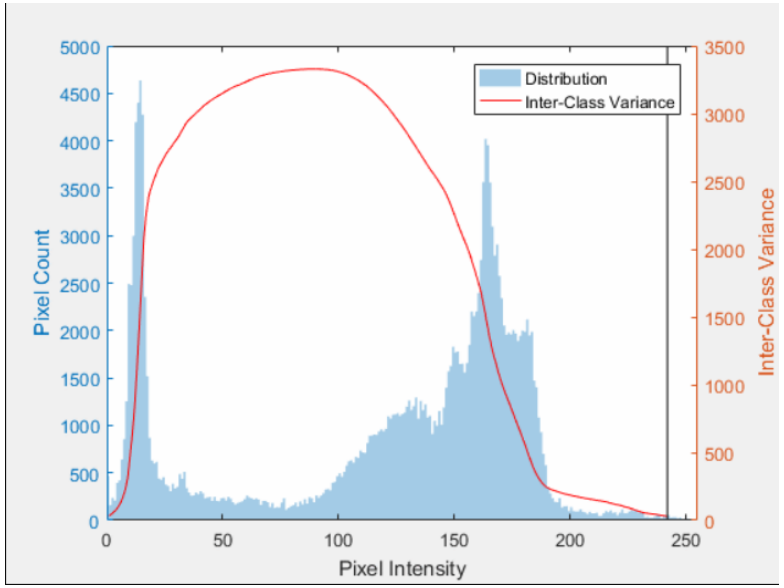
Localized and Sequential Thresholding (LoST) Algorithm



Step 1: convert images to an 8-bit grayscale, increase the contrast by normalizing the intensity, and blur the image through a Gaussian filter to alleviate the noise

Step 2: isolate spots from the background using Otsu's method





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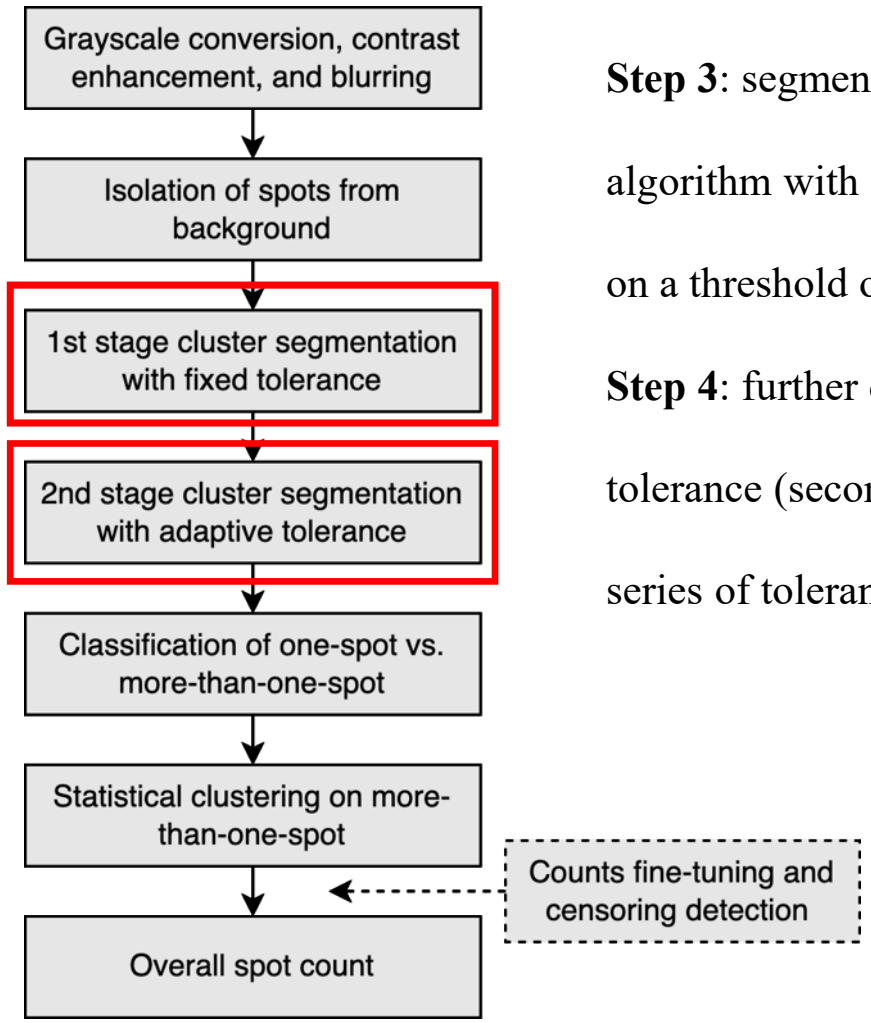
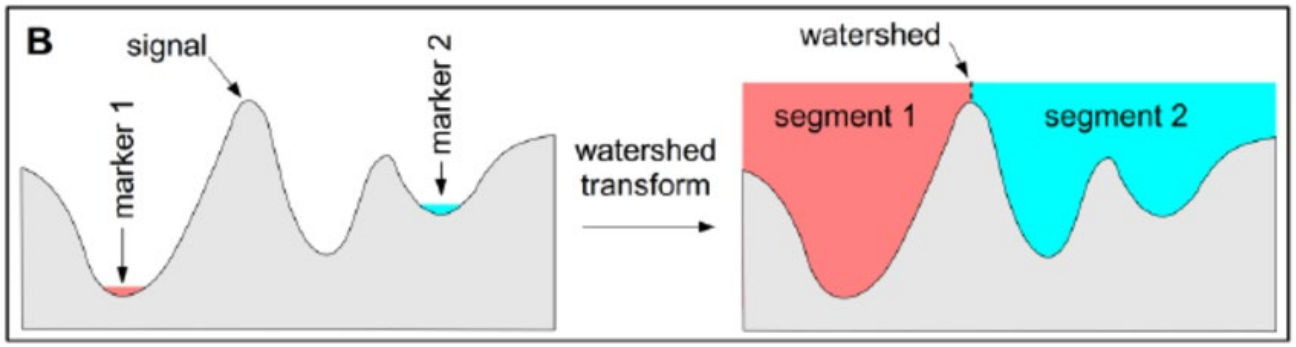


Step 3: segment the apparent (and relatively large) clusters of overlapping spots using a watershed algorithm with a specific tolerance (first-stage segmentation), followed by image denoising based on a threshold of cluster size.

Step 4: further decompose large clusters by the watershed algorithm with a lower clustering tolerance (second-stage segmentation). The second-stage tolerance is adaptive and scans through a series of tolerances until all clusters are below a set size.



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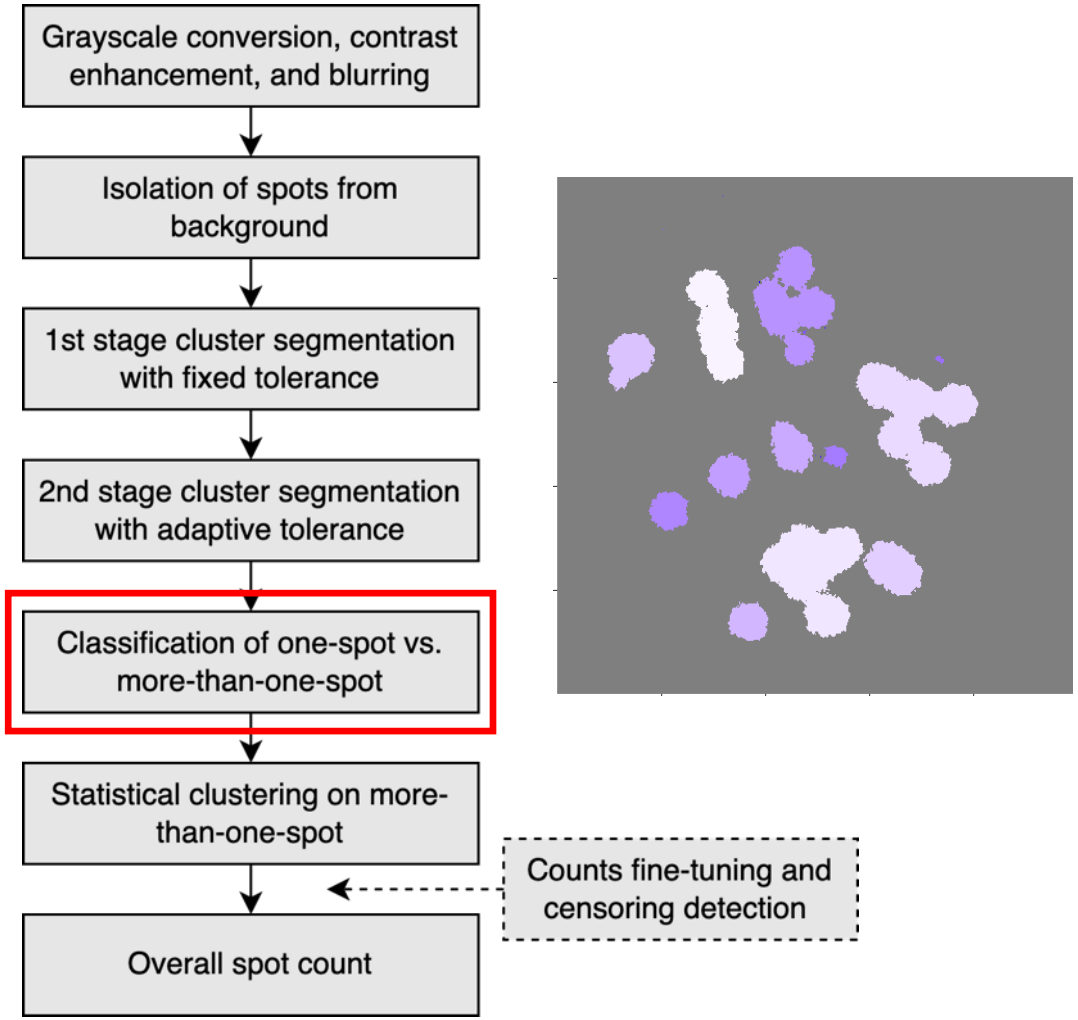
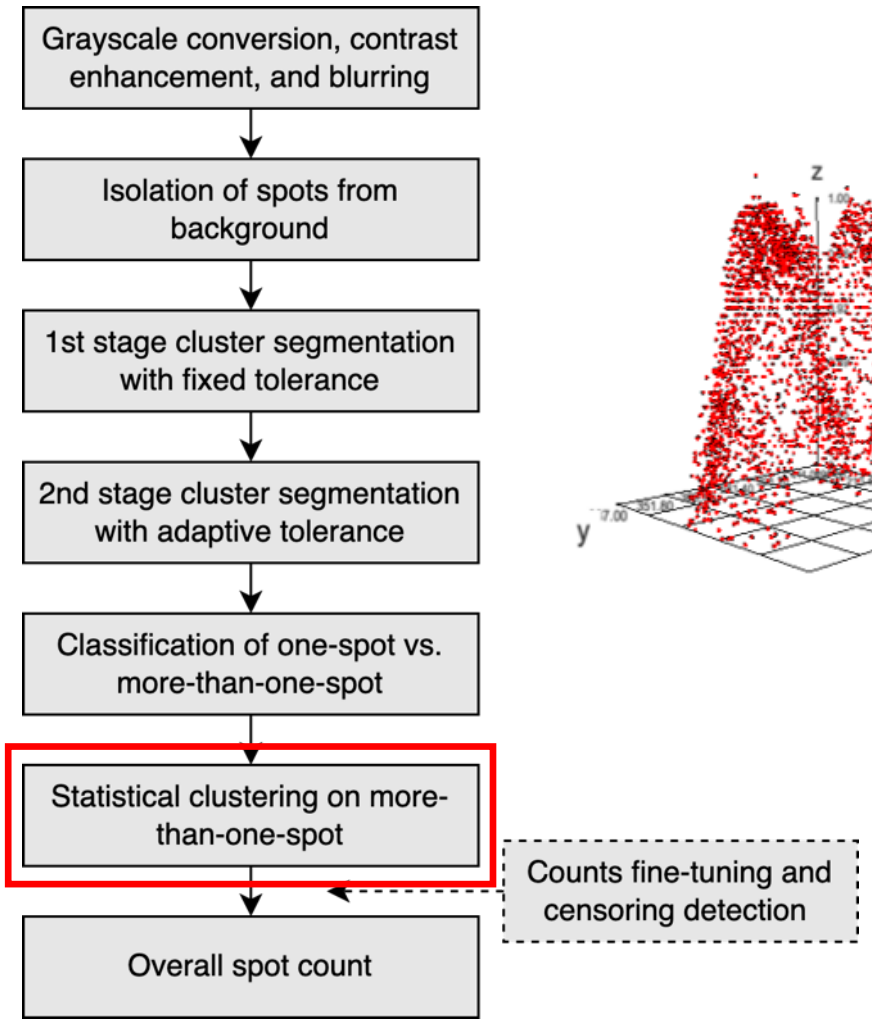
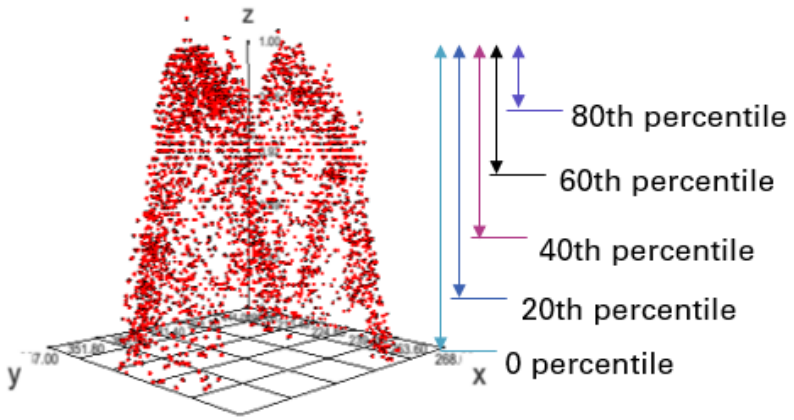


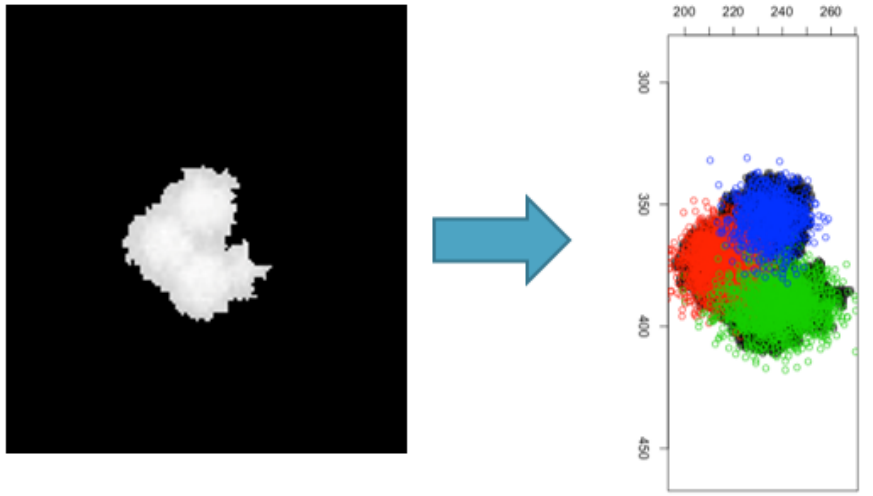
Step 5: use a decision trees to classify all the clusters into two categories: one or more than one spot.

Based on circularity attributes such as ratio of the smallest to the largest eigenvalues of the covariance matrix of the cluster points, sample correlation coefficient of the cluster edge points, coefficient of variation of the distance between cluster edge and the center (i.e., cluster radius), and cluster area.

Localized and Sequential Thresholding (LoST) Algorithm







Step 6: apply Gaussian mixture model (GMM) clustering to those clusters consisting of more than one spot.

Comparison of PFU Counting Tools

- *LoST* was tested against 3 commercially available tools
- Manual counting was used as the gold standard
- *LoST* performed as well as human counters, and better than all 3 tools on images with overlapping spots

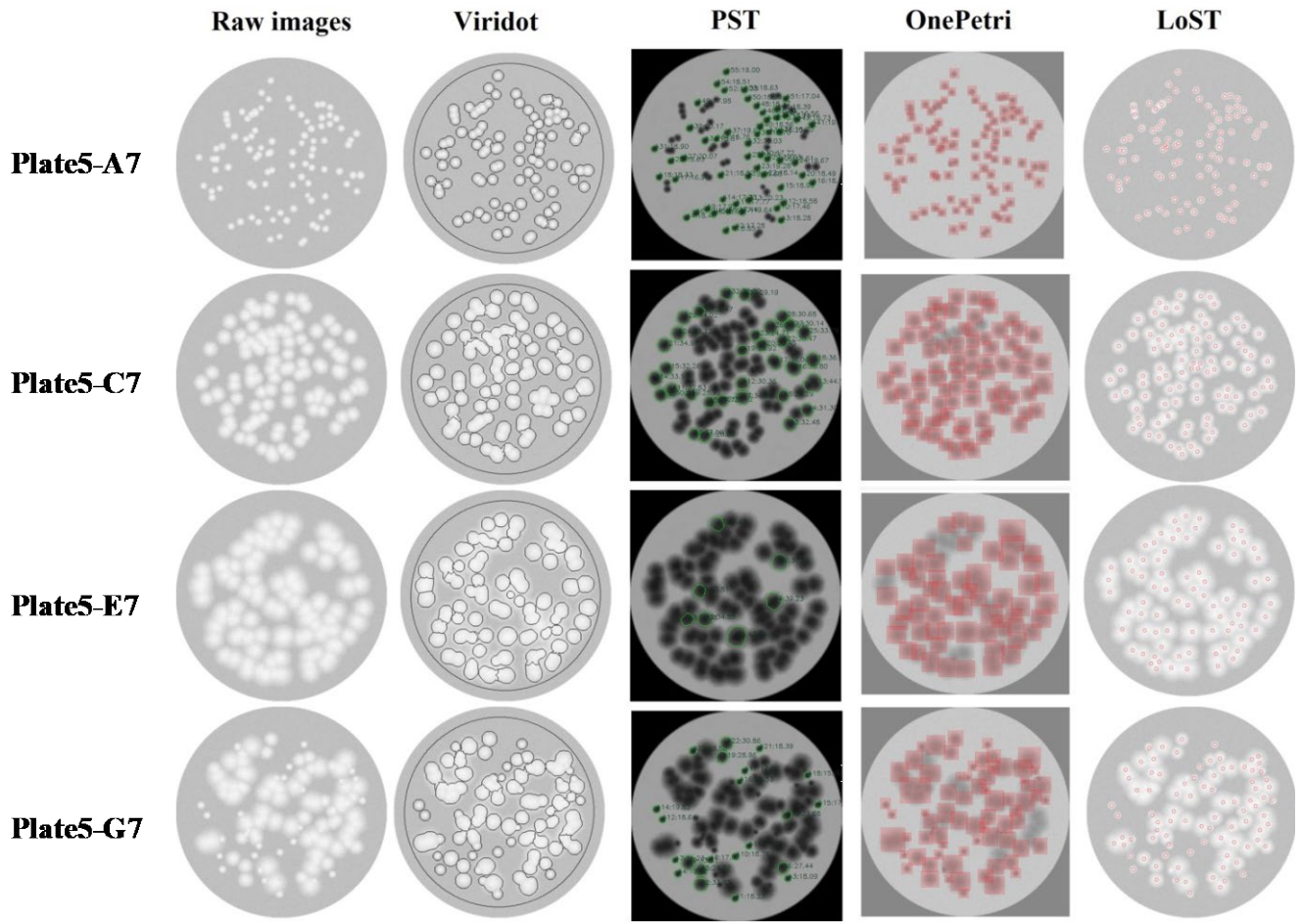


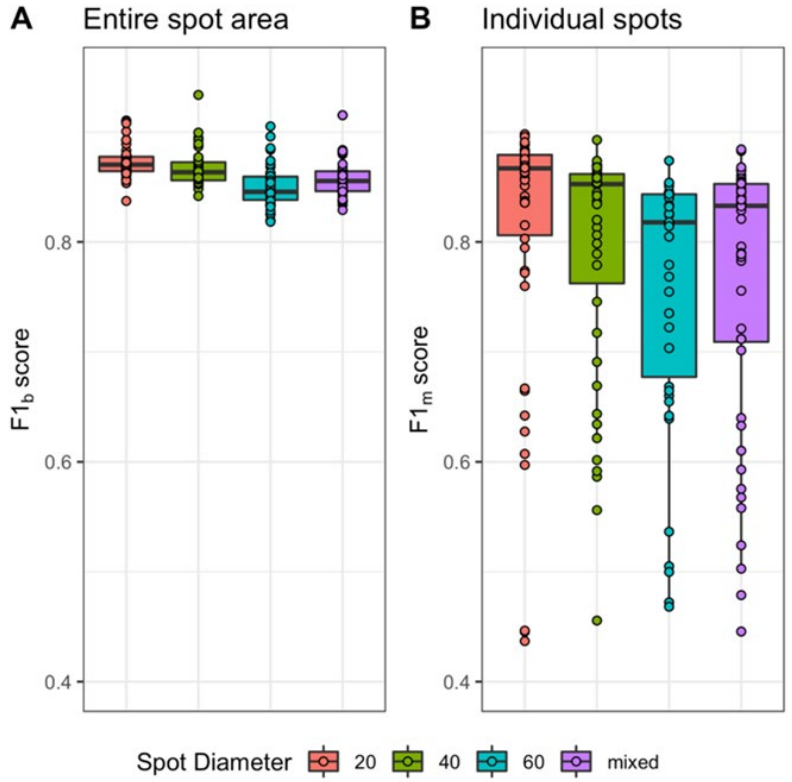
Image	Spot diameter	Simulated count	Observer 1	Observer 2	Viridot	PST	OnePetri	LoST
Plate5-A7	20	96	95	95	67	55	90	94
Plate5-C7	40	110	93	95	58	32	80	93
Plate5-E7	60	104	75	78	54	7	60	77
Plate5-G7	mixed	116	89	95	59	22	70	89

LoST Accuracy

- the $F1_b$ scores (assessing the performance of isolating the spot area from the background) from *LoST* are above 0.8 for all spot tested sizes

- $F1_m$ scores (assessing the performance of identifying an individual spot at a correct location) ranged from 0.5 to 0.89, with a median above 0.8, for all spot sizes

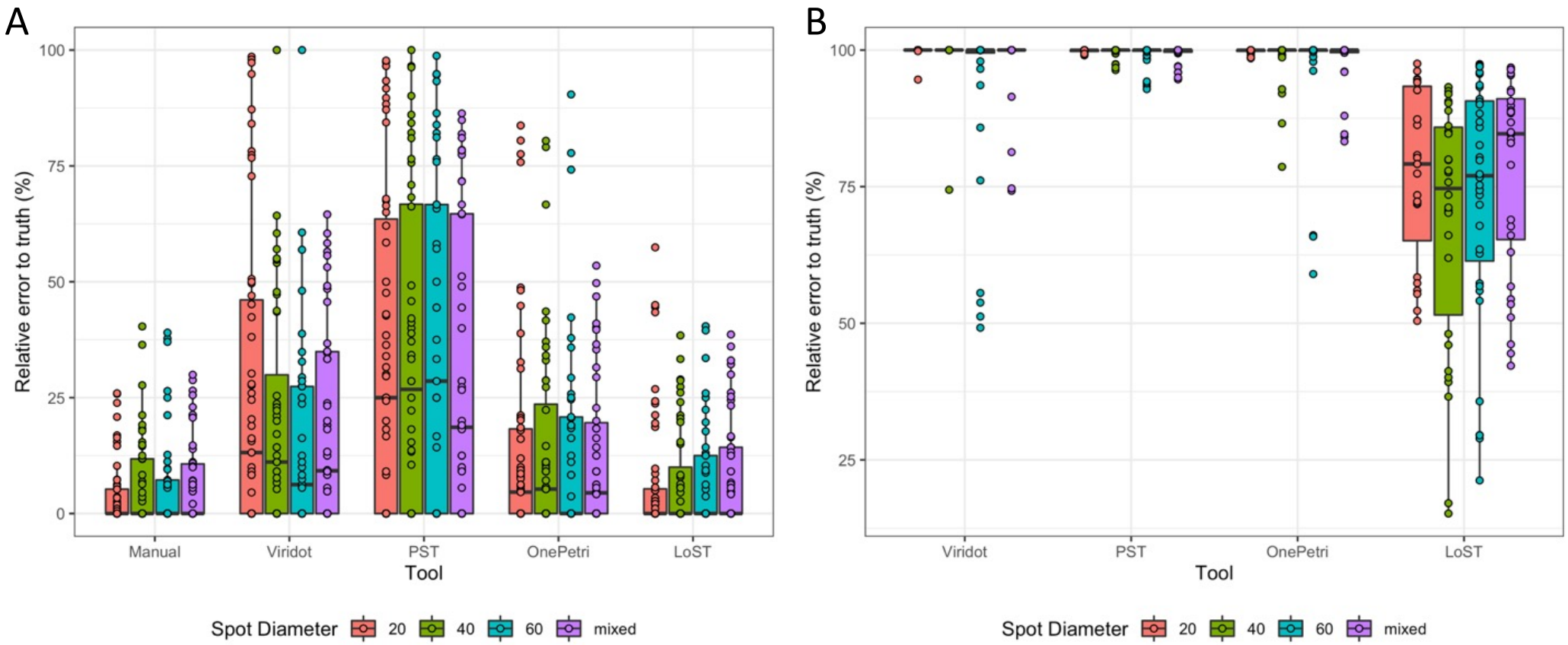
		Spot diameter			
		20	**40**	**60**	**mixed**
Entire spot area ($F1_b$)	median	0.87	0.863	0.846	0.855
	90% prob. interval	0.856-0.903	0.851-0.894	0.825-0.885	0.836-0.883
Individual spots ($F1_m$)	median	0.867	0.853	0.818	0.833
	90% prob. interval	0.544-0.89	0.588-0.87	0.5-0.853	0.51-0.867



$$F1_m = \frac{\sum_{n=1}^{N} w_n F_n}{\sum_{n=1}^{N} w_n} \qquad F1 = \frac{2TP}{2TP + FN + FP}$$

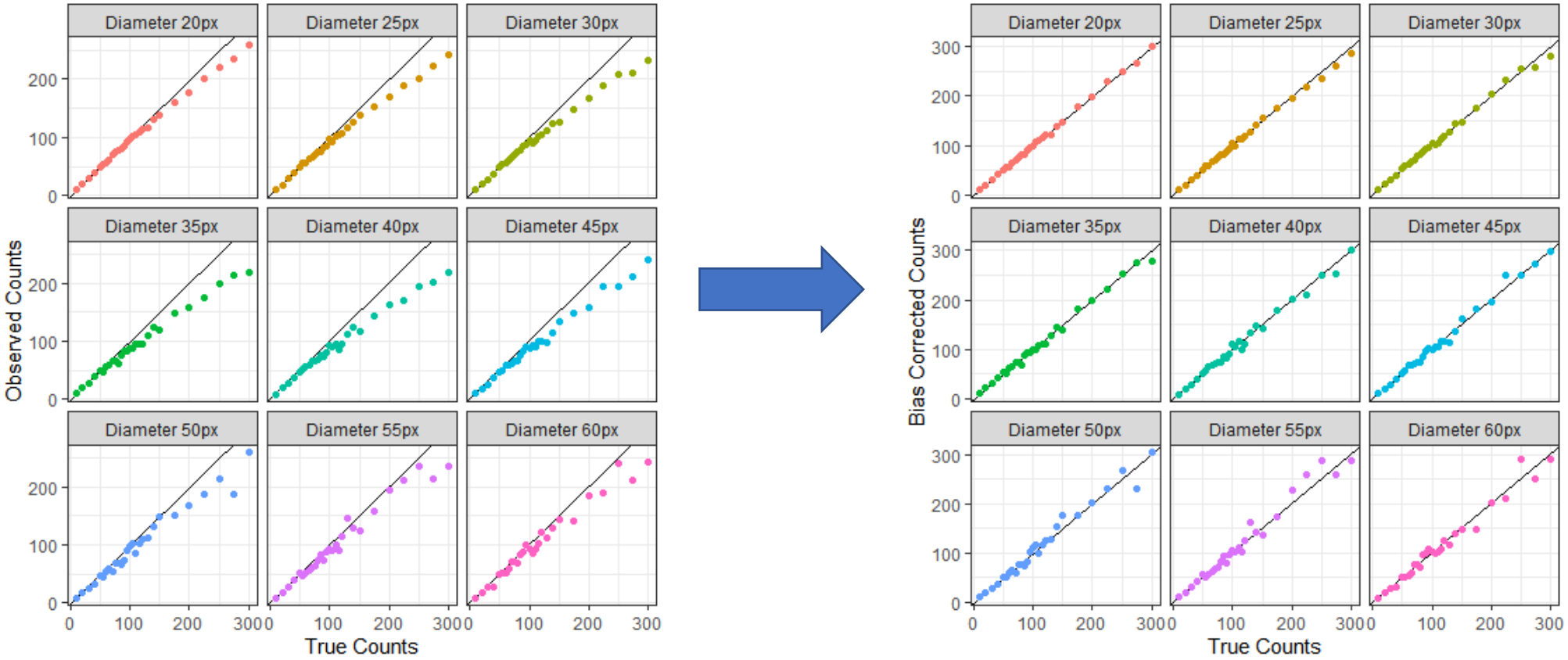
3. Bias correction

Boxplot of the relative error ratio to truth (%) for images with(A) and without (B) manual counts available, grouped by spot size.



Both, human observers and algorithms were unable to separate spots that were almost completely overlapped

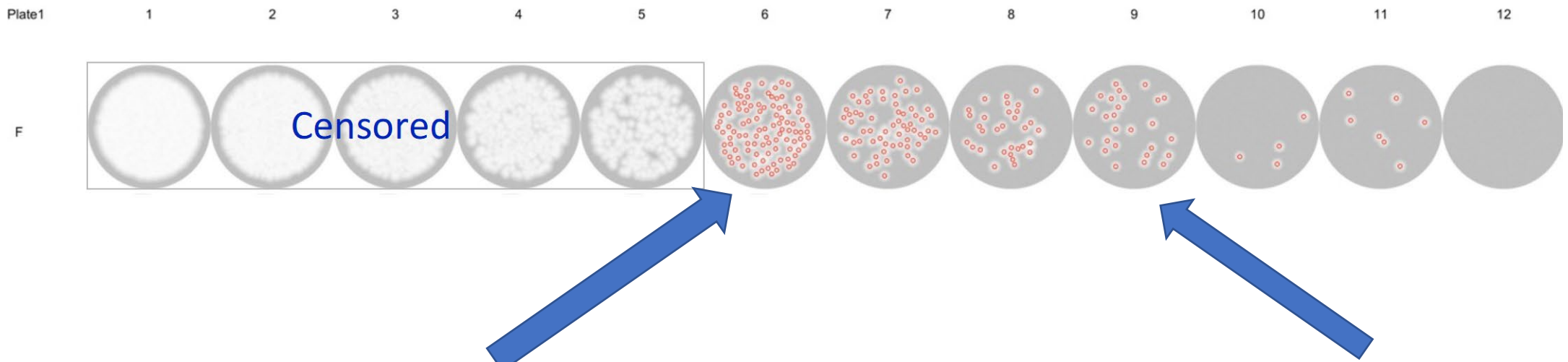
Bias Correction



Bias correction is done by computing probabilities of spot centers being separated by a small distance (delta) given the spot size and observed counts

4. Dilution series modeling

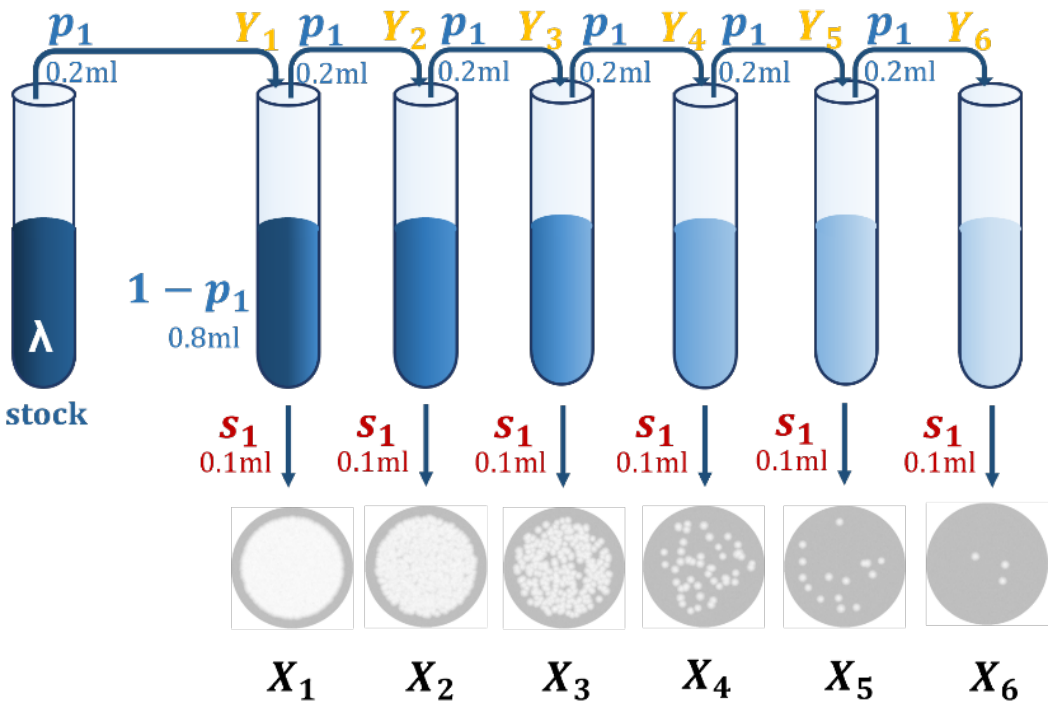
PFU Counts: Single Best and Single First



First countable well

"Best" countable well

PFU Counts Modeling



$$Y_1 \sim Bin(\lambda, p_1), Y_2|Y_1 \sim Bin(Y_1, p_1), \ldots, Y_N|Y_{N-1} \sim Bin(Y_{N-1}, p_1)$$

and

$$X_1|(Y_1 - Y_2) \sim Bin(Y_1 - Y_2, p_2), X_2|(Y_2 - Y_3) \sim Bin(Y_2 - Y_3, p_2), \ldots,$$

$$X_{N-1}|(Y_{N-1} - Y_N) \sim Bin((Y_{N-1} - Y_N, p_2), X_N|Y_N \sim Bin(Y_N, s_1)$$

$$X_1 \sim Bin(\lambda, q_1), X_2 \sim Bin(\lambda, q_2), \ldots, X_N \sim Bin(\lambda, q_N)$$

Approximated by:

$$X_1 \sim Pois(\lambda q_1), X_2 \sim Pois(\lambda q_2), \ldots, X_N \sim Pois(\lambda q_N)$$

Log-Likelihood Solution

$$l_{Bin}(x_1, x_2, x_3, \dots, x_N;\ \lambda) = \sum_{i=1}^{n} log\big(1 - F_{Bin}(x_i; \lambda; q_i)\big) + \sum_{j=n+1}^{N} log\ f_{Bin}\big(x_j; \lambda; q_j\big)$$

$$\hat{\lambda}_{Pois} = \underset{\lambda \in S}{argmax}\ l_{Poi}(x_1, x_2, x_3, \dots, x_N;\ \lambda)$$

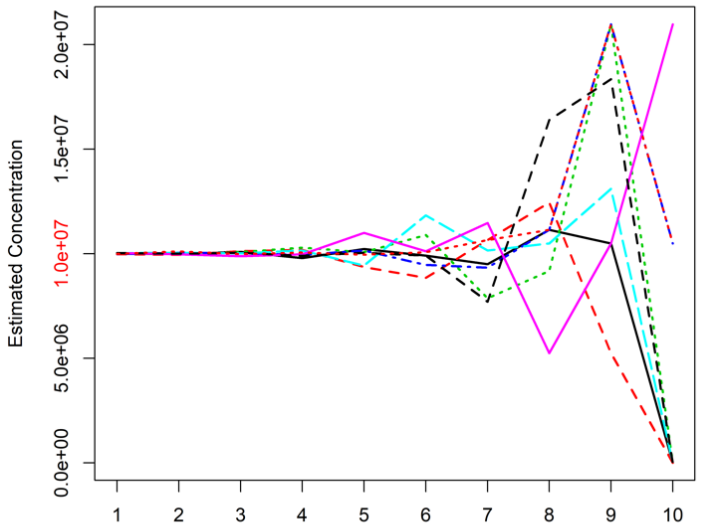
$$l_{Pois}(x_1, x_2, x_3, \dots, x_N;\ \lambda) = \sum_{i=1}^{n} log\big(1 - F_{Pois}(x_i; \lambda q_i)\big) + \sum_{j=n+1}^{N} log\ f_{Pois}\big(x_j; \lambda q_j\big)$$

$$\hat{\lambda}_{Bin} = \underset{\lambda \in S}{argmax}\ l_{Bin}(x_1, x_2, x_3, \dots, x_N;\ \lambda)$$

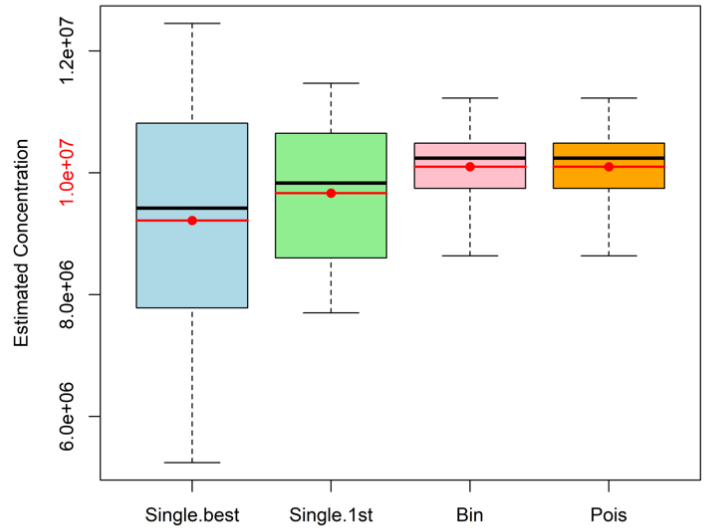
Series Modeling vs. Single Best and Single First

Method	Median	Mean	Mean Bias/λ	SD/λ	MAE/λ	MSE/λ^2
Bin	**10,239,033**	**10,100,252**	**0.0100**	**0.076**	**0.031**	**0.005**
Pois	**10,239,036**	**10,100,254**	**0.0100**	**0.076**	**0.031**	**0.005**
Single, 1st	9,830,400	9,666,560	-0.0333	0.135	0.066	0.017
Single, Best	9,420,800	9,216,000	-0.0784	0.226	0.164	0.051

A



B



5. LoST R package and Shiny application

Automated Spot Counting and Dilution Series Modeling

Counting

Analysis on experiment

Perform automated counting

GO

Choose image files

Browse... No file selected

Select image to be displayed:

A8.CTL

Choose meta file

Browse... No file selected

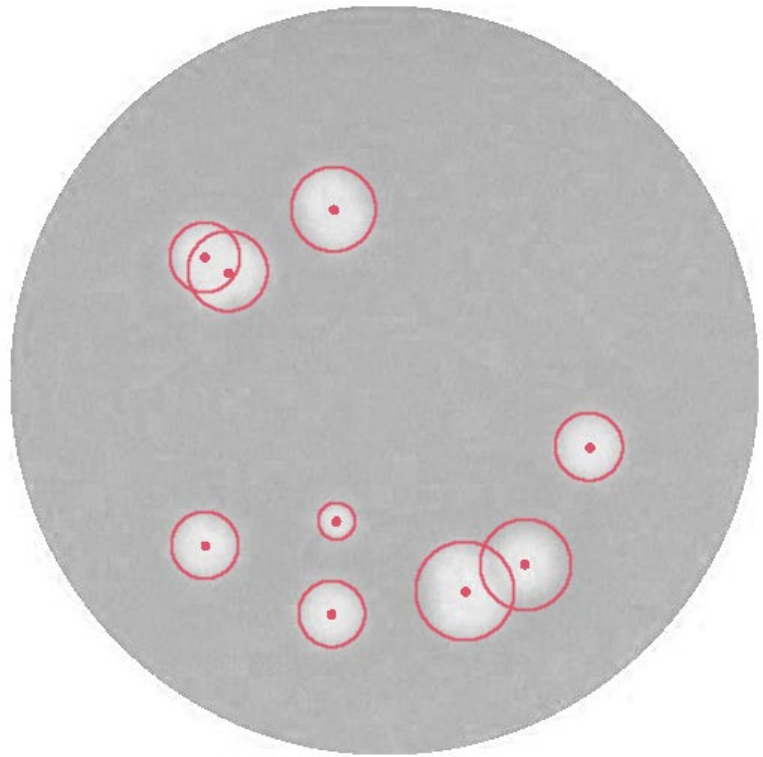
Load demo file

Image | Meta file | Counting result | Neat sample estimate

Analysis with weighted regression

Image name:

A8.CTL



Neat Sample Estimates

Image | Meta file | Counting result | Neat sample estimate | Analysis with weighted regression

Download the data

plate	sample.number	Lambda.est	Bootstrap.CI_L	Bootstrap.CI_R	experiment.group
1.00	1.00	19505.00	12353.00	27957.00	1
1.00	2.00	22756.00	14954.00	30557.00	1
1.00	3.00	105326.00	58514.00	157989.00	2
1.00	4.00	105326.00	55442.23	157989.00	2

Automated Spot Counting and Dilution Series Modeling

Counting | Analysis on experiment

Perform analysis [Analyze]

Select reference/control group:

1

Select variables as additional factors for analysis

group_index

Description:

Perform **weighted t test** when there are only two experimental groups;

Perform **weighted F test** when there are multiple experimental groups;

Perform **weighted linear regression** when there are multiple factors;

Image | Meta file | Counting result | Neat sample estimate | Analysis with weighted regression

Download the data

```
Call:
lm(formula = reformulate(c("experiment.group", names(gp_Addfactor)),
    response = "Lambda.est"), data = df2, weights = df2$weights)

Weighted Residuals:
       1         2         3         4
-0.009710  0.009709  0.061899 -0.063811

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)        16367.6      776.4  21.082   0.0302 *
experiment.group2  77893.3     1894.0  41.126   0.0155 *
Addfactor1          3175.2      490.4   6.474   0.0976 .
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 0.08995 on 1 degrees of freedom
Multiple R-squared:  0.9996,    Adjusted R-squared:  0.9989
F-statistic:  1364 on 2 and 1 DF,  p-value: 0.01915
```

Conclusion

- Automated plaque counting improves data quality and reduces manual workload
- Bias correction accounts for overlaps, hence, improves over “gold standard” manual counting
- Novel approach to combine PFU data from multiple dilutions to back-calculate the concentration in the original sample improves point estimates and confidence intervals
- Shiny web application provides an intuitive, user-friendly interface to the R package to count PFUs, estimate neat sample concentrations, and test hypothesis with weighted regression
- Future: combining data from multiple studies using meta-analysis further increases our confidence in the experimental results

Co-Authors

Special thanks to everyone working on this project and whose work went into this presentation:

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Willem Talloen
Helena Geys
Evangelos Kanoulas
Surya Mohanty

Scientific Team:

Christine Livingston

Robert Vogel

Jack Hartman

Mayukh Das

Q&A

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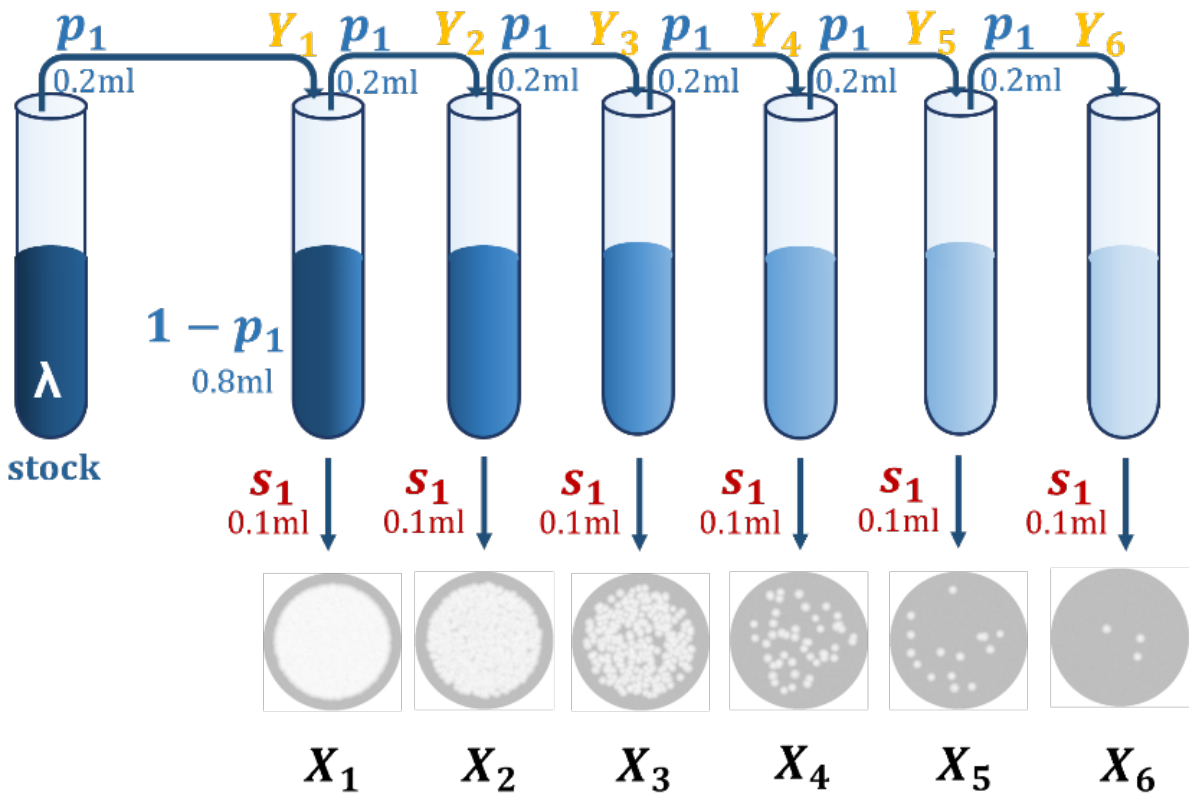
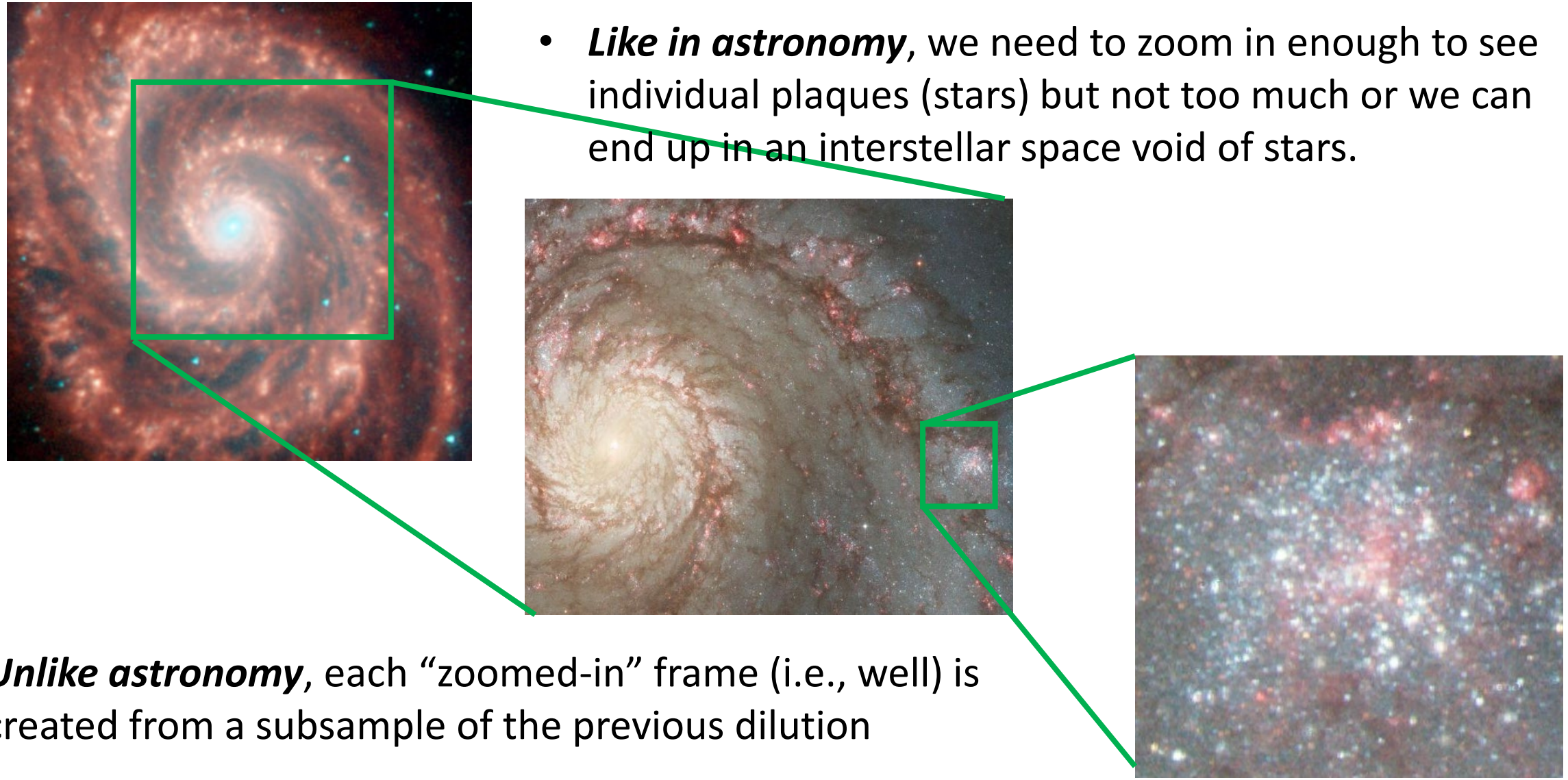
Plaque Counting Assay



- Stock (or neat) samples can be highly concentrated
- The neat samples is serially diluted
- Diluted samples are transferred into petri dishes or multi-well plates pre-populated with the host cells
- After incubation period, the number of plaques (circular spots) in each well is counted.
- Each plaque represents 1 plaque forming unit (PFU) and assumed to come from 1 active viral particle.

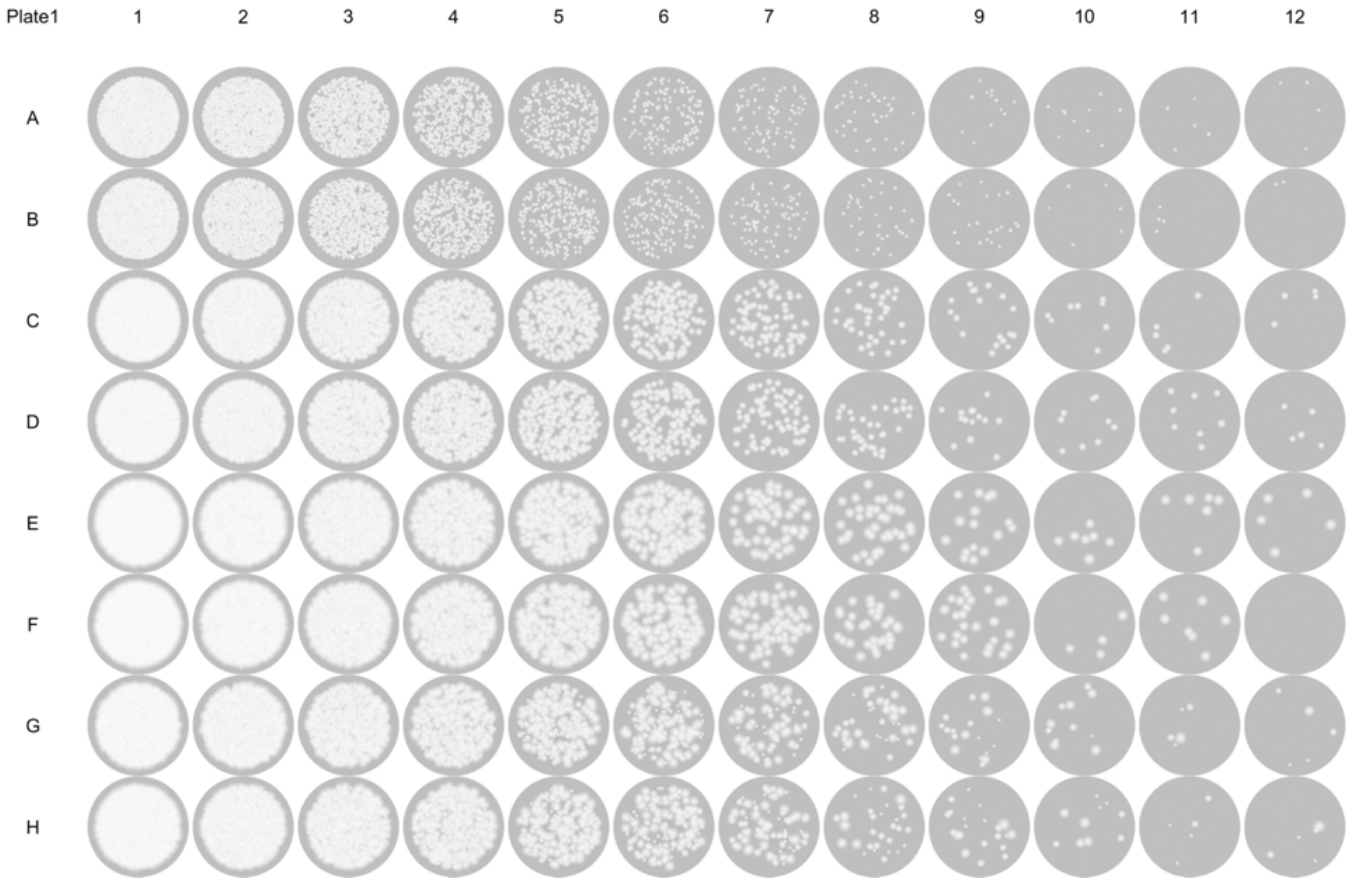
- **M51** Galaxy is 23 million light years away from us

- ***Like in astronomy***, we need to zoom in enough to see individual plaques (stars) but not too much or we can end up in an interstellar space void of stars.



Unlike astronomy, each "zoomed-in" frame (i.e., well) is created from a subsample of the previous dilution

Example: 96-Well Plate Design



- If samples are too concentrated (columns 1:5), the plaques are fused and indistinguishable
- If samples are too diluted (columns 10 to 12), the chances of finding an active particle are slim
- The concentration is “just right” in columns 6 to 9 to be able to count individual PFUs