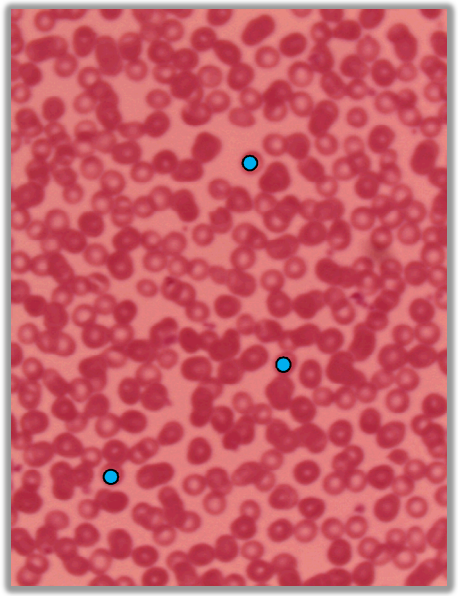
SAND2015-3959C

# Unknown Pathogen Detection in Clinical Samples: A Novel Hyperspectral Imaging and Single Cell Sequencing Approach (165607)

**Primary Objective: to identify and isolate infected cell subpopulations from clinically relevant samples using novel technology based on high-content, high throughput imaging, analysis, and sorting.**



Blood

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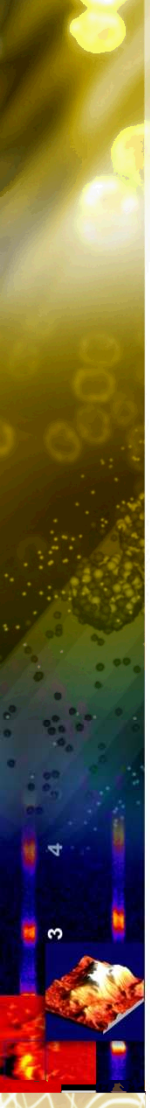
# Project Details

**Principal Investigator: Bryan D. Carson (8631), Program Manager: James Carney (8631)**

**Key Team Members:**

Jerilyn Timlin (8631) --- Spectral data analysis
Stephen Anthony (8631) --- Classification/data analysis
Michael Sinclair (1816) --- Hyperspectral optics
Matt Moorman (1714) --- Microfluidics
Sadie La Bauve (8631) --- Virology, Microbiology
Jaclyn Murton (8631) --- Biological experiments
Kunal Poorey (8623) --- Bioinformatics, data processing
Kelly Williams (8623) --- Bioinformatics, metagenomics



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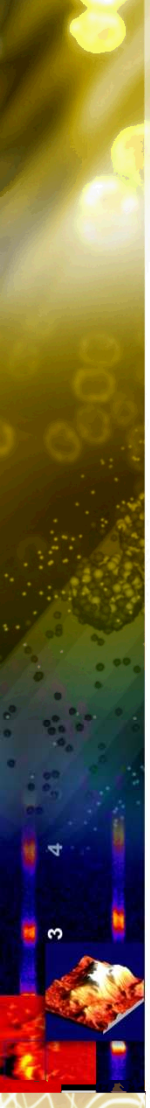
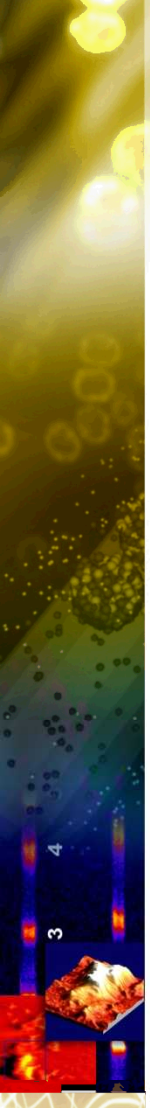
# Who Cares?

- Agnostic method with potential for delivering valuable information about disease state
- Enrichment of rare cells, infected populations
  - Biomarker development
  - Presymptomatic diagnosis
  - Aid in countermeasures and response
- Great interest to all health offices (NIH, DTRA, DHS, etc.)
- Broader impact:
  - Synthetic biology, high content screening applications



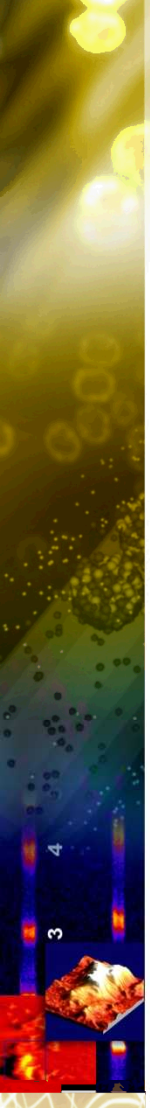
# FY15 Milestones and Progress

- Investigate additional dyes
  - Did a brief look at other dyes, AO is more complicated than we thought, behaves like several dyes in one, chose to focus on AO.
- Hyperspectral images of moving cells
  - Delays in system construction, demands for Mike's time
- Investigate additional viruses
  - Imaged a complete time course of cells infected with adenovirus and cowpox, replicates to follow.
- Sequencing of low input samples
  - Successfully sequenced 1-100 cells, w & w/o virus
- Integrate sorting into microfluidics
  - Manual cell selection via a LabVIEW interface demonstrated, automated cell selection will be completed by the end of May



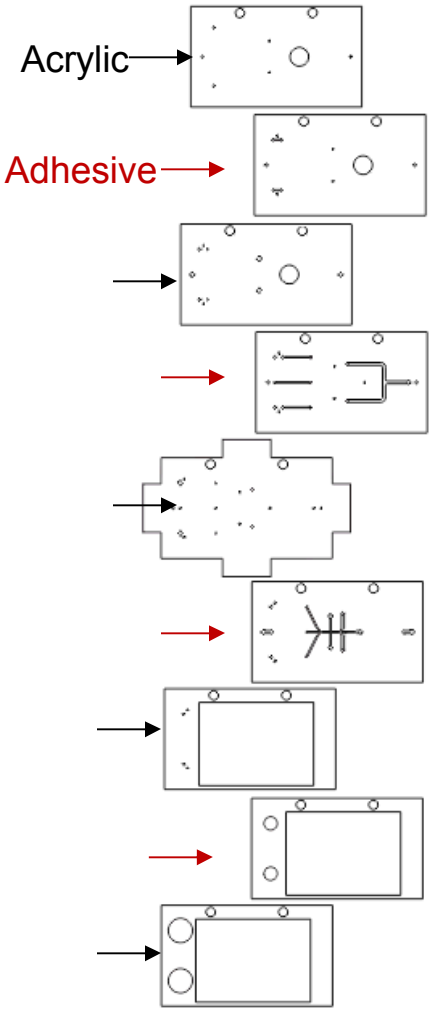
# FY15 Accomplishments

- Microfluidics
  - Refined microfluidics design
  - Demonstrated cell-sorting (manual accomplished, automated in progress)
- Microscope development
  - Constructed and aligned custom prism spectrometer
- Classification of viral infected cells
  - Implemented logistic regression classification methods
  - Expanded to Adeno and Cowpox viruses
  - Developed understanding of spectral behavior of dye
- Single Cell Transcriptomics
  - Successful analysis of 100 cells with detection of virus
  - Single cell sequencing accomplished, not as successful



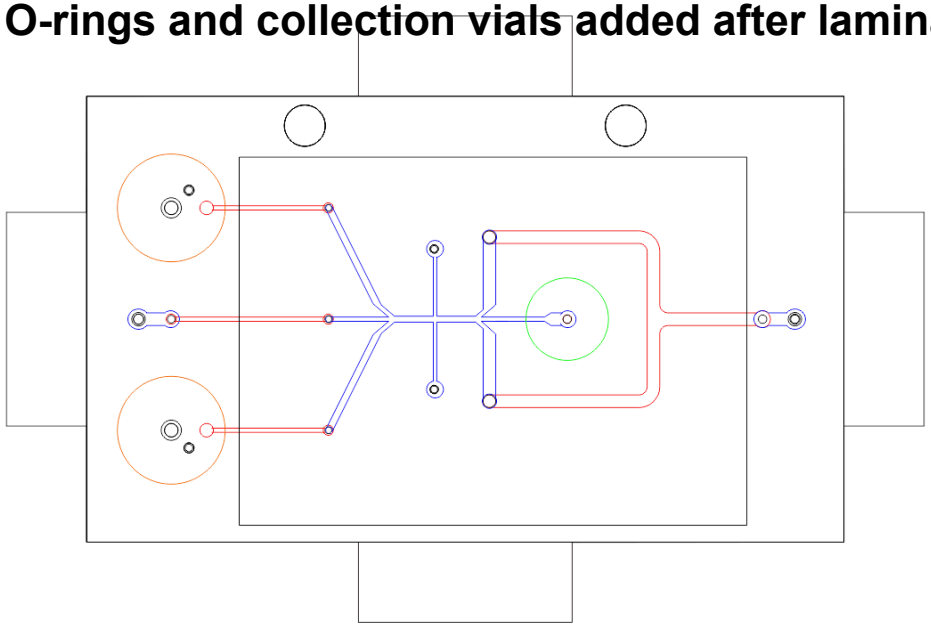
# Microfluidic Chip Design Refined

Nine Layer Design




**Microfluidic chip made from laser cut polymer, glass, and medical-grade adhesive films stacked and pressed into a laminate.**

- **Flexible fabrication technique.**
- **Relatively inexpensive compared to silicon or glass manufacturing processes.**
- **O-rings and collection vials added after laminate**



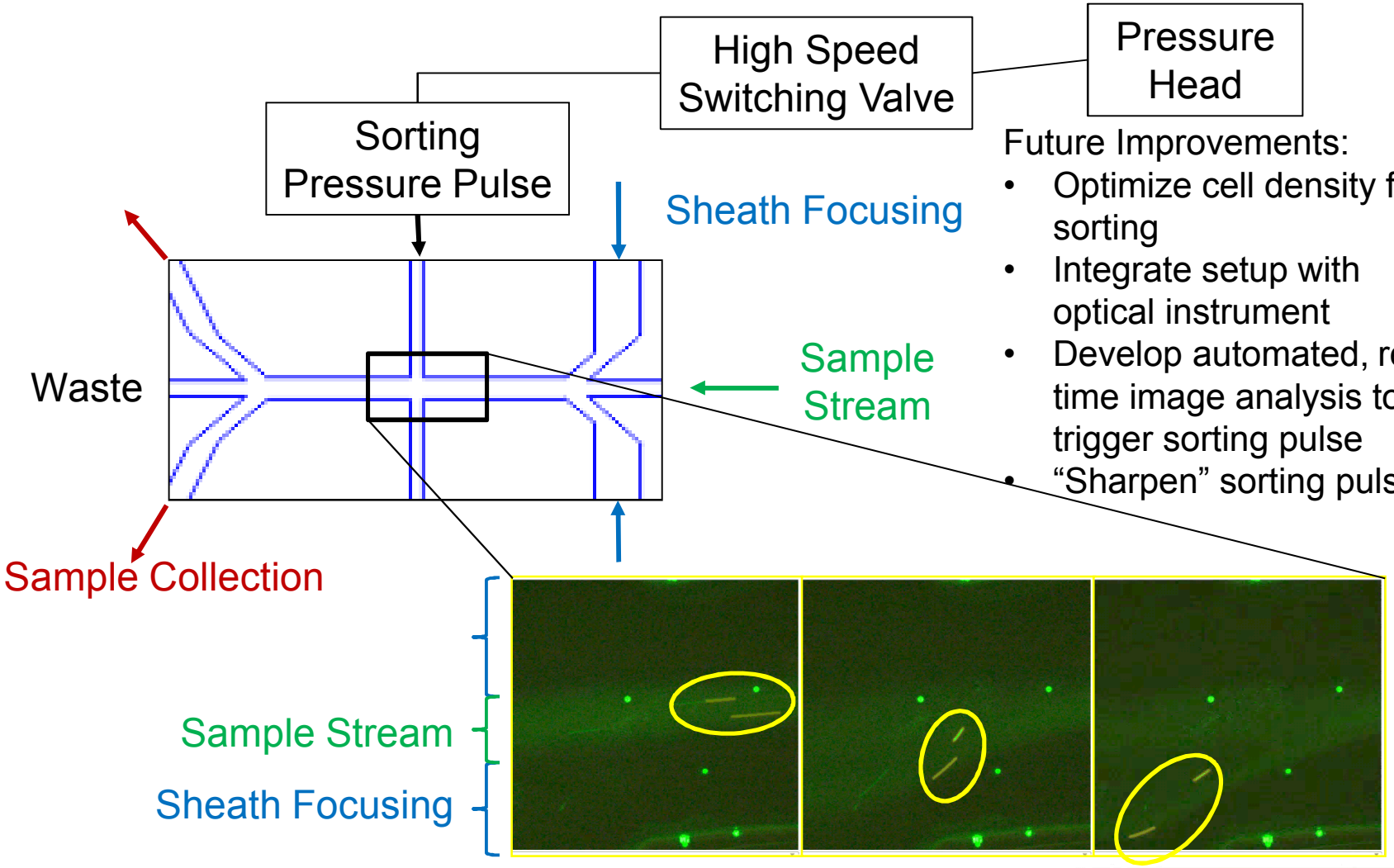
Sample and waste collection

Fluid inlets

Sorting and focusing channels

Cell introduction

# Cell Sorting Demonstrated




Future Improvements:

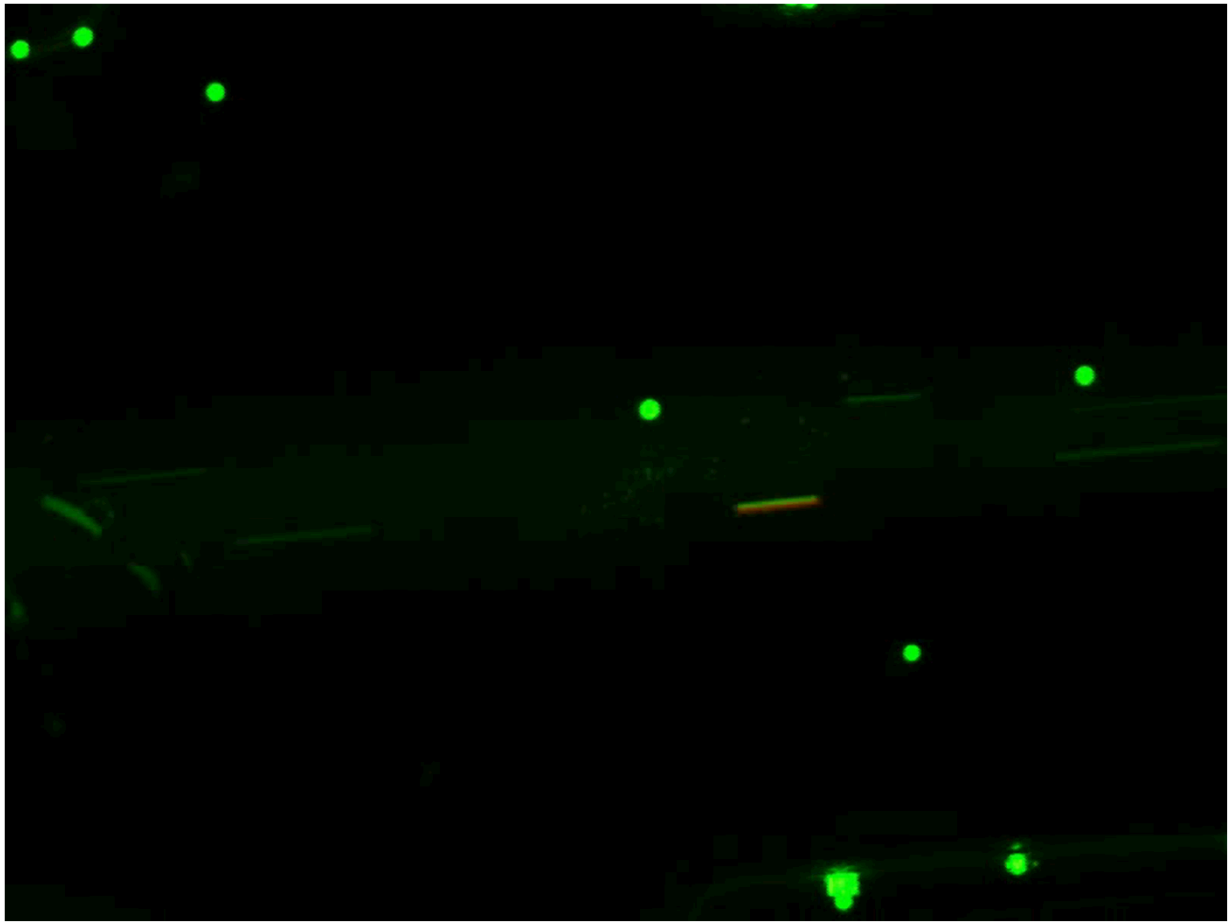
- Optimize cell density for sorting
- Integrate setup with optical instrument
- Develop automated, real-time image analysis to trigger sorting pulse
- "Sharpen" sorting pulse

# Cell Sorting Demonstrated

Sheath

Core

Sheath



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# GUI-enabled Automated Cell Sorting

Current sorting based on color, but easy to add different discriminators such as spectra or MCR results

User defined ROI selection & thresholds in real-time

Stores original and processed images for cells selected


Performs several image processing in real-time, capable of >50 fps

GUI controls color camera acquisition, expandable to other cameras

Greg Poulter, 1814



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# HI-Sort Instrument Status

**Much progress has been made:**

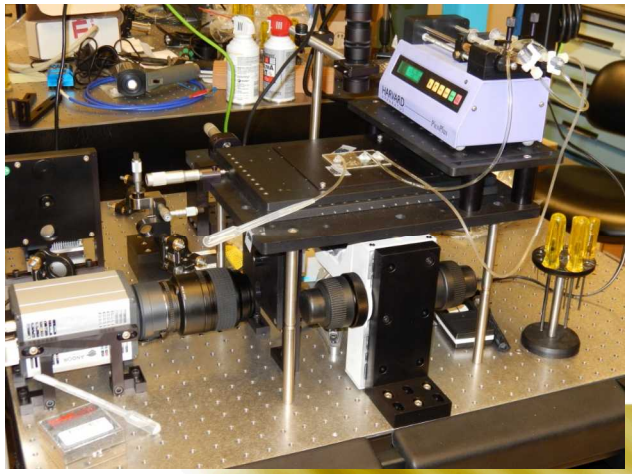
- High performance prism spectrometer completed
  - Fully custom design to match HI-Sort instrument needs
- High speed strobe illumination converted to Epi configuration
- Custom galvo-mirror controller completed

**But … development has fallen behind schedule:**

- Optics house had problems fabricating custom prism --- 6 weeks delay
- Troubles with hyperspectral confocal consumed much time
- Unanticipated project (now finished) decreased bandwidth

**Now progressing at a rapid pace:**

- Final integration is commencing
- Should have first flowing cell images in 3wks
- Cell tracking --- 7wks
- Full system operational by August



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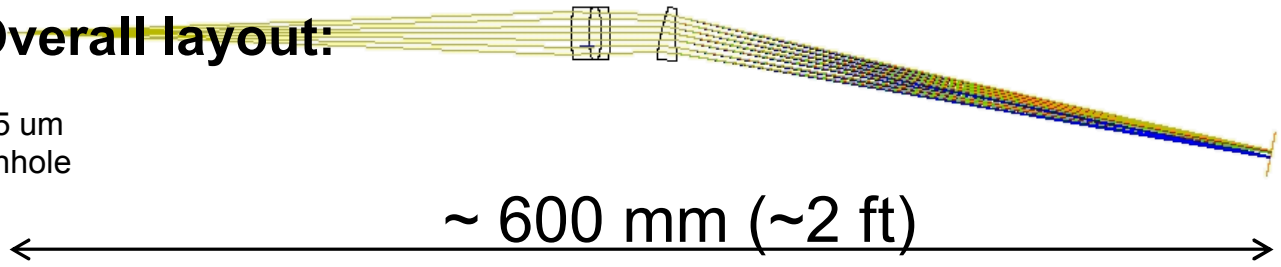
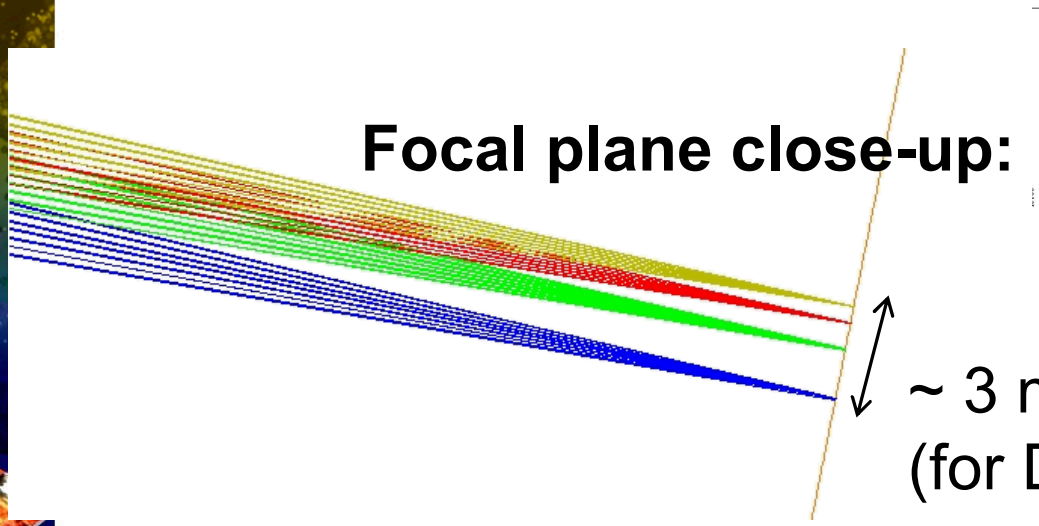
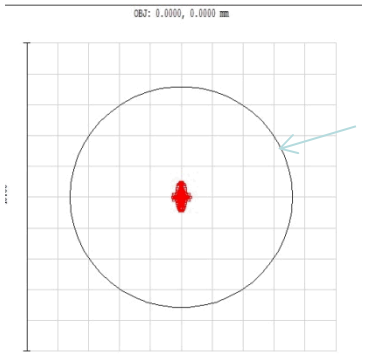
# High Performance Prism Spectrometer: Optical Design

## Overall layout:




## Focal plane close-up:

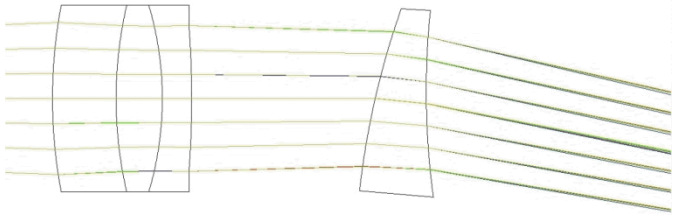




Airy disk ~= pixel size

~ 3 mm
(for DU860 detector)

## Custom fabricated optics:



triplet lens  meniscus wedge prism

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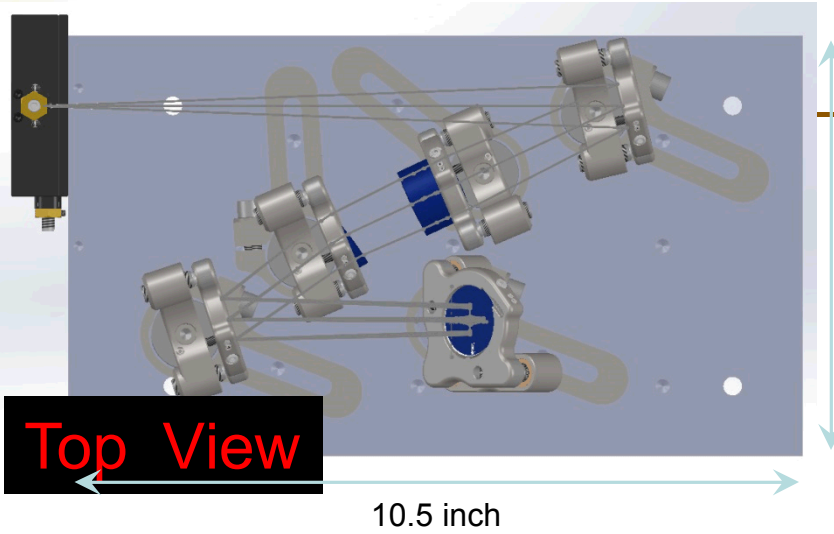
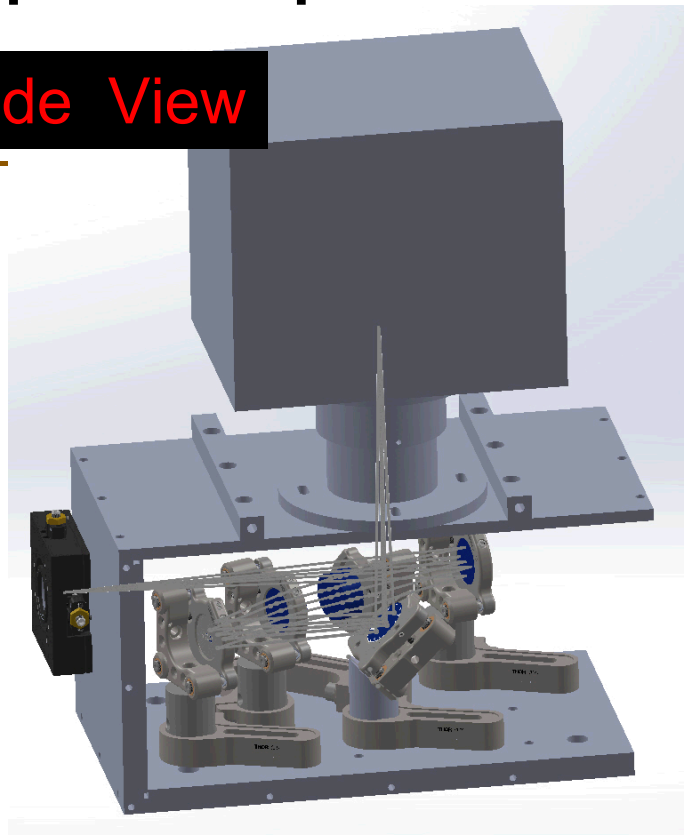
# Optomechanical Design: Folded Beam Path for Compact Footprint




## Completed Spectrometer


- Alignment is tricky!
- Initial characterization confirms as-designed performance

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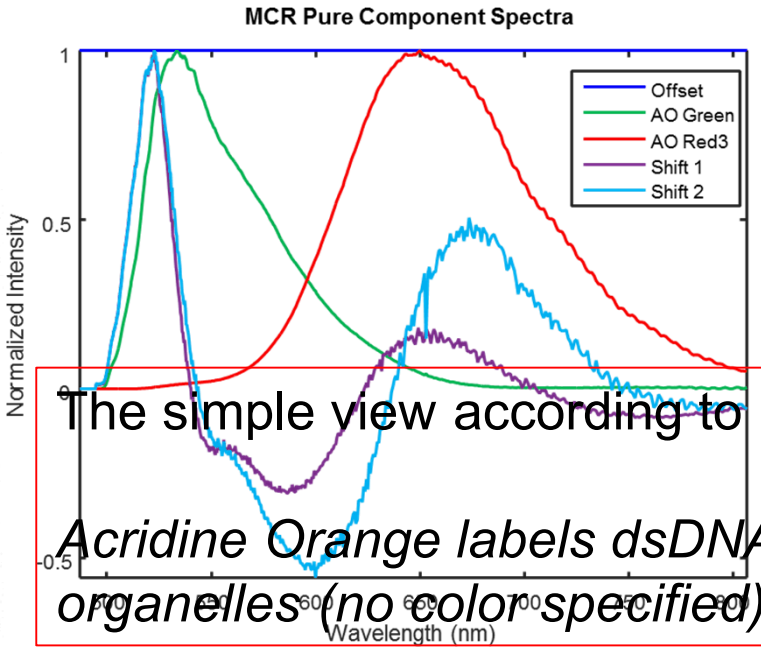
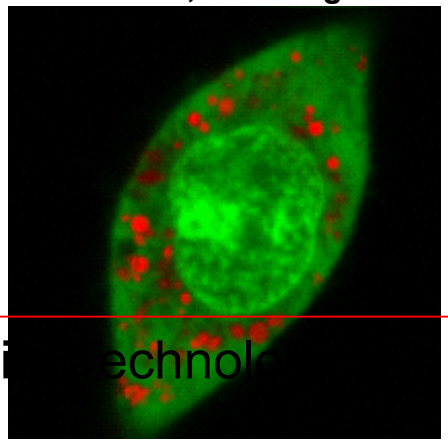
# Analysis: Adeno Virus Infected Cells

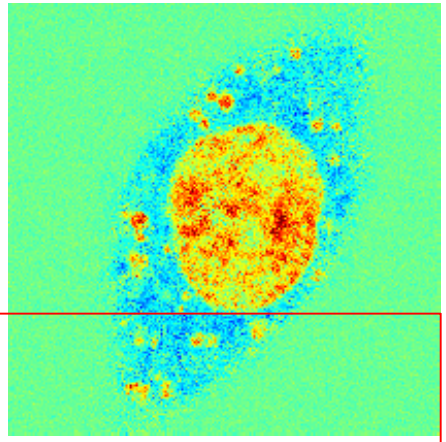
## 18 hrs post infection, MOI = 12.5




R= AO red, G = AO green



Sum of Shift 1 & Shift 2




The simple view according to Li[illegible]echnol

*Acridine Orange labels dsDNA green, RNA red, and acidic organelles (no color specified)*

- Acridine orange is a solvato-chromatic dye
- Spectral shifts related to local environment
  - Charge and concentration sensitive

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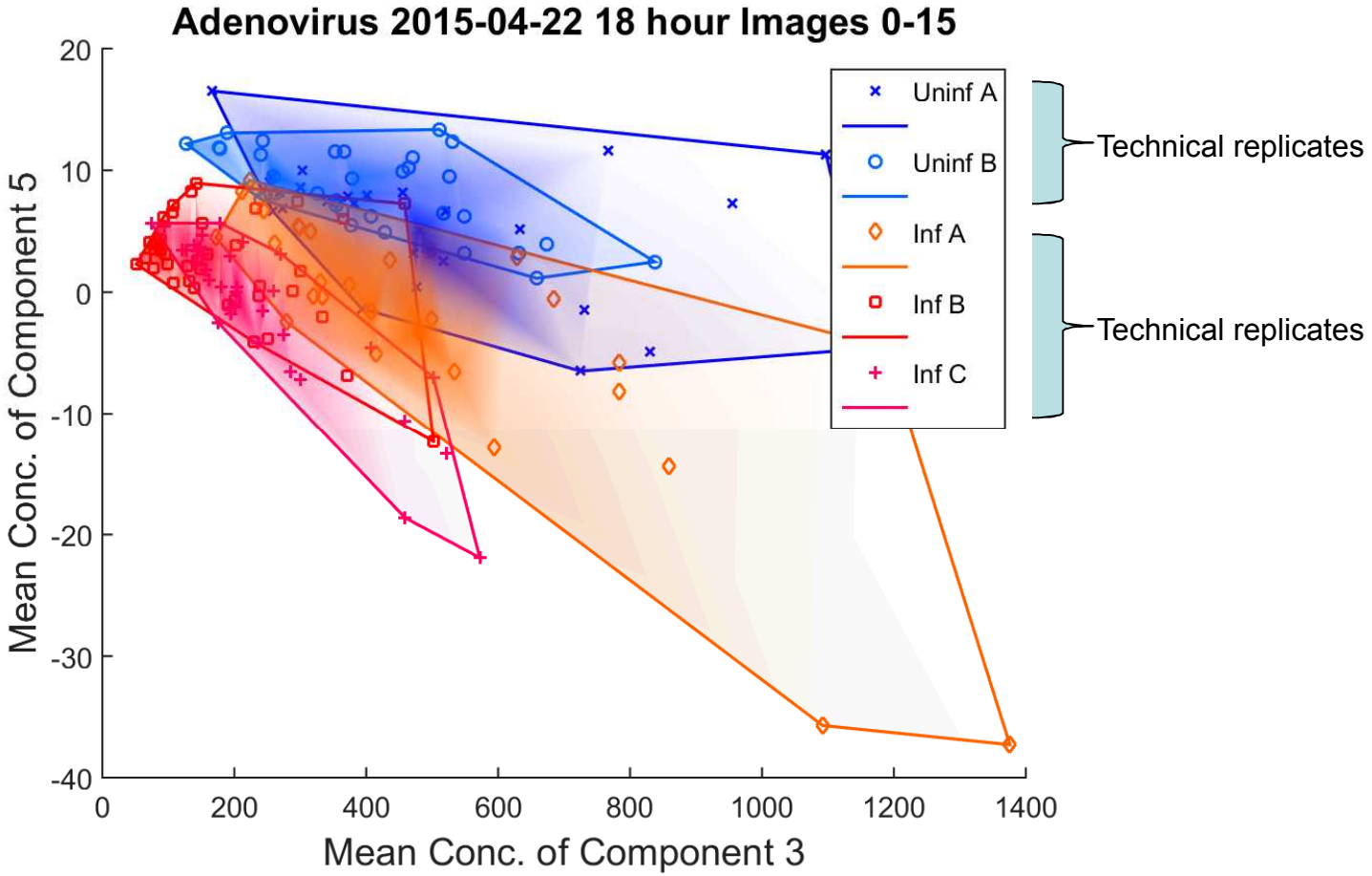
# Classification: Adenovirus Infected Cells

## 18 hrs post infection, MOI = 12.5




- Several possible classifiers were investigated
- Higher order classification not beneficial
- High degree of repeatability over technical replicates

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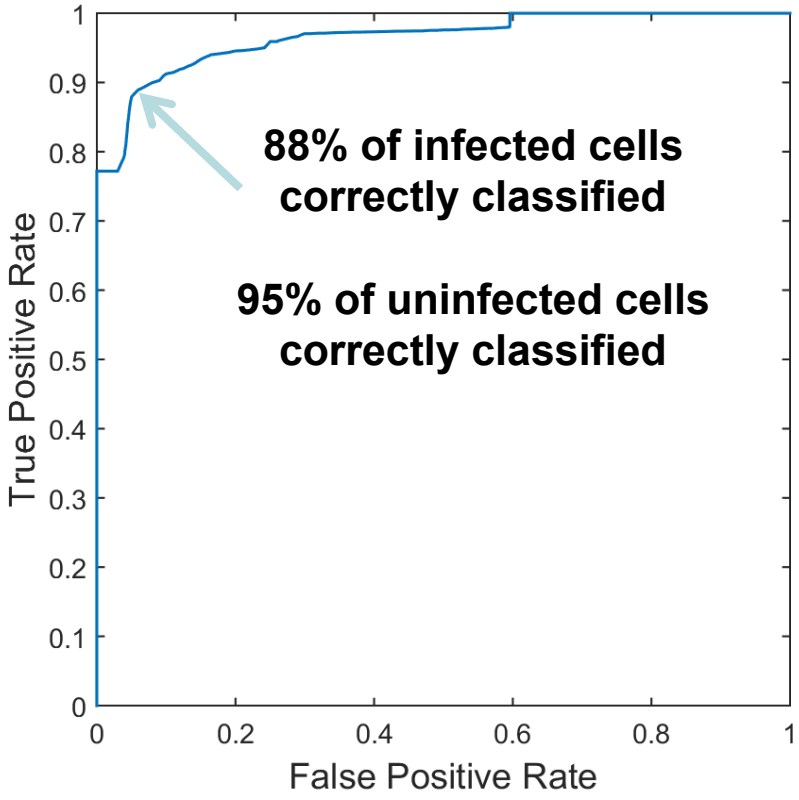
# Classification: Adenovirus Infected Cells

## 18 hrs post infection, MOI = 12.5

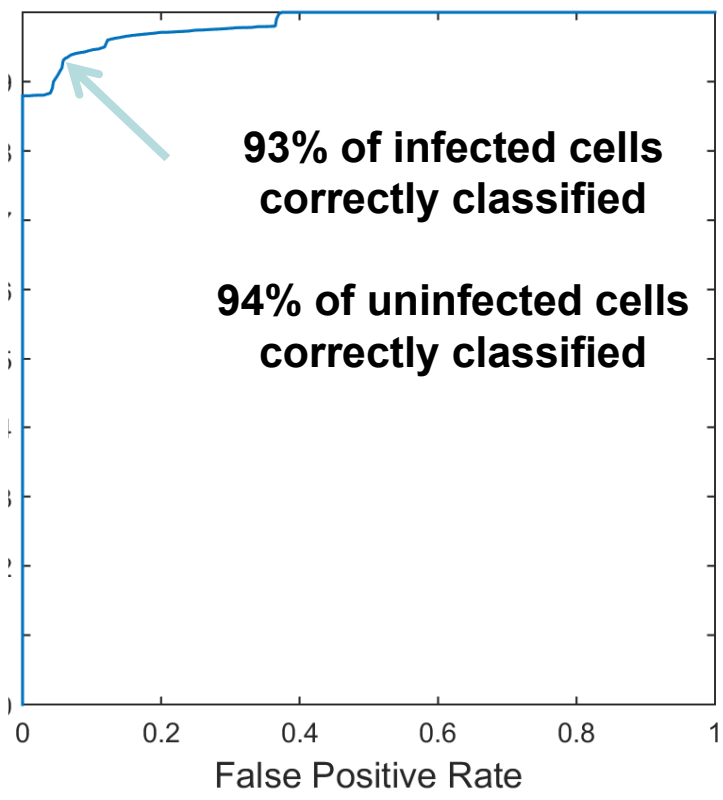
### Infected/Uninfected

**Use all cells**




### Infected/Uncertain/Uninfected

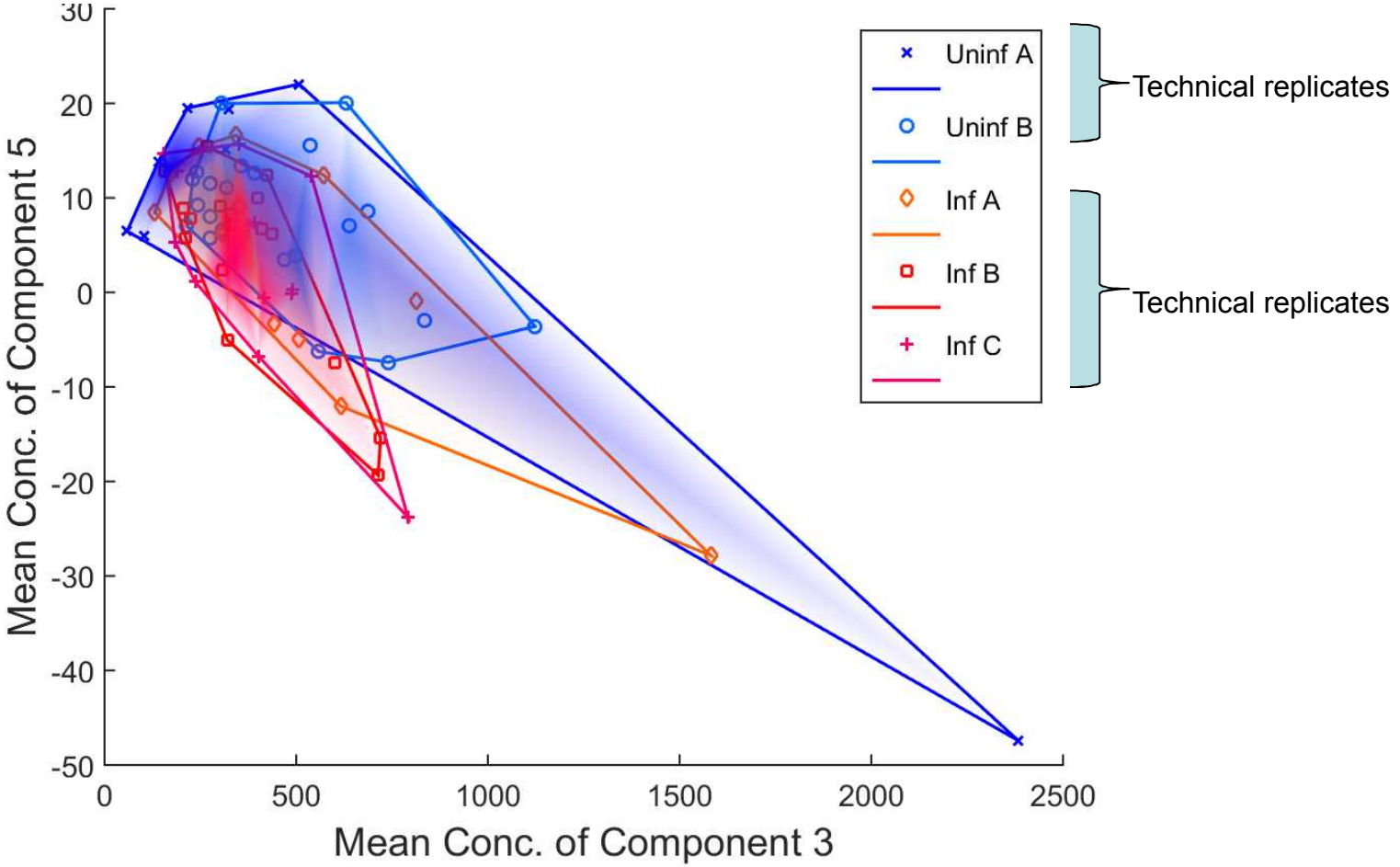
**Discard 13% of cells with uncertain classification**




Logistic regression, *uninfected n=61 cells, infected n=103 cells*

# Classification: Cowpox Infected Cells

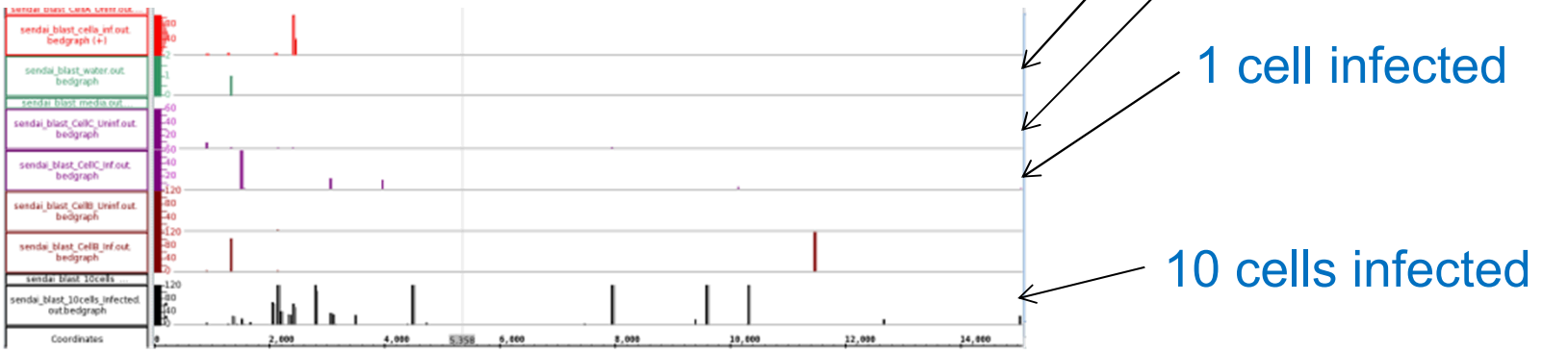
## 18 hrs post infection, MOI = 12.5




- Additional classifiers under investigation
- High degree of repeatability over technical replicates

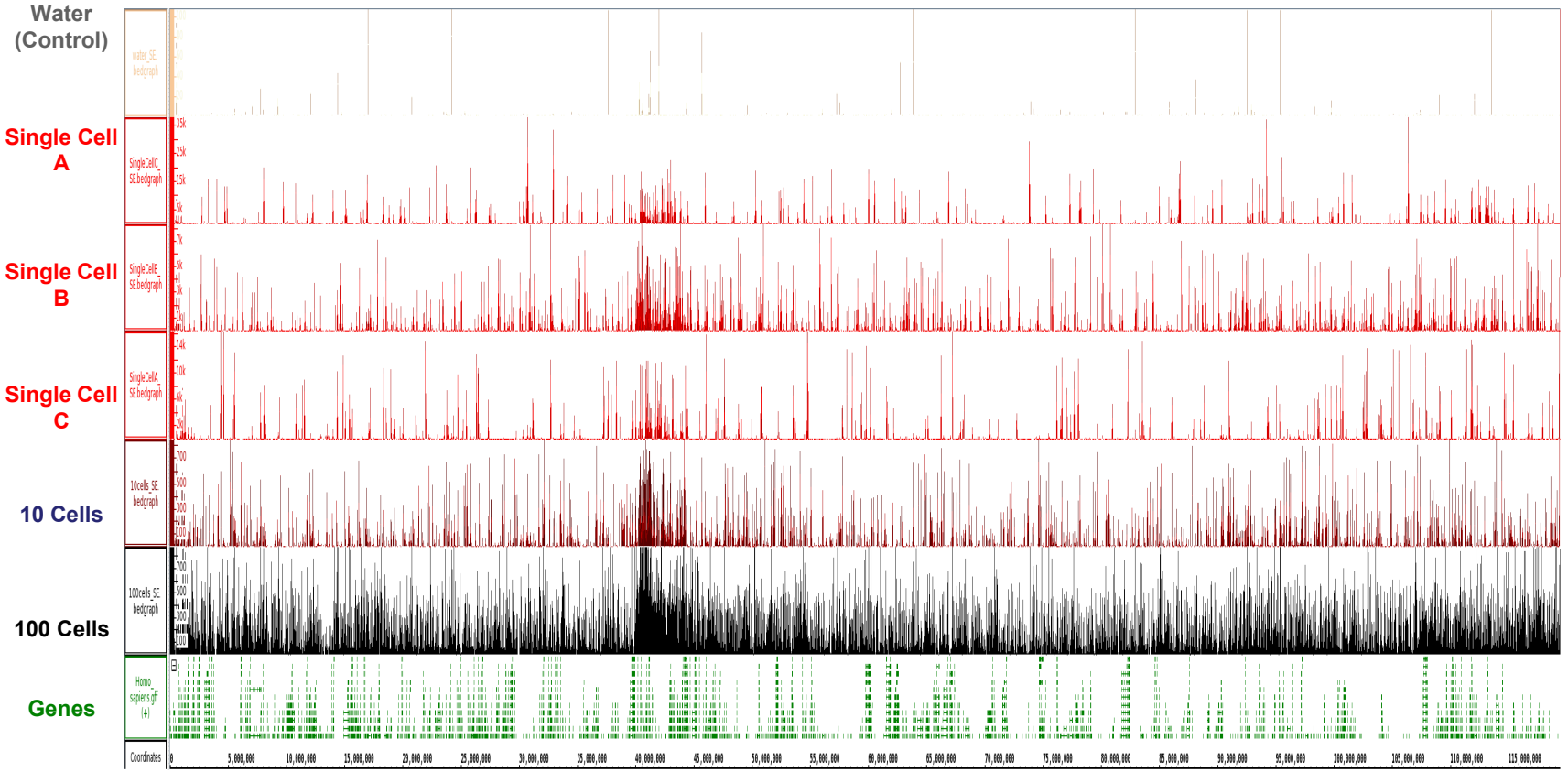
# Single and Few Cell Sequencing Results Summary

- Fewer hits to known infecting virus (Sendai) than expected (but 10-100 cells much better than 1 cell)
- Good transcriptome coverage from 100 cell input with NuGen and HiSeq → good prelim data for what can be done with feasible numbers of sorted cells.



# 1-100 Cell Coverage by HiSeq

## Coverage

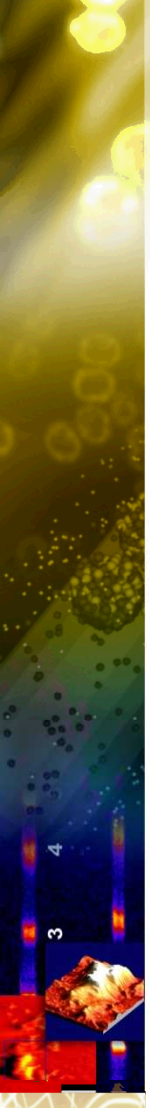



Coordinate along 1st 120 Mbp of Human Chromosome 1

# Publications, Presentations, Awards, External Proposals …

FY 15

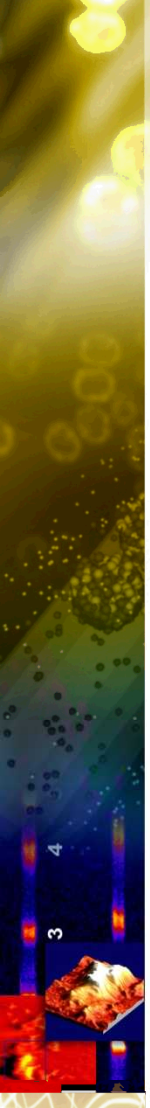
- Poster presentation @ 2015 CBD S&T conference in St. Louis
- Anthony, Carroll-Portillo, Timlin “Dynamics and Interactions of Individual Proteins in the Membrane of Single, Living Cells” in Single Cell Protein Analysis: Methods and Protocols, Springer Press, 2015, in press. (partial support)
- Publication in progress on the classification of viral infected cells using non-specific dyes; *need biological replicates*
- Other publications under consideration:
  - Instrument design
  - Technical note on the photophysics of Acridine orange; need to conduct control experiments under known crowding and charge conditions to lend credibility to findings
  - Improvements in classification methods, need careful assessment of literature and determine uniqueness aspects of approach



# Path Forward

- Complete construction of the instrument
- Complete automated cell sorting integration
- Complete experiments with Adeno and Cowpox viruses (biological replicates)

- Key preliminary data for external proposals – hyperspectral images + classification of flowing cells



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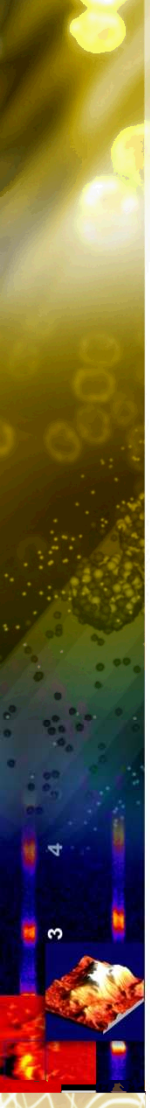
# Extras



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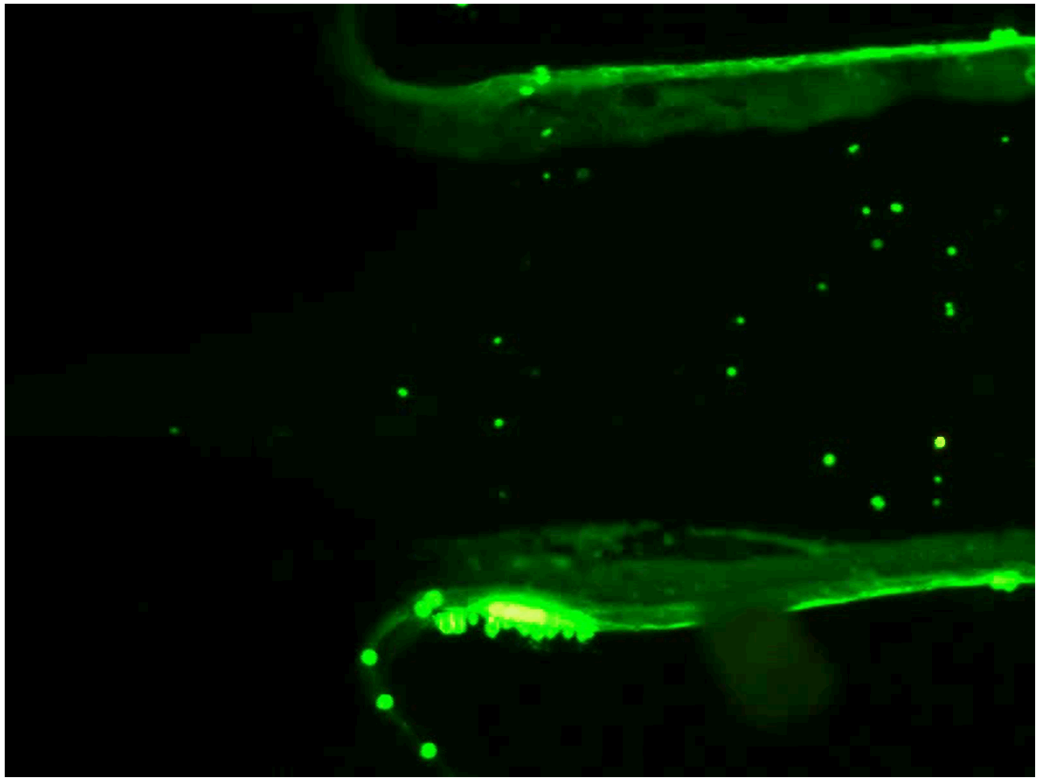
# Cells Get in Line for Interrogation

Observations:

- Cells tumble prior to entering the main stream
- Moderate speed variations and variations in spacing
- Camera speed insufficient for capturing good cell pictures in the flow stream
- 19 frames/sec at 800 x 600 pixels



Cells_test10longdilute.avi

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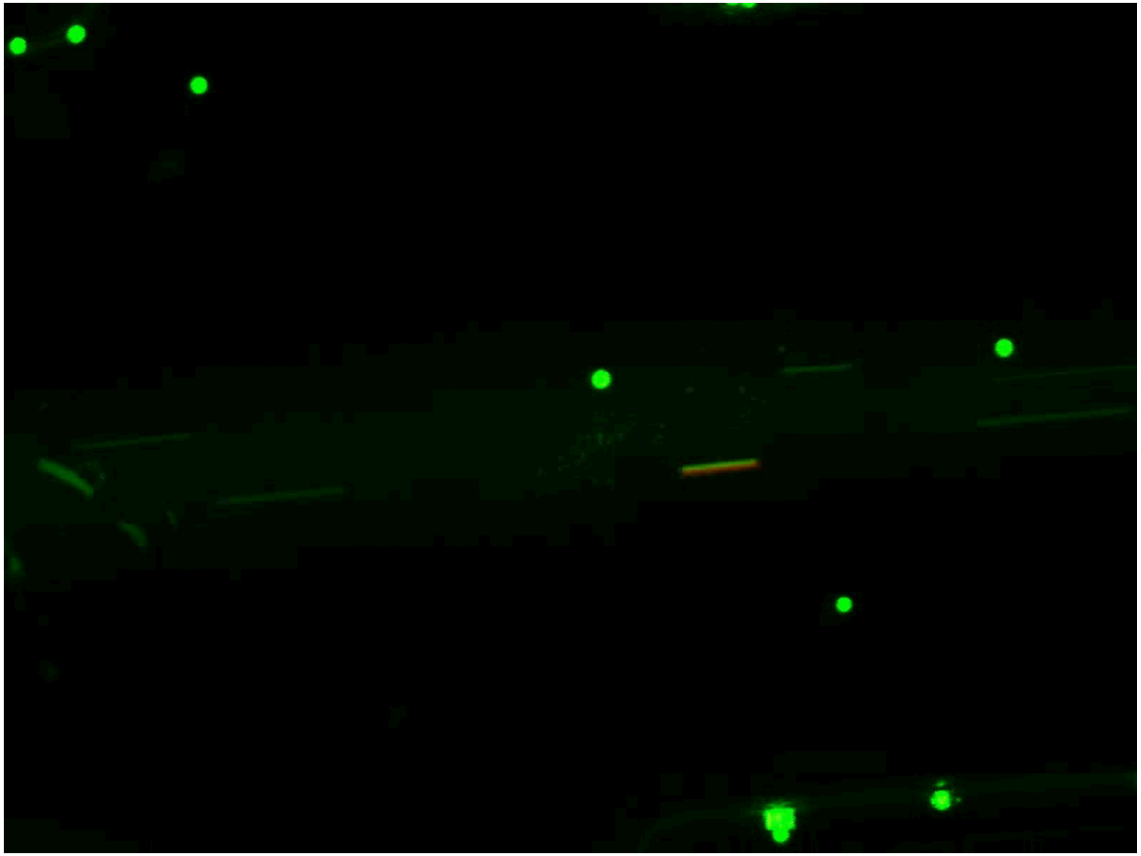
# Cells are Sorted into Diversion Channel

Observations:

- Unmoving solid circles are beads stuck to channel from previous runs
- Cells are not in single file in stream
- Nice clear diversion
- Moderate to extend halting of motion following a trigger is possible, but not a guarantee



Cells_test7longdilute.avi

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# Cell Sorting

Acquisition time + time between frames is approximately 50 msec.

19 frames/sec at 800 x 600 pixels

Cells_test6longdilute.avi – last 3 frames

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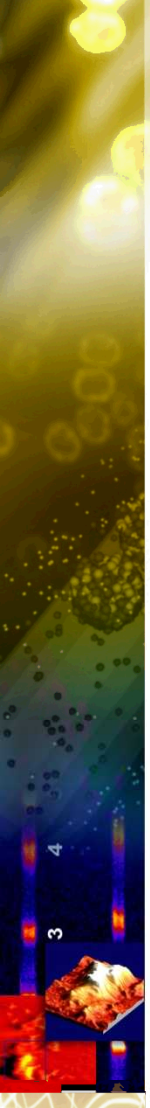
# Next Steps

- Need to calibrate microscope field of view so we have a scale bar
- Need to optimize the concentration of cells to get close to single file as possible
- Experiments to understand the range of speed available.
  - What is the slowest reliable speed?
  - Can we achieve sufficient distance between cells to ensure only one sorts?



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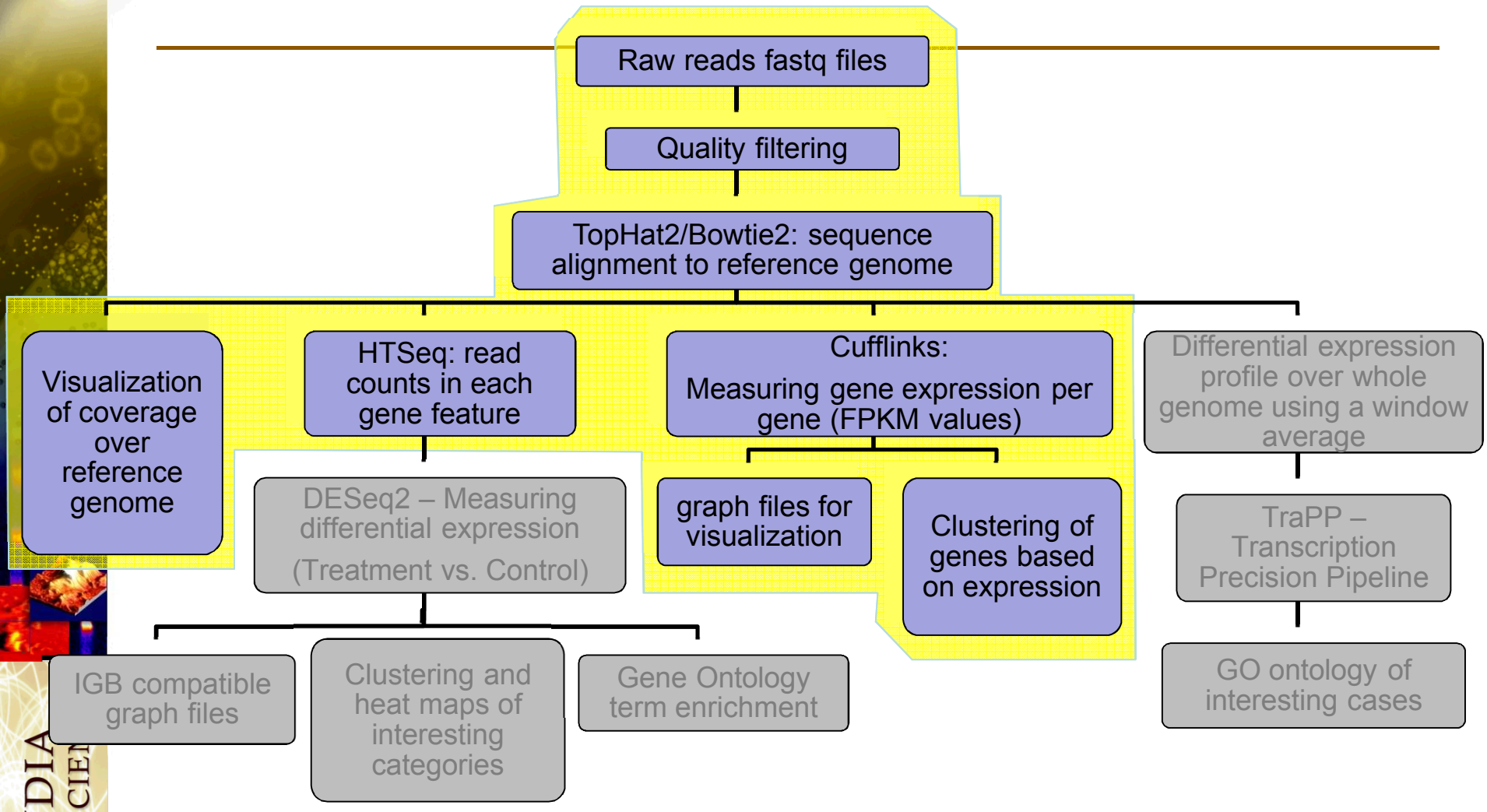
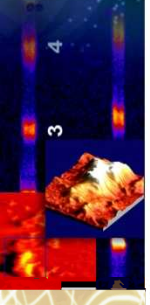
# Transcriptomics Pipeline



# Four Experiments

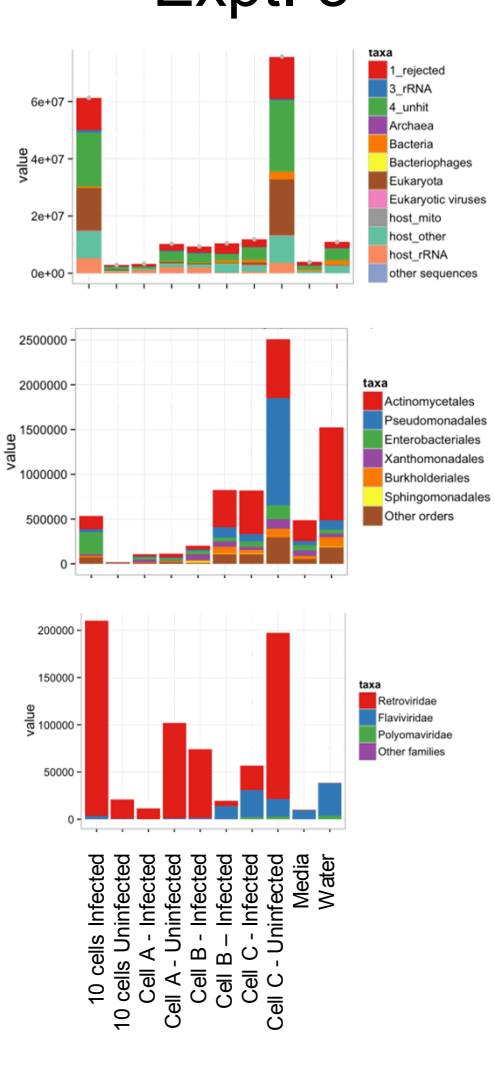
- Exp. 1. Uninfected Human Cells, MiSeq
  - Lesson: need more reads, turn to HiSeq
- Exp. 2. Uninfected Human Cells, HiSeq
- Exp. 3. Mouse macrophage line infected with Sendai virus, June 2014
- Exp. 4. Mouse macrophage line infected with higher MOI Sendai virus, Oct 2014
  - Both Expts. 3 and 4 show (with no improvement in Expt. 4)
    - Low Sendai virus counts
    - Contamination by Dengue virus and bacteria

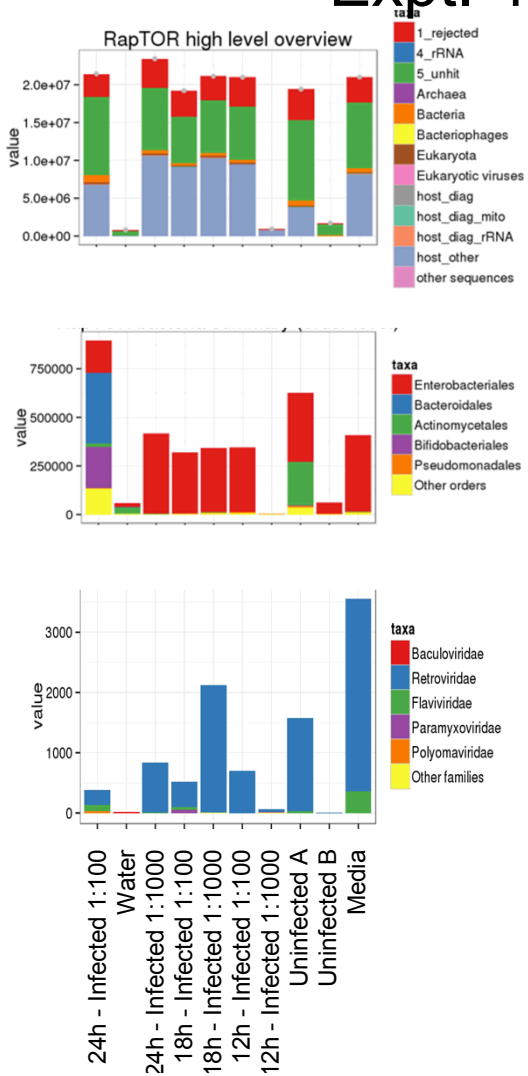


# Sendai Infecting Mouse Macrophages

Expt. 3

Expt. 4 (higher MOI)




High-level overview

Bacterial breakdown

Viral breakdown

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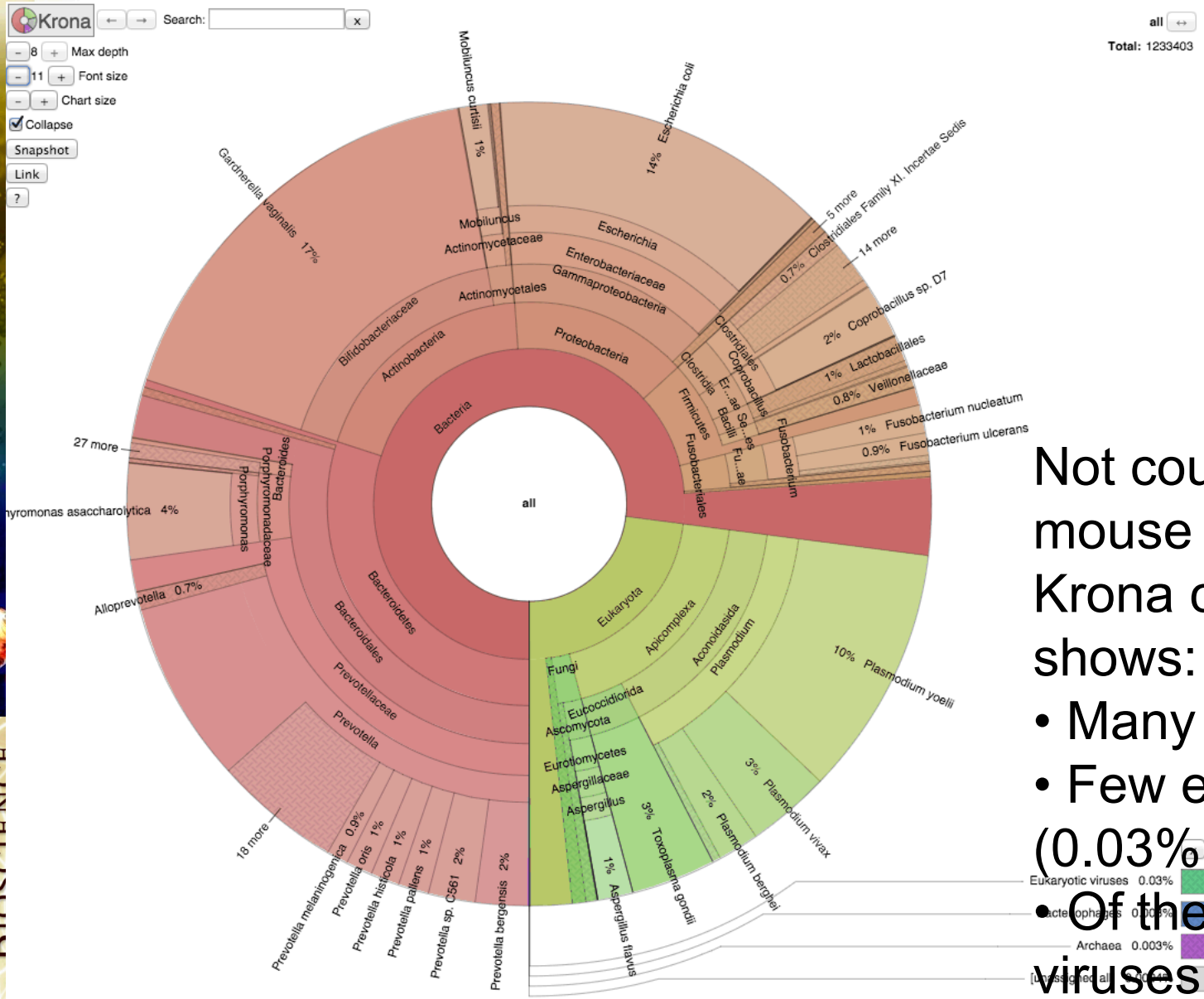
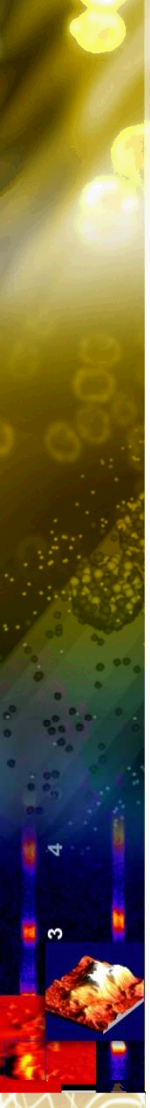
# Expt. 4: Taxa Detected




Not counting mouse hits, Krona chart shows:

- Many bacteria
- Few euk viruses (0.03%)
- Of the euk viruses, only 0.5%

# Expts. 1 & 2 – Human data



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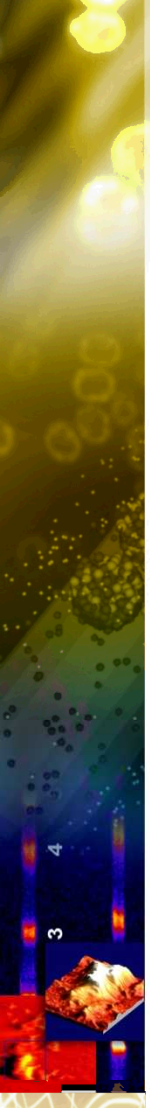

# Exp 3-4
# Mouse Infected high-sort data
# June & Oct run

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# Summary of alignment to mouse genome

Reads aligned to Mouse Genome

Uninfected10cell: Mapped : 2123374 (74.1% of input)
Infected10cell: Mapped : 37620736 (61.4% of input)

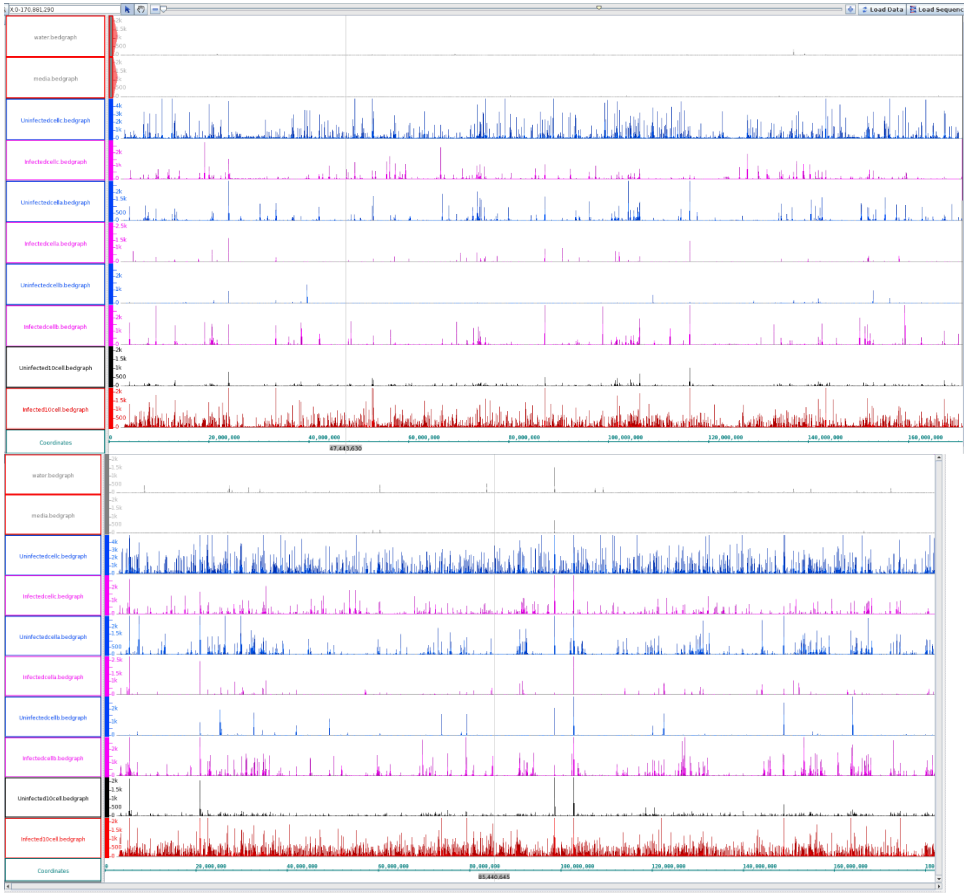
Uninfectedcella: Mapped : 7077073 (68.5% of input)
Infectedcella: Mapped : 1709098 (51.4% of input)

---

Uninfectedcellb: Mapped : 628865 ( 6.0% of input) ← Bad run (I will check what went wrong with this sample)
Infectedcellb: Mapped : 6062065 (64.5% of input)

Uninfectedcellc: Mapped : 43933878 (58.0% of input)
Infectedcellc: Mapped : 3402168 (28.6% of input)

media: Mapped : 48829 ( 1.3% of input)
water: Mapped : 109820 ( 1.0% of input)



The RapTOR

There are traces of Sendai virus both in RapTOR output and also the quick and dirty analysis, which I performed with blast. see the table below

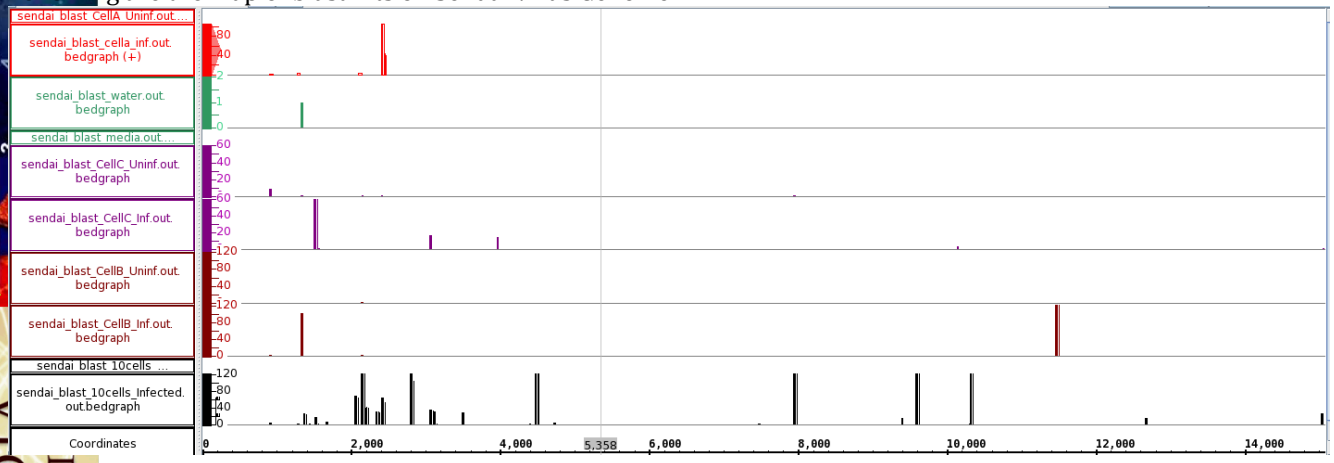
| RapTOR analysis | 10 Infected | 10 Un Inf | Cell A Inf | Cell A Un Inf | Cell B Inf | Cell B Un Inf | Cell C Inf | Cell C Un Inf | media | water |
|---|---|---|---|---|---|---|---|---|---|---|
| Eukaryotic viruses;Retro-transcribing;Retroviridae;Gammaretrovirus;Murine leukemia virus | 100865 | 9746 | 4262 | 52192 | 38935 | 1995 | 10069 | 100446 | 71 | 106 |
| Eukaryotic viruses;ssRNA positive-strand;Flaviviridae;Flavivirus;Dengue virus | 3117 | 517 | 160 | 929 | 937 | 13730 | 28480 | 18811 | 9552 | 34906 |
| Eukaryotic viruses;Retro-transcribing;Retroviridae;Gammaretrovirus;Abelson murine leukemia virus | 14479 | 1509 | 1270 | 6873 | 7965 | 1717 | 1625 | 14367 | 19 | 27 |
| Eukaryotic viruses;Retro-transcribing;Retroviridae;Betaretrovirus;Mouse mammary tumor virus | 10339 | 678 | - | 2053 | 2957 | 158 | 3524 | 614 | 3 | 4 |
| Eukaryotic viruses;Retro-transcribing;Retroviridae;Gammaretrovirus;Moloney murine sarcoma virus | 4366 | 263 | 8 | 616 | 1450 | 9 | 527 | 9345 | 15 | 10 |
| Eukaryotic viruses;Retro-transcribing;Retroviridae;Gammaretrovirus;Spleen focus-forming virus | 7693 | 870 | 51 | 2190 | 1326 | 3 | 842 | 843 | 1 | 1 |
| Eukaryotic viruses;Retro-transcribing;Retroviridae;Gammaretrovirus;Murine leukemia-related retroviruses | 5842 | 297 | 427 | 1519 | 950 | - | 202 | 2318 | 1 | - |
| Eukaryotic viruses;dsDNA;Polyomaviridae;Polyomavirus;Simian virus 40 | 110 | 52 | - | 62 | 278 | 245 | 2122 | 2394 | 55 | 3376 |
| Eukaryotic viruses;Retro-transcribing;Retroviridae;Gammaretrovirus;Murine osteosarcoma virus | 2160 | 482 | 6 | 217 | 564 | 192 | 665 | 2407 | - | 1 |
| **Eukaryotic viruses;ssRNA negative-strand;Mononegavirales;Paramyxoviridae;Respirovirus;Sendai virus** | **489** | **-** | **4** | **-** | **-** | **-** | **52** | **-** | - | **-** |
| Eukaryotic viruses;Retro-transcribing;Retroviridae;Betaretrovirus;Mason-Pfizer monkey virus | - | - | - | - | - | - | 50 | - | - | - |
| Eukaryotic viruses;Retro-transcribing;Hepadnaviridae;Orthohepadnavirus;Hepatitis B virus | - | - | - | - | - | - | 27 | - | - | - |
| Eukaryotic viruses;ssRNA positive-strand;Luteoviridae;Polerovirus;Potato leafroll virus | - | - | - | - | - | 13 | - | - | - | - |
| Eukaryotic viruses;Retro-transcribing;Retroviridae;Lentivirus;Human immunodeficiency virus 1 | 2 | - | - | 2 | - | - | 2 | 4 | - | 1 |
| Eukaryotic viruses;dsDNA;Adenoviridae;Mastadenovirus;Human adenovirus F | - | - | - | - | - | - | - | | 6 | - |
| Eukaryotic viruses;dsDNA;Ascoviridae;Ascovirus;Trichoplusia ni ascovirus 2c | 5 | - | - | - | - | - | - | | - | - |
| Eukaryotic viruses;Retro-transcribing;Retroviridae;Gammaretrovirus;Feline leukemia virus | 3 | - | - | - | - | - | - | 1 | - | - |
| Eukaryotic viruses;ssRNA positive-strand;Flaviviridae;Flavivirus;Yokose virus | - | - | - | - | - | - | 1 | 2 | - | - |
| Eukaryotic viruses;dsDNA;Phycodnaviridae;Chlorovirus;Paramecium bursaria Chlorella virus 1 | - | - | - | - | - | - | 3 | - | - | - |
| Eukaryotic viruses;ssRNA positive-strand;Virgaviridae;Tobamovirus;Tobacco mosaic virus | - | - | - | - | - | - | - | - | - | 2 |
| Eukaryotic viruses;dsDNA;Herpesvirales;Herpesviridae;Varicellovirus;Bovine herpesvirus 1 | 2 | - | - | - | - | - | - | - | - | - |
| Eukaryotic viruses;dsDNA;Herpesvirales;Herpesviridae;Macavirus;Alcelaphine herpesvirus 1 | - | 1 | - | - | - | - | - | - | - | - |
| Eukaryotic viruses;dsDNA;Baculoviridae;Alphabaculovirus;Bombyx mori NPV | - | - | - | - | 1 | - | - | - | - | - |
| Eukaryotic viruses;dsDNA;Herpesvirales;Herpesviridae;Rhadinovirus;Ateline herpesvirus 3 | - | - | - | - | - | 1 | - | - | - | - |
| Eukaryotic viruses;Retro-transcribing;Retroviridae;Gammaretrovirus;Woolly monkey sarcoma virus | - | - | - | 1 | - | - | - | - | - | - |
| Eukaryotic viruses;ssRNA negative-strand;Arenaviridae;Arenavirus;Lassa virus | - | - | - | - | - | 1 | - | - | - | - |

Method 2 Blast of Genome seq to sequensing run

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Dengue | 2505! | 131! | 131! | 725! | 685! | 10097! | 24636! | 16423! | 7090! | 29610! |
| Sendai | 1901! | 0! | 114! | 0! | 255! | 1! | 108! | 13! | 0! | 1! |

!

Following are the map of blast hits on Sendai virus Genome



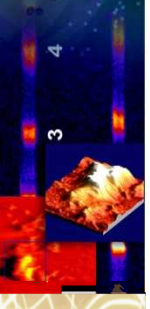

# Sequence mapping to Sendai virus were very low.

# High Sort with higher MOIs

Exp 5

Oct run

Kunal

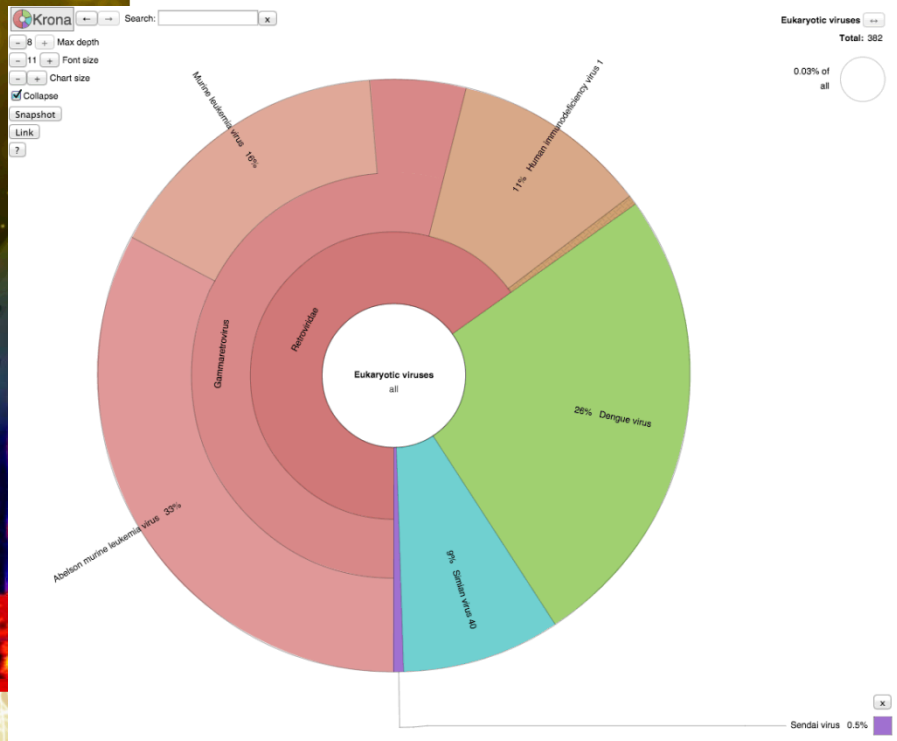


SANDIA BIOSCIENCE

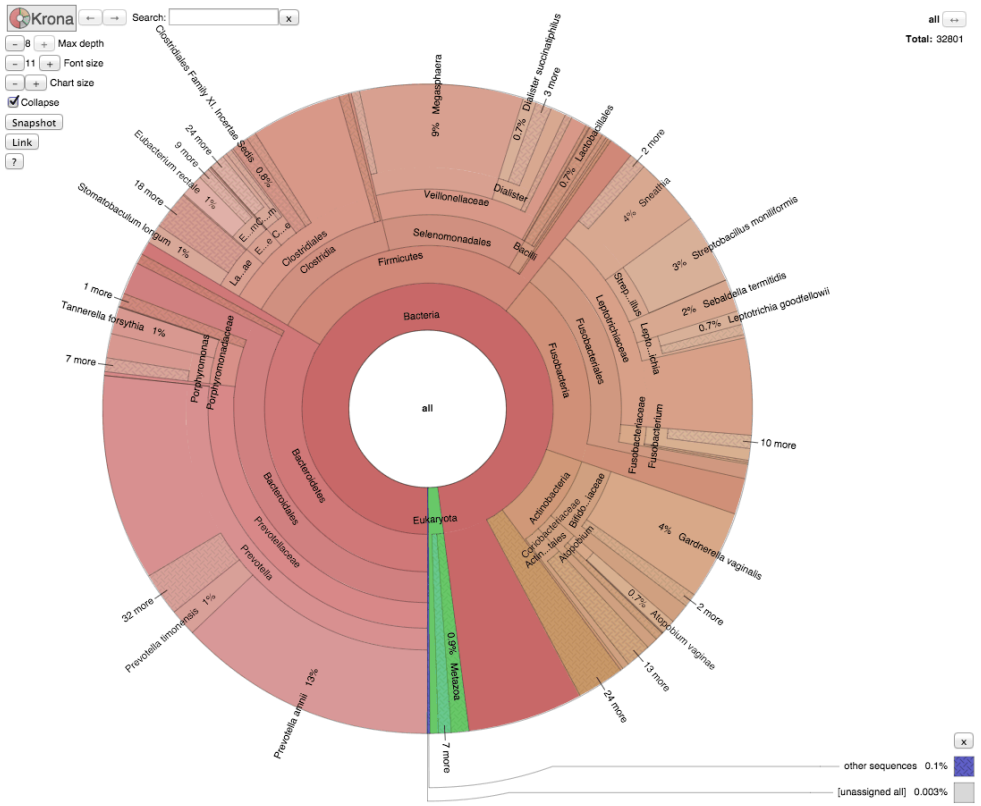
Sandia National Laboratories

# Infected 24h 1:100

## Virus hits




## rRNA hits




Sendai virus in very low quantities and many bacterial hits.