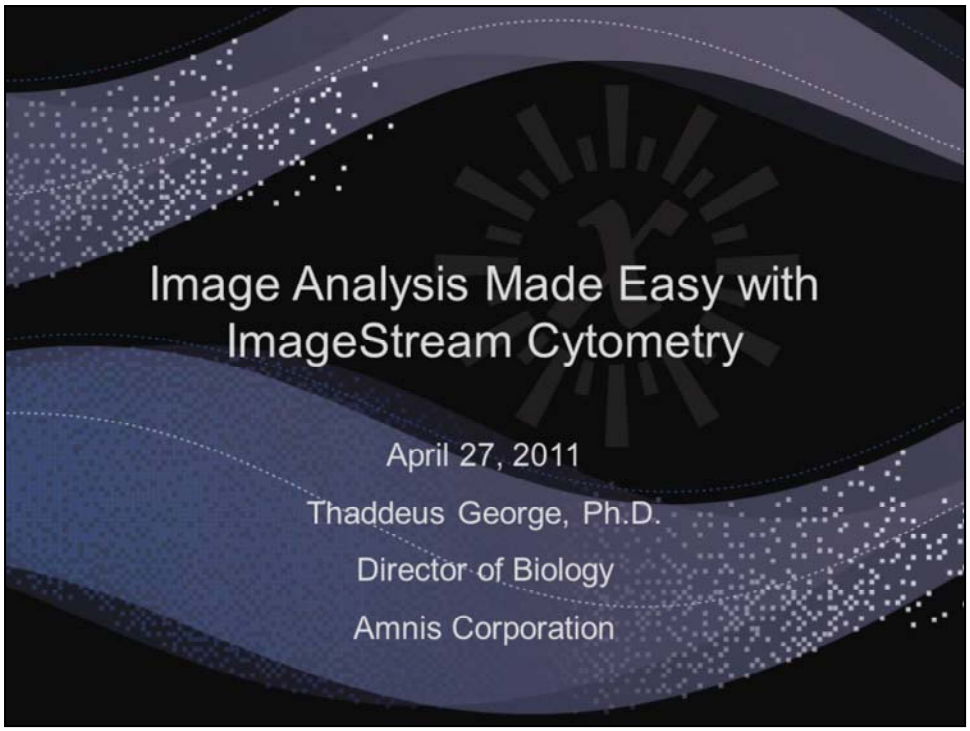
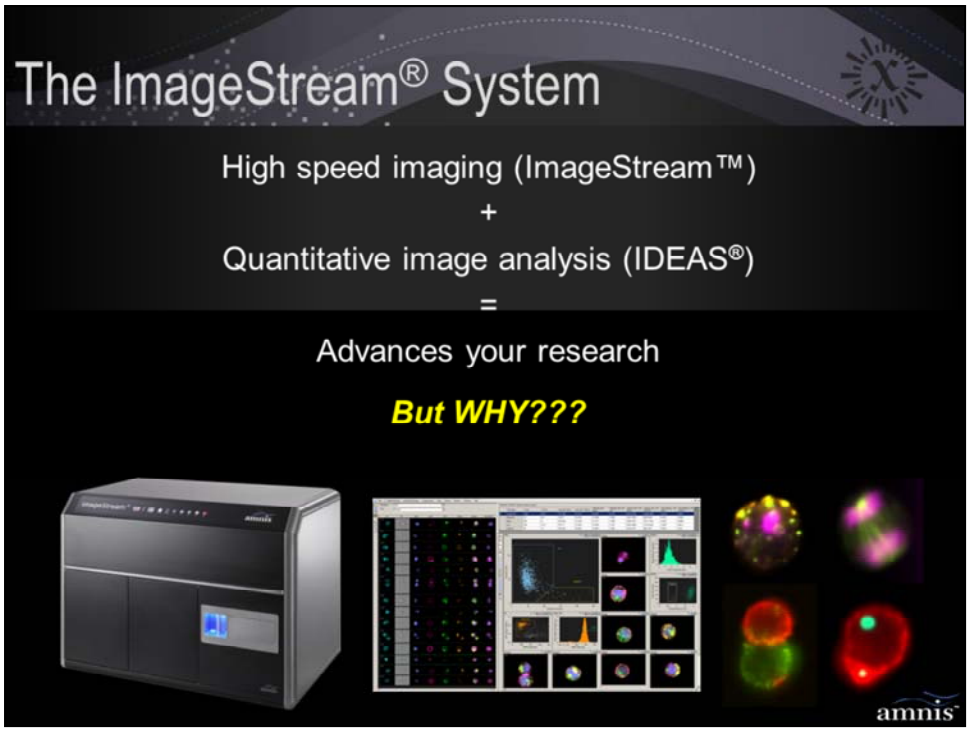


Image Analysis Made Easy with ImageStream Cytometry
April 27, 2011
Thaddeus George, Ph.D.
Director of Biology
Amnis Corporation



Key advantages over microscopy:

1 – objective image collection (avoids selection bias)

2 – high speed (provides statistics even for rare events)

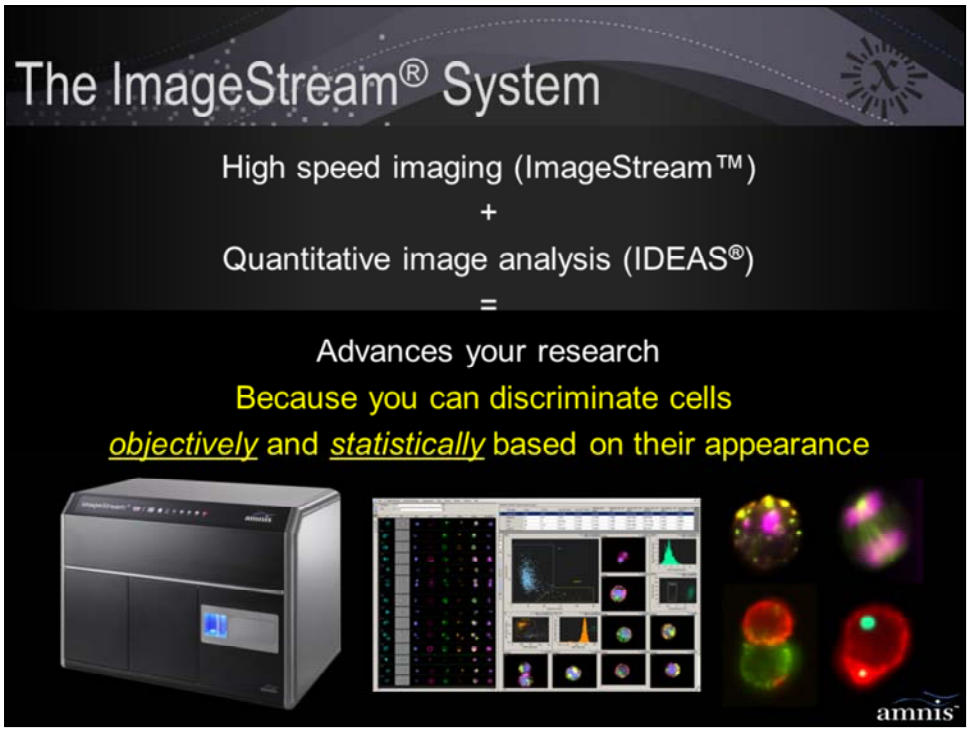
3 – simultaneous multispectral imaging (avoids requirement to take sequential pictures of each cell)

4 – quantification of imagery by IDEAS (provides standard objective repeatable measurements of imagery)

Key advantages over flow cytometry:

1 – high information content provided by images (features related to size, shape, texture, location, co-location)

2 – images linked to quantitative features (can see the cell when you click on a dot)



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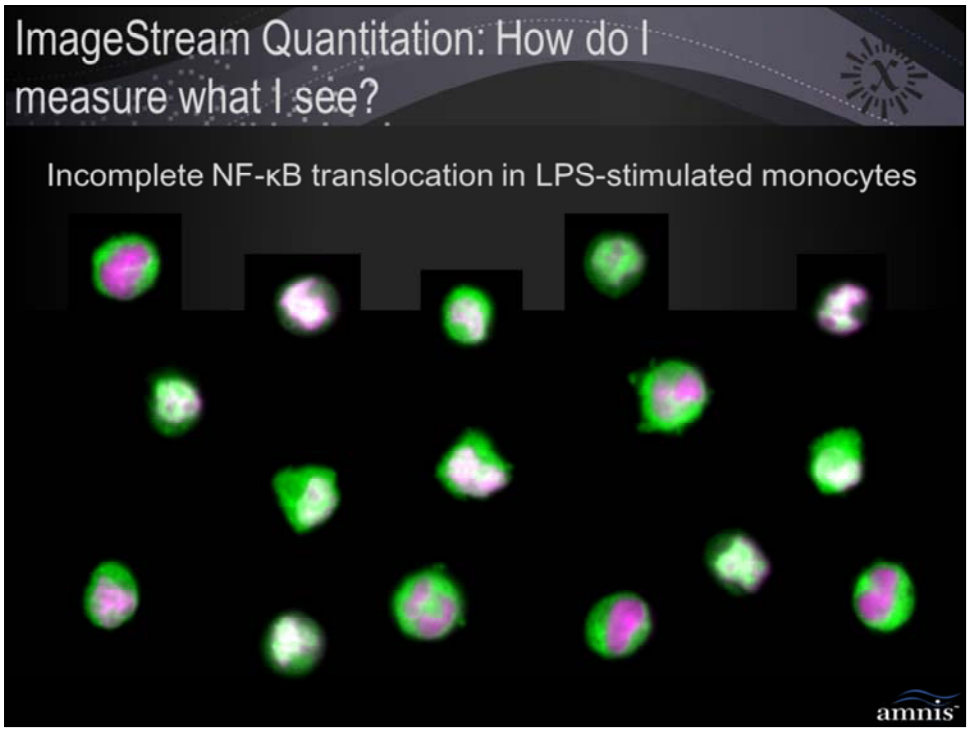


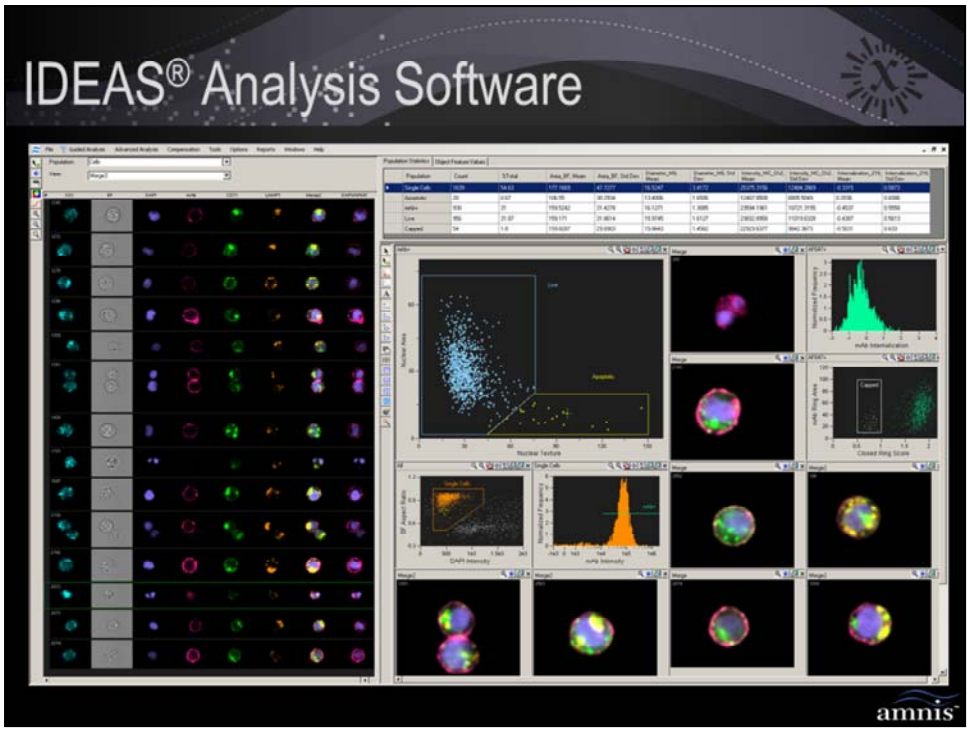
These are human monocyte line THP-1 cells treated with an intermediate dose of LPS to activate nuclear translocation of NFkB, then stained with FITC NFkB (green) and 7-AAD (nuclear dye, red). The imagery shows that the population contains cells that span a continuum from the untranslcoated to the translocated state.

Qualitative analysis is difficult: someone expecting translocation and another person expecting inhibition of translocation will both see cells that meet their expectations…

Manually quantifying this sample would require observation and scoring of many cells – too time consuming and subjective to feasible perform quantitative analysis. Also note that the nucleus occupies most of the cell, and the cells and nuclei are irregular, making it difficult to accurately quatify the amount of NFkB in the cytoplasmic and nuclear compartments.

Bio2:\2010 Data\061410 X101 THP-1 NFkB 60X 40X Mag EDF


ImageStreamX
• 1,000 cells per second
• 12 image channels per cell: SSC, brightfield, fluorescent
• Up to 5 lasers (488, 405, 561, 592, 658 nm)
• 430-800 nm imaging bandwidth
• Multiple magnifications (60X/.9NA, 40X/.75NA, 20X/.5NA)
• AutoSampler for 96 well plates
• Extended depth of field optics (EDF)
ImageStream
amnis



IDEAS performs high content morphometric analysis on tens of thousands of images

Cell boundaries are automatically determined.

Identify area of interest on the cell of interest – then apply to all cells

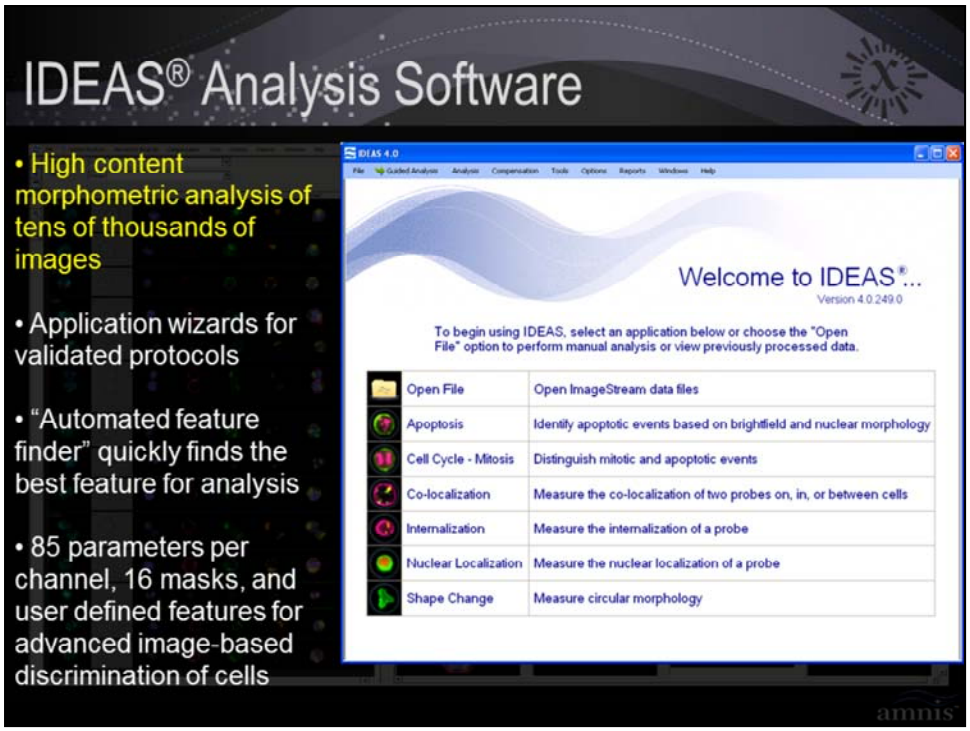
Obtain individual cell data as well as population statistics

Identify subtle shifts in staining patterns

Features are what IDEAS uses to quantify cell morphology.

- 85 features per image
- 16 function masks
- Unlimited number of user defined features
- New features are continually being developed

Multiple levels of analysis – beginner to expert



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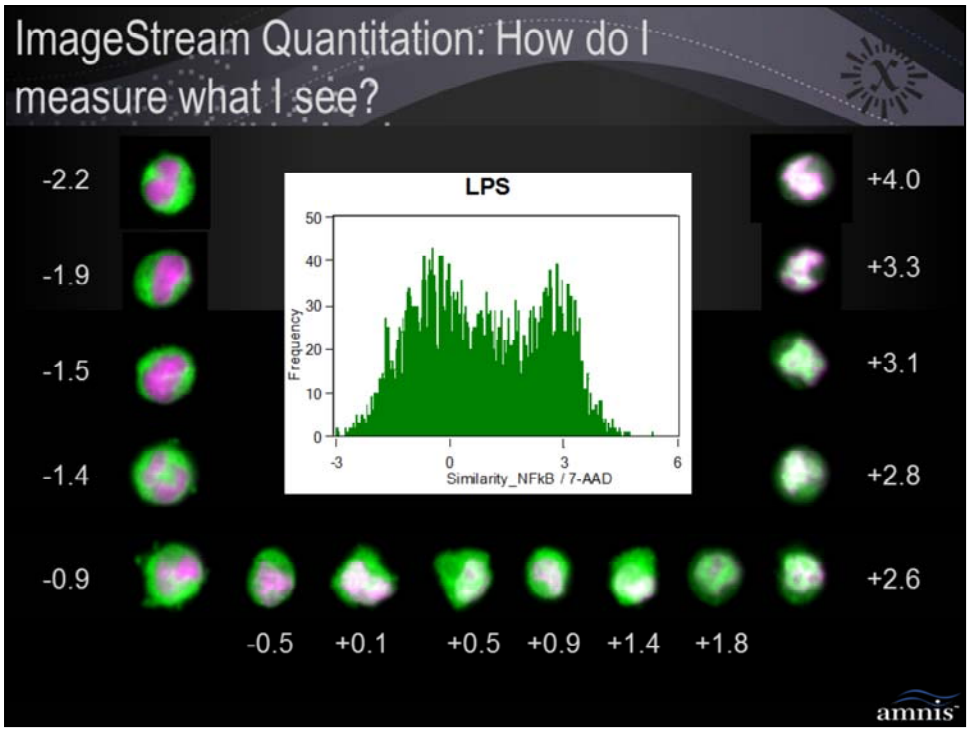
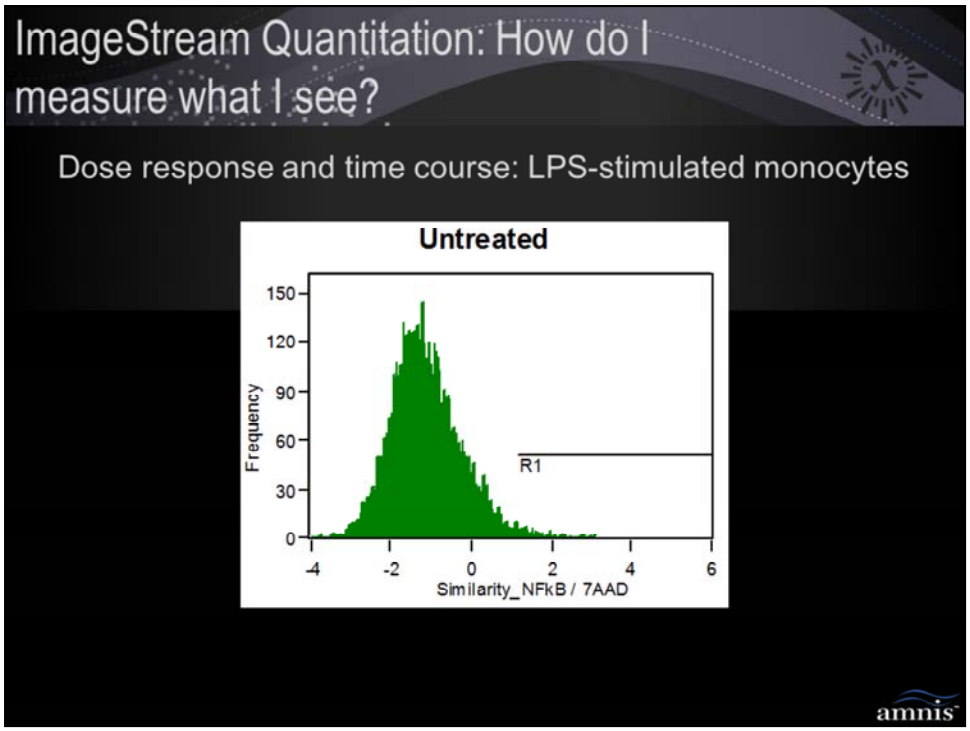


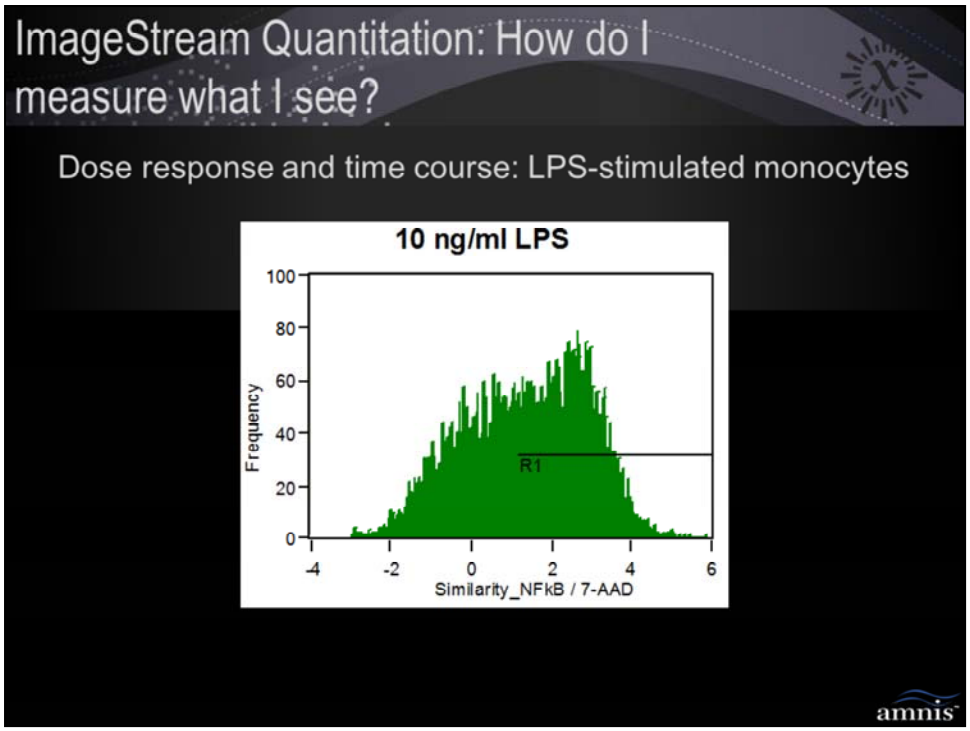
THP1 human monocyte cell line treated with intermediate dose of LPS

Here the NFkB / 7-AAD similarity scores are plotted for the sample, and cells with different values are lined up according to their score.

Note that despite the irregularity of the cells, the similarity score accurately reflects the relative nuclear localization of the NFkB in the cells shown, which span a continuum from completely untranslocated (upper left) to nearly completely translocated (upper right)



Because we quantify the extent of translocation on a per cell basis, and do this on large sample sizes, we can gate on the percentage of cells translocated per sample. This allows translocation scoring for dose responses and time courses…



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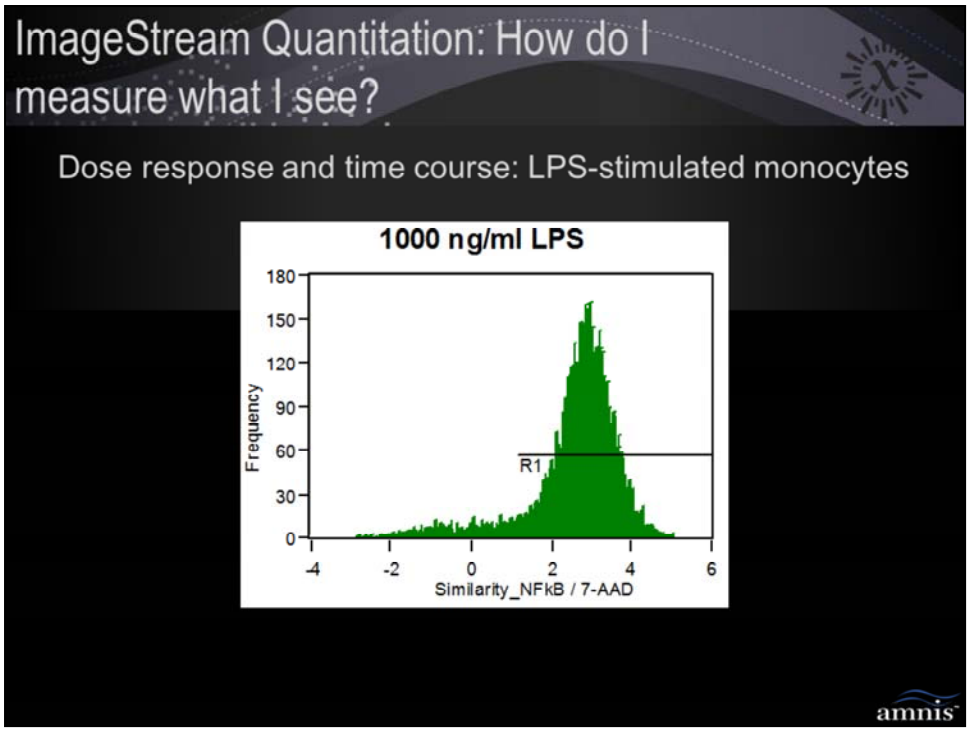

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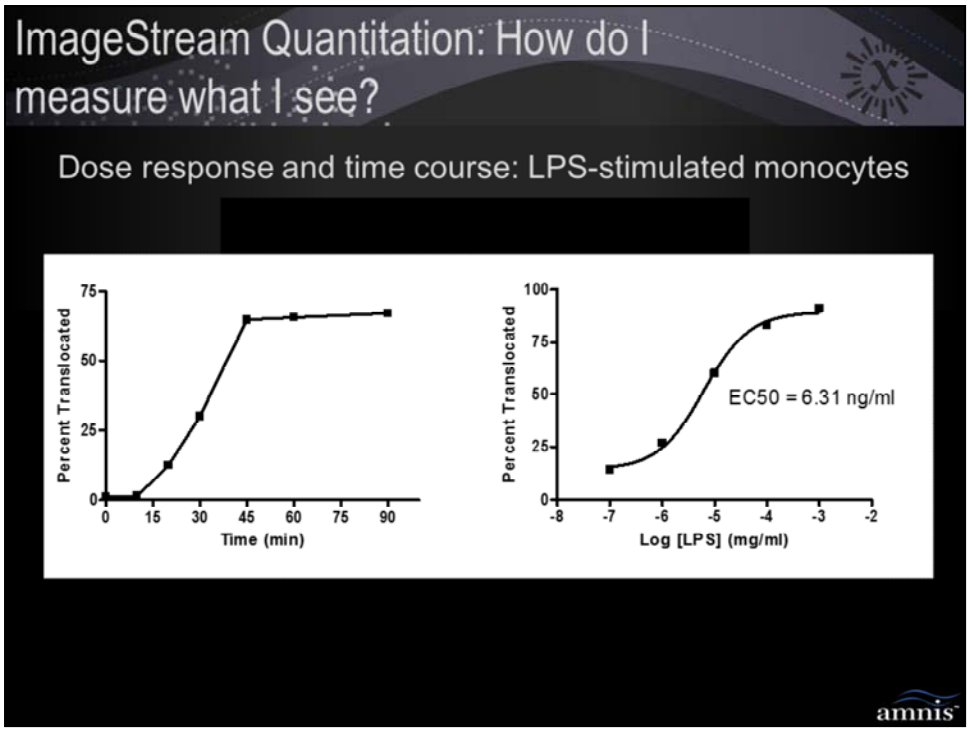

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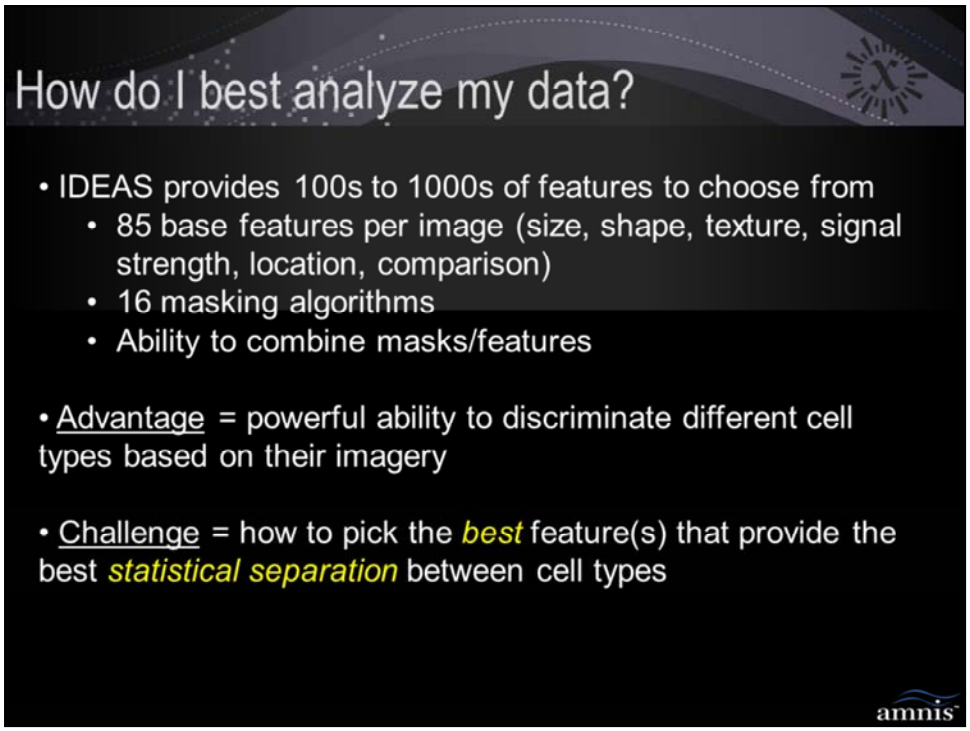
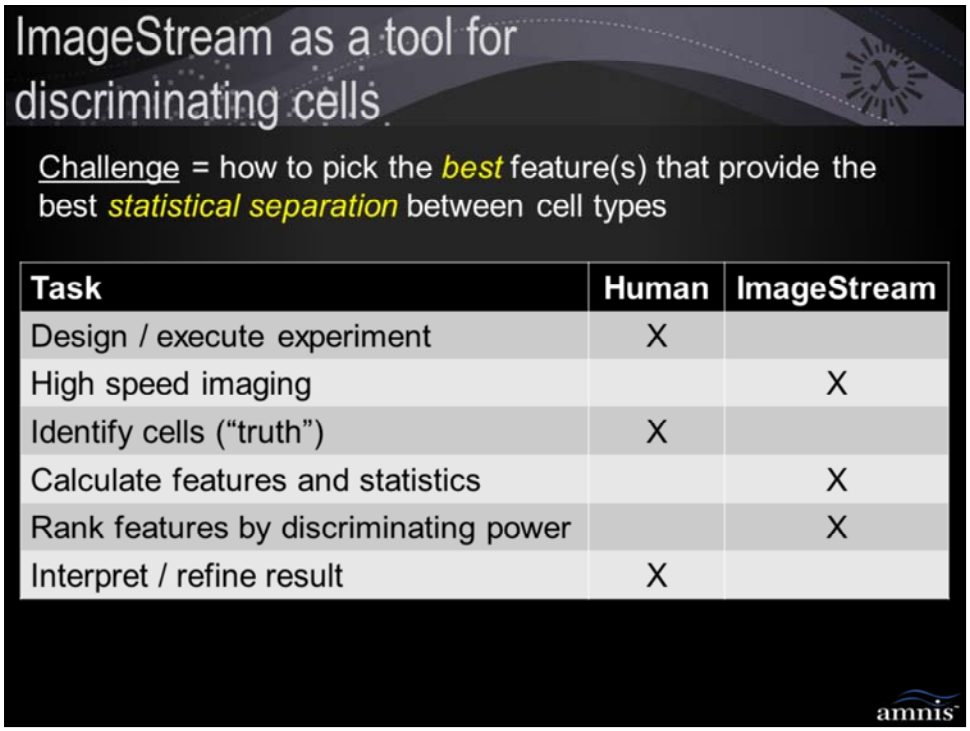


Dilemma for new user: While IDEAS provides powerful tools to discriminate cells based on their imagery, it is difficult to imagine:

a) Learning how all the features/mask work
b) Learning how to apply them to different applications
c) Choosing the best analysis for an experiment

This is a barrier to adoption of the ImageStream platform



To solve the dilemma, it is important to remember that the ImageStream is a TOOL that researchers use.

On a basic level, a tool is a device with properties that enable humans to do a job better than they could by themselves. When you use a tool, you 'collaborate' with it to get the job done. This simply means that you do what you do best and you let the tool do what it does best, and together the job gets done more effectively.

The job = discriminate cells based on their appearance

Tasks:

1 – design and execute experiment = HUMAN; leverage knowledge of the biological system, experimental design, staining protocols, etc

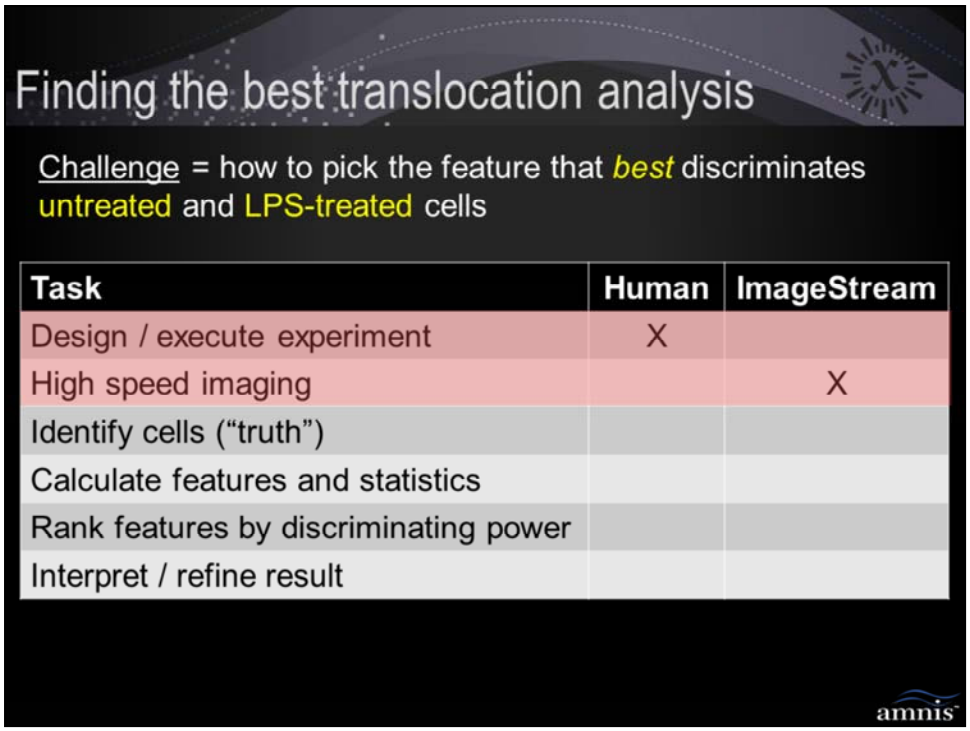
2 – high speed imaging= IMAGESTREAM; leverage high speed camera to take statistically large number of images per sample

3 – identify phenotypes and select truth sets = HUMAN; humans are outstanding at discriminating even subtle differences in imagery. The researcher also usually knows what is expected based on the experiment and can pick them out by hand (ie select 'truth sets' in IDEAS).

4 – Quantify imagery = IMAGESTREAM; humans are horrible at quantifying the differences they see. IDEAS has an extensive set of image-based algorithms and masks designed to discriminate cells based on their appearance; because of the large data sets, IDEAS also provides robust statistics (mean, standard deviation, etc)

5 – Quantify statistical discrimination power of the features, and rank to choose best one = IMAGESTREAM; Use the Rd statistic in IDEAS to measure the discriminating power of each feature; use excel to rank order to choose the best one

6 – Evaluate / refine the result = HUMAN; apply the feature to the whole sample and look for trends in false pos/neg; repick truth if necessary; add advanced features (as you learn the system); protocol development if necessary



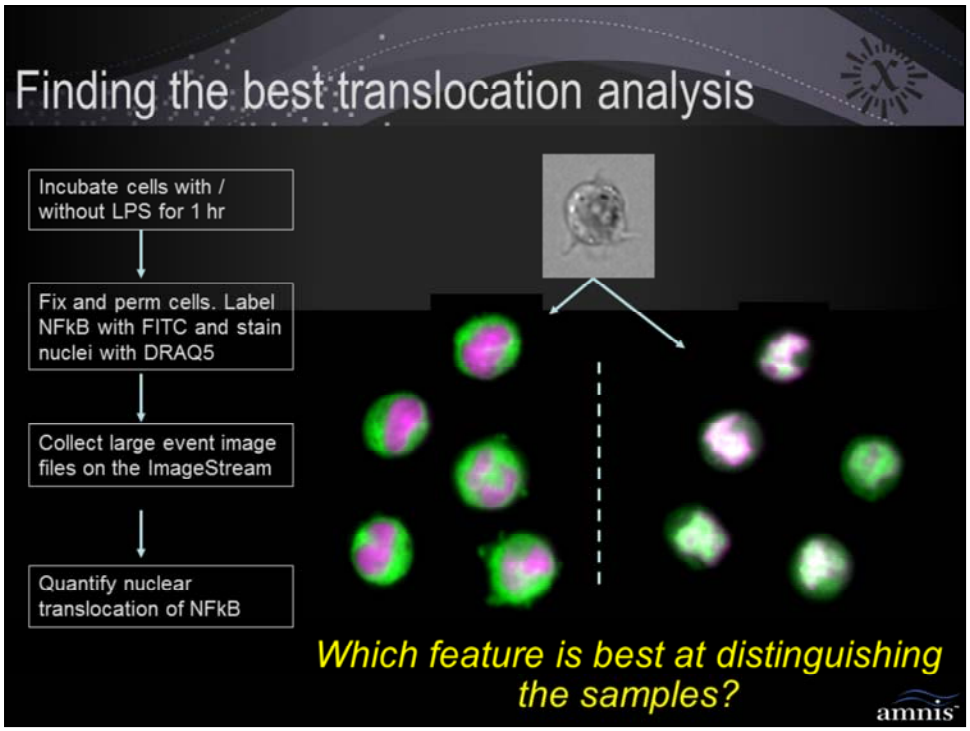
Example number 1: LPS-induced nuc translocation.

The job = Find the best nuclear translocation feature

Tasks:

1 – design and execute experiment = HUMAN; THP-1 cells are incubated with or without a high dose of LPS to activate nuclear translocation of NFkB, then stained with FITC NFkB (green) and DRAQ5 (nuclear dye, red).

2 – high speed imaging= IMAGESTREAM; leverage high speed camera to take statistically large number of images per sample



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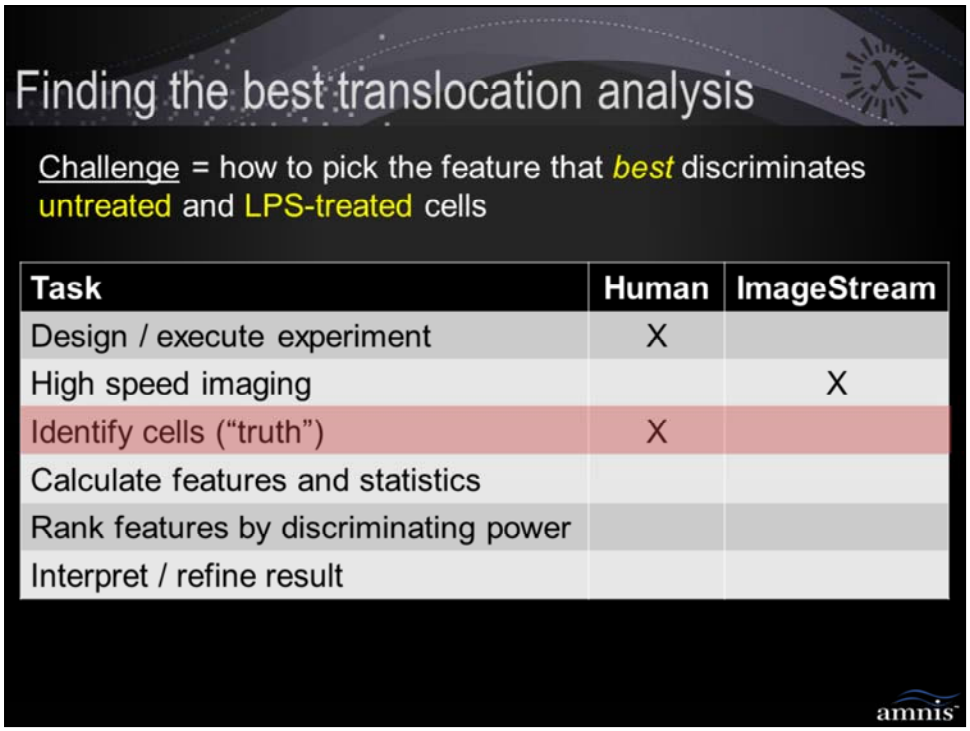

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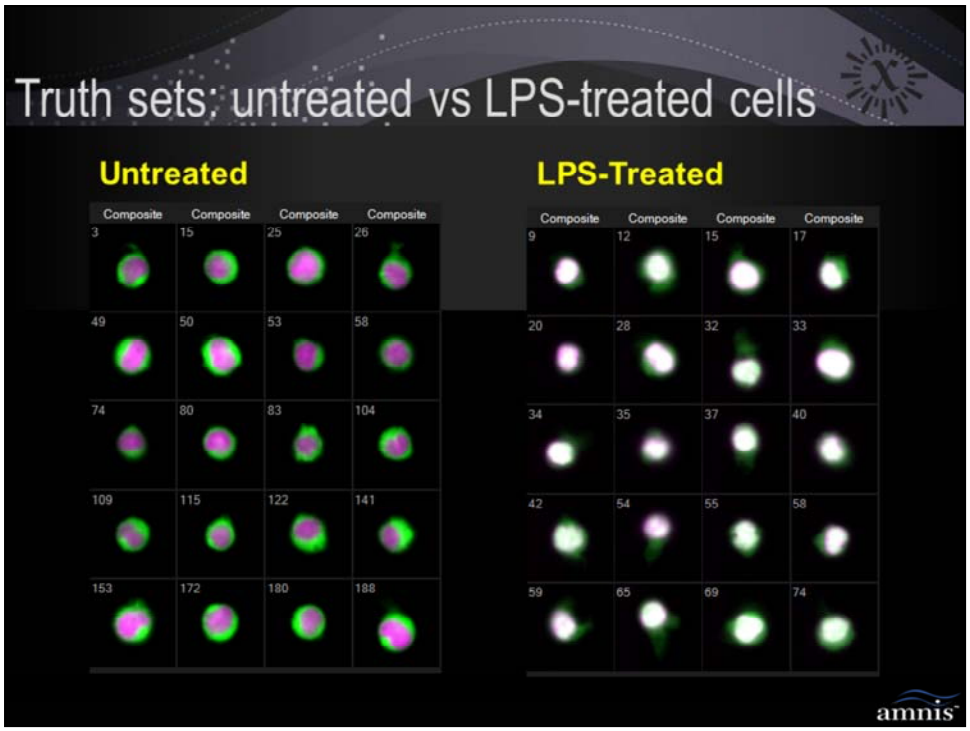

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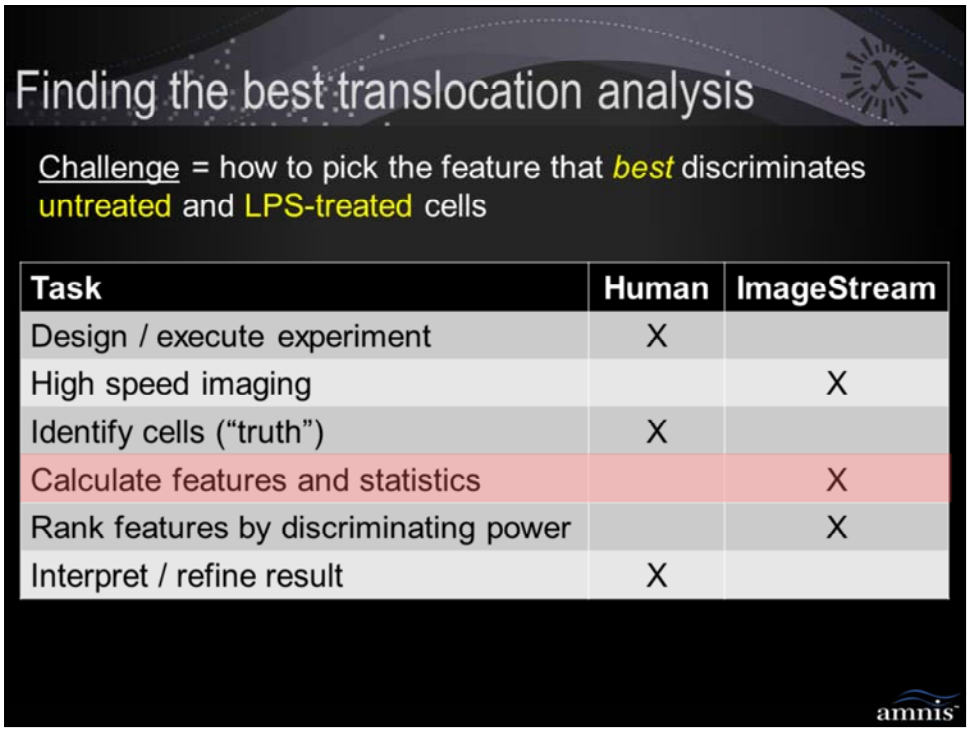

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The job = Find the best nuclear translocation feature

Tasks:

4 – Quantify imagery = IMAGESTREAM; in this example we are hypothetically assuming that IDEAS only has four features (Area, H Variance Mean, Nuc:Cyt NFkB ratio, and Similarity) – this is so we can cover the Rd concept without having to also introduce concept of how to create/handle huge feature sets right off the bat

5 – Quantify statistical discrimination power of the features, and rank to choose best one = IMAGESTREAM; Use the Rd statistic in IDEAS to measure the discriminating power of each feature; use excel to rank order to choose the best one

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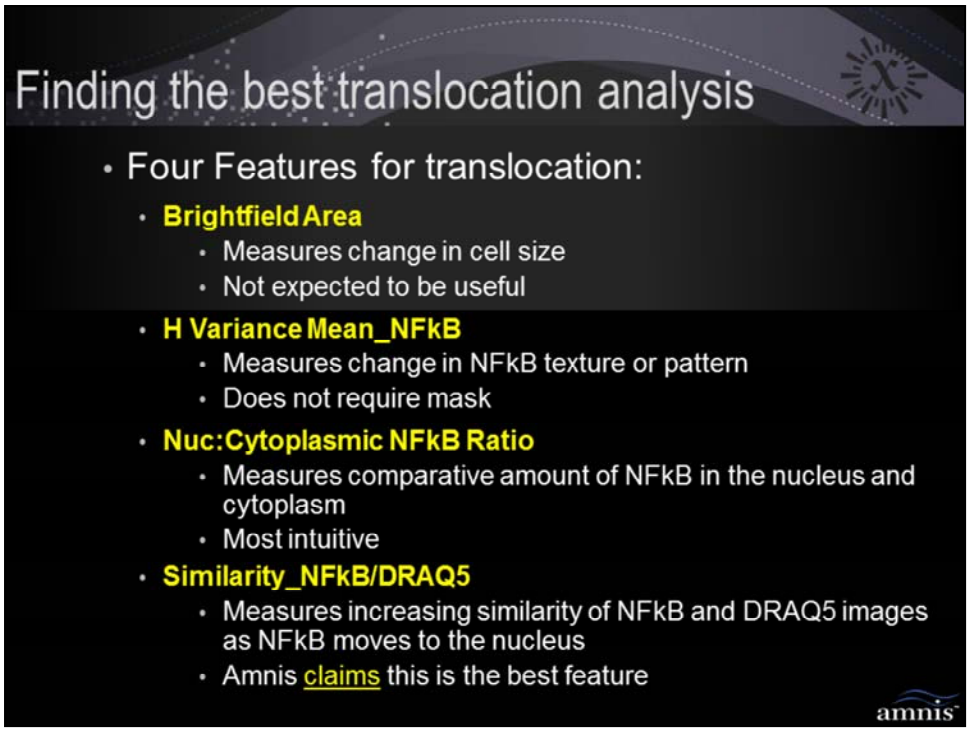

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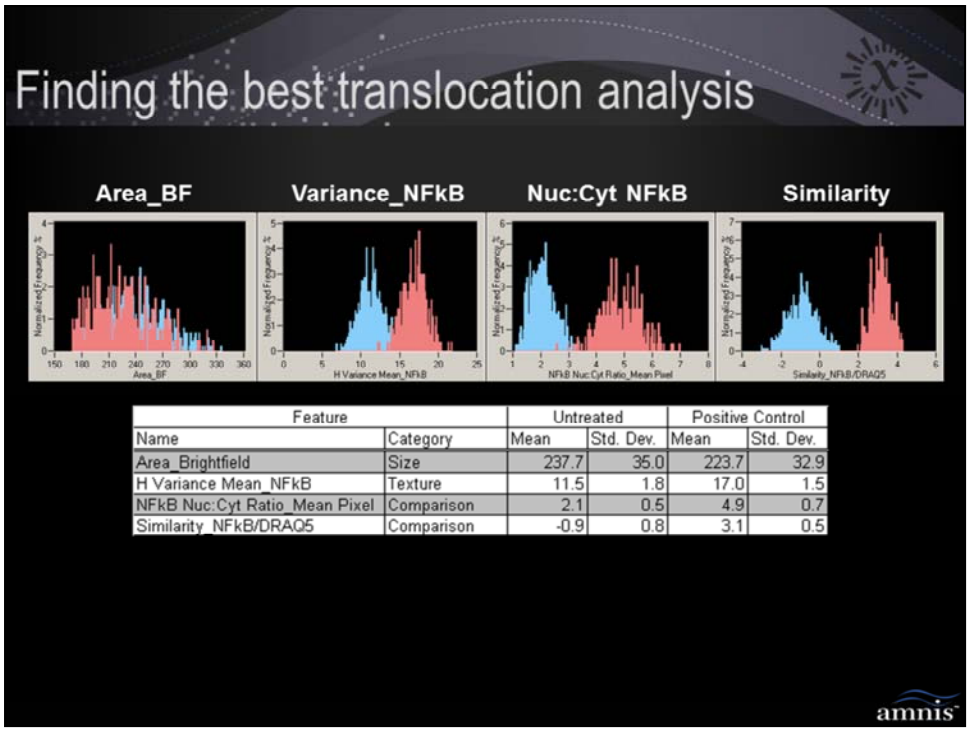


Feature		Untreated		Positive Control	
Name	Category	Mean	Std. Dev.	Mean	Std. Dev.
Area_Brightfield	Size	237.7	35.0	223.7	32.9
H Variance Mean_NFkB	Texture	11.5	1.8	17.0	1.5
NFkB Nuc:Cyt Ratio_Mean Pixel	Comparison	2.1	0.5	4.9	0.7
Similarity_NFkB/DRAQ5	Comparison	-0.9	0.8	3.1	0.5

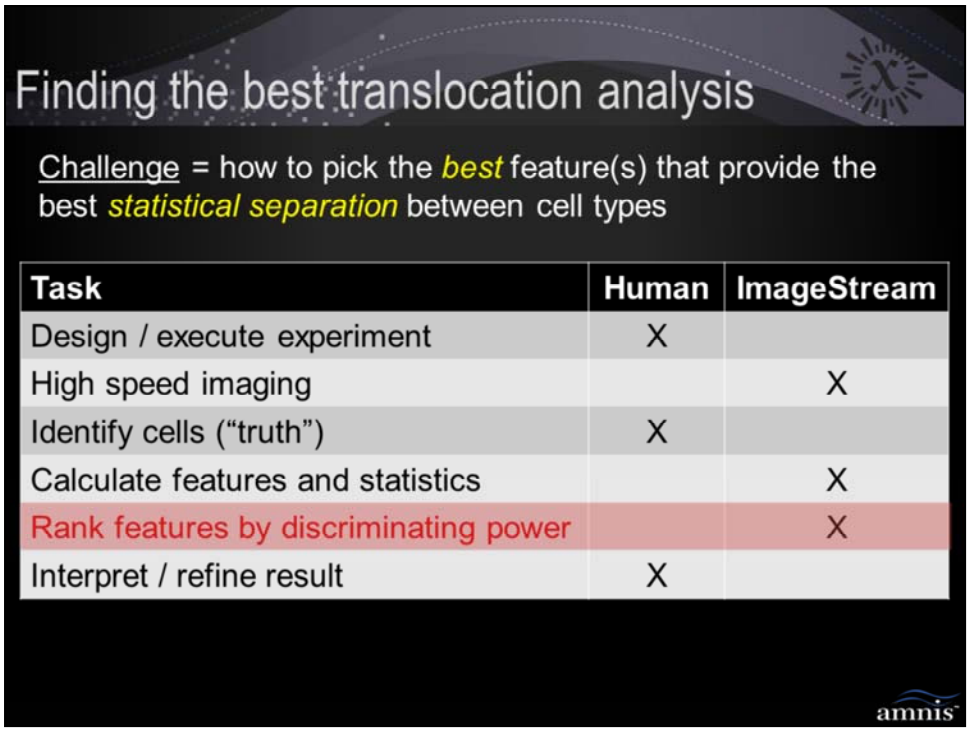
Example number 1: LPS-induced nuc translocation.

The job = Find the best nuclear translocation feature

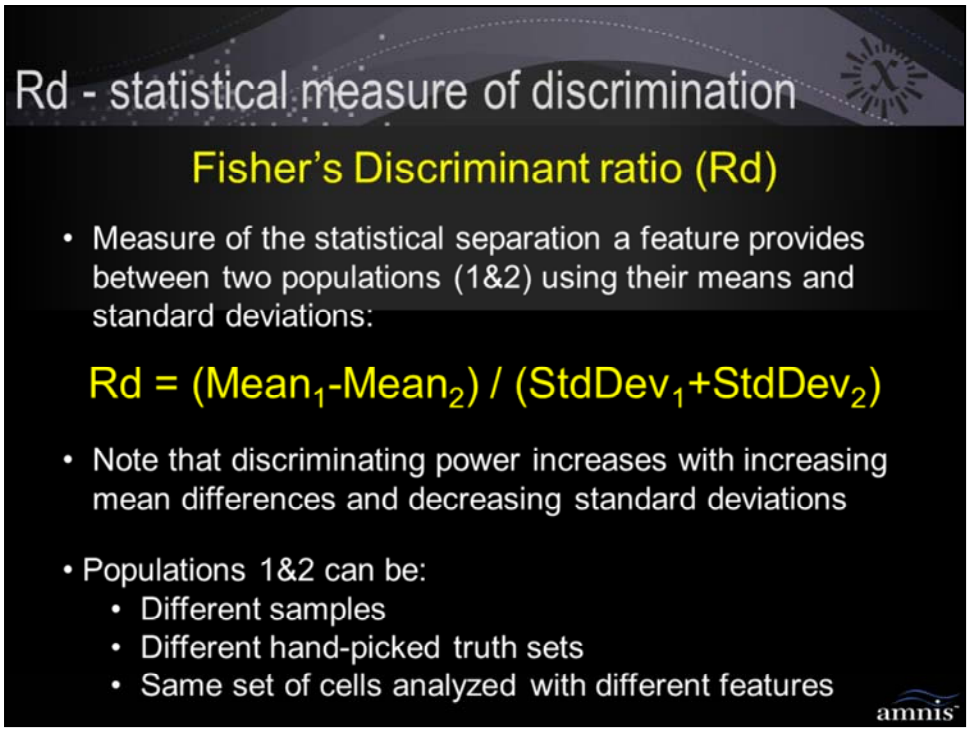
Tasks:

5 – Quantify statistical discrimination power of the features, and rank to choose best one = IMAGESTREAM;

Here we can QUALITATIVELY assess the discrimination power of the 4 features by looking at the overlap between the untreated (blue) and treated (red) histograms. Area looks the worst; Variance is pretty good but not as good as Nuc:Cyt Ratio or Similarity, and it is difficult to tell which of the latter two is the best



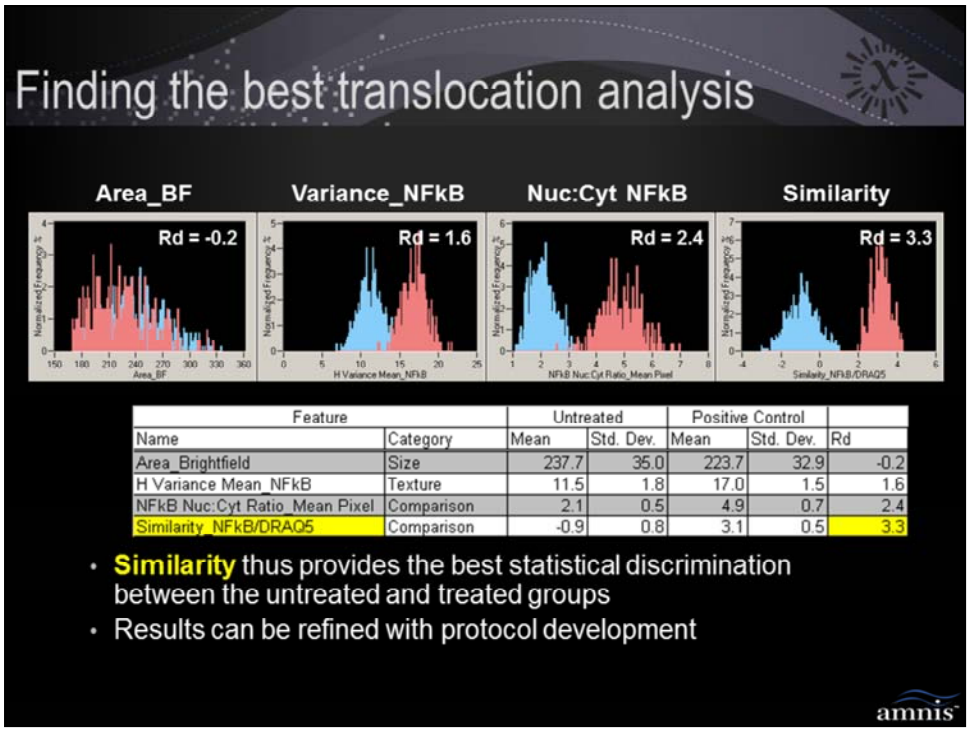
Key to statistically determining the best feature that discriminates the hand-picked cells is the discrimination statistic (Rd) which is explained on the next slide…



Key to statistically determining the best feature that discriminates the hand-picked cells is the discrimination statistic which is called:

Fisher's Discriminant Ratio (Rd):

This statistic measures how much separation a particular feature provides between two populations



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The job = Find the best nuclear translocation feature

Tasks:

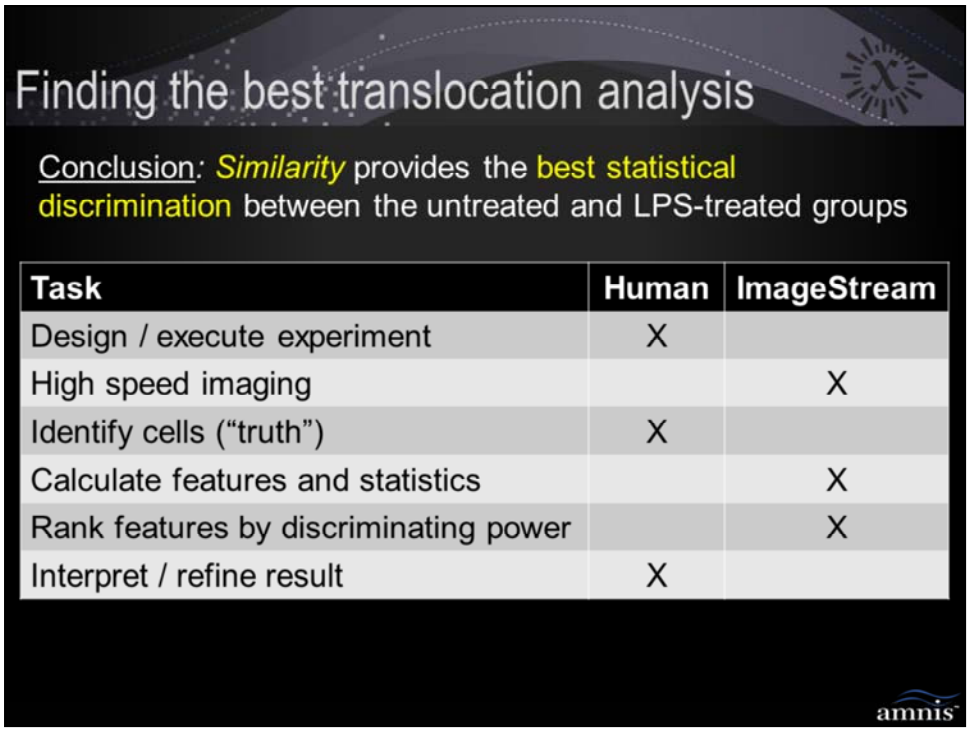
5 – Quantify statistical discrimination power of the features, and rank to choose best one = IMAGESTREAM;

Here we can QUANTITATIVELY assess the discrimination power of the 4 features by ranking the Rd. Doing so allows us to determine that Similarity is the best

6 – Evaluate / refine the result = HUMAN; in this example, there are no other possible features, so unless there is a concern with the truth populations, there is no need to refine those. Changes to the protocol (diff stains, diff fix/perm, diff incubation times, ete) could be tried to improve the separation, and in these cases, the protocol that provided the greatest Rd Similarity would be chosen as the best protocol.

Thus, the ImageStream is an excellent tool for protocol development, as it provides you with tools to visualize, hand select, gate, quantify your cells of

interest and can measure statistical separation



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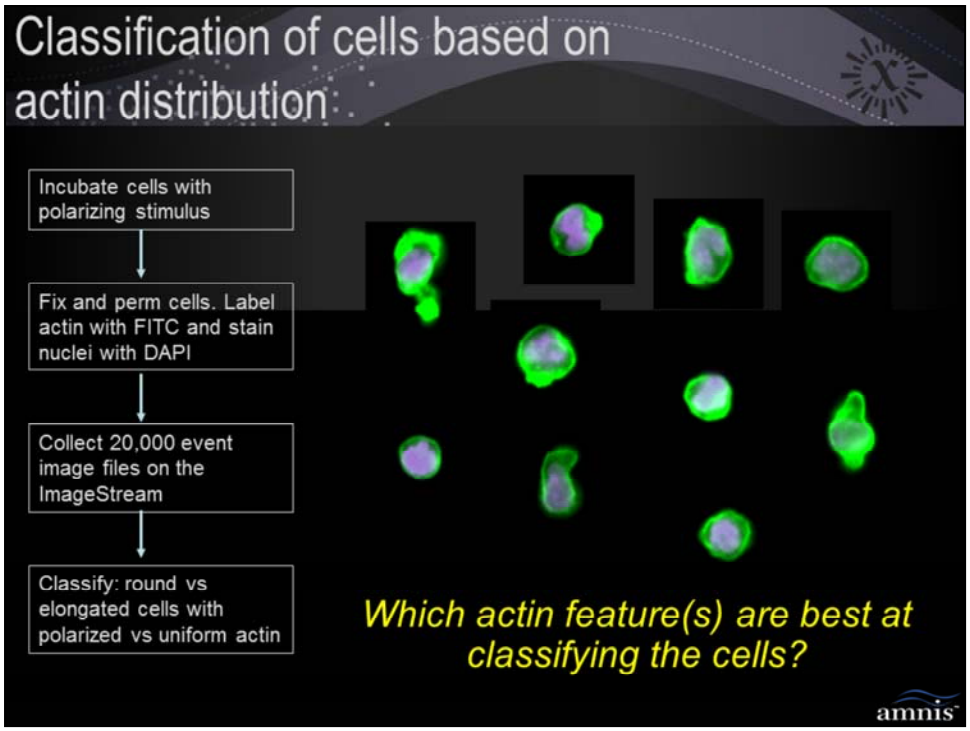
Conclusion = SIMILARITY



Example number 2: Treatment-induced change in actin distribution

Cell line incubated with compound to induce changes in actin, then stained with FITC phalloidin (actin, green) and DAPI (nuclear dye, purple).

The job = classify cells based on their actin morphology

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1 – design and execute experiment = HUMAN; leverage knowledge of the biological system, experimental design, staining protocols, etc

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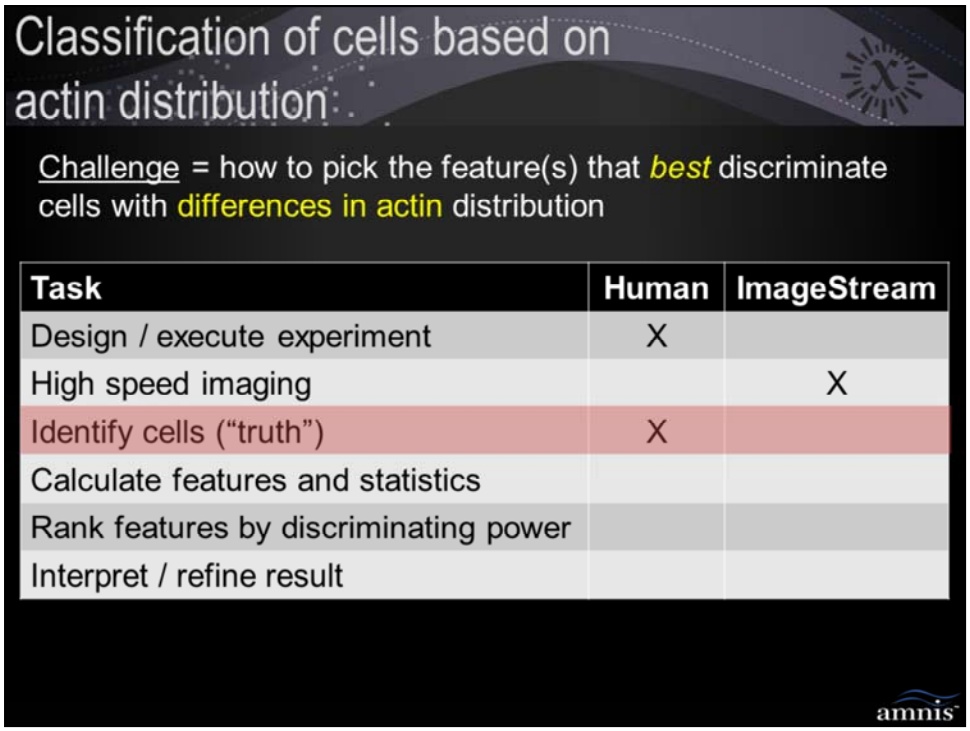

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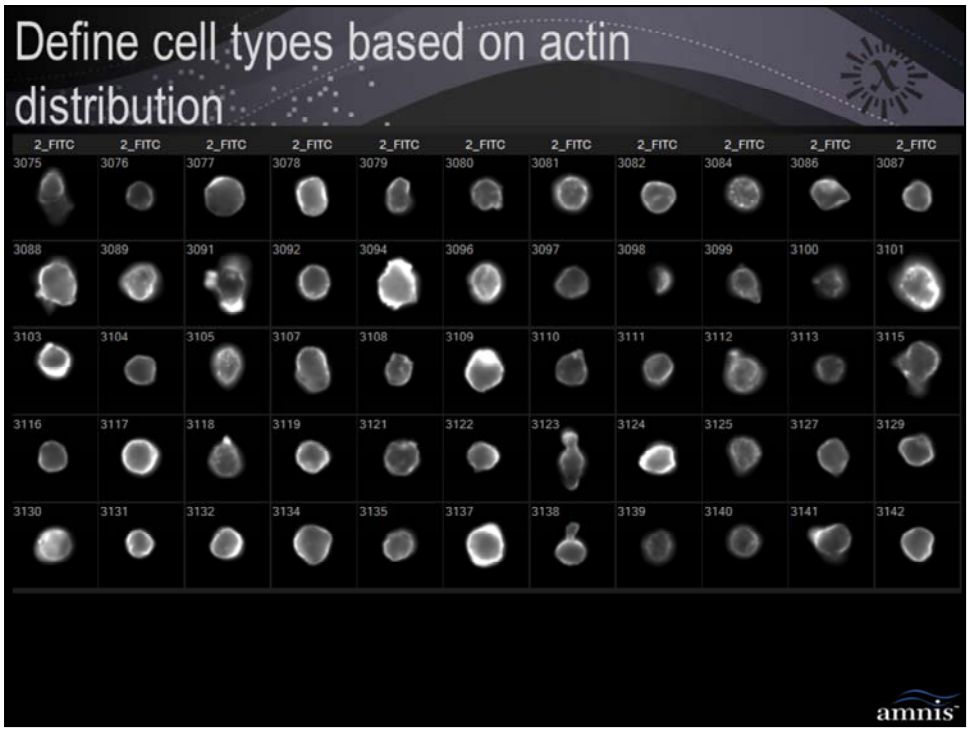

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3 – identify phenotypes and select truth sets = HUMAN; Panel of images showing some differences. Can see a couple of patterns:

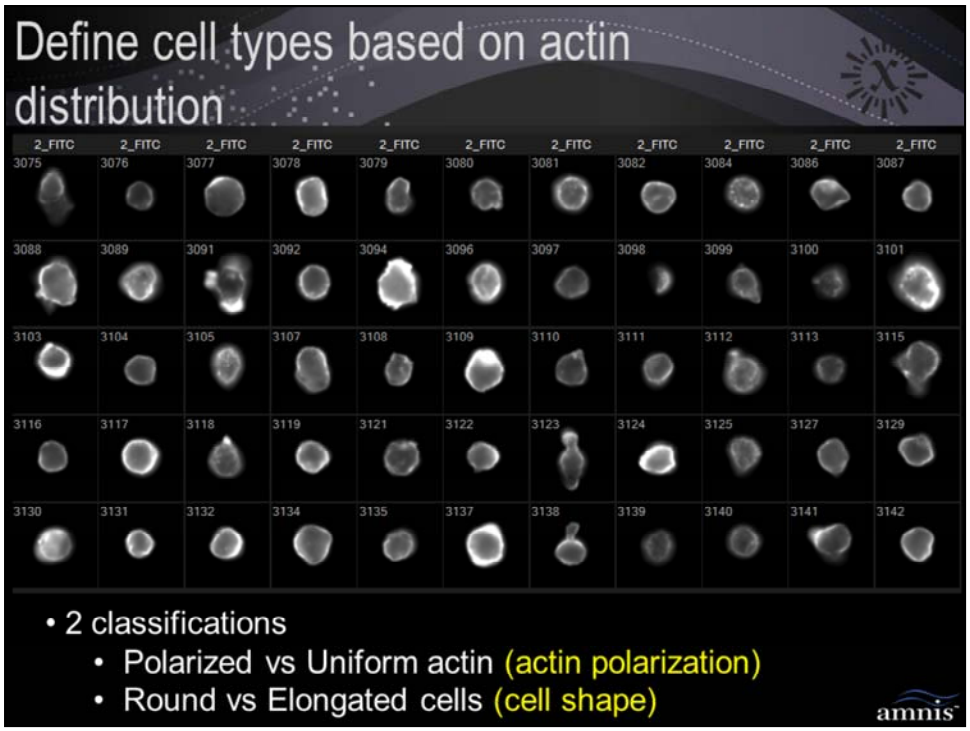
1 – shape: some cells are round (3077, 3087, 3117, 3137, etc) while some are elongated (3123, 3138).

2 – polarity: some have uniformly distributed actin (3076, 3078, 3242, etc) while some have polarized actin (3103, 3109, etc)

Thus, we will create 4 truth populations, and pair them to find a best feature to discriminate shape and a best feature to discriminate polarity

THIS IS AN IMPORTANT FIRST STEP TO ANY IMAGESTREAM

EXPERIMENT as it gives you an idea of how well the experiment worked, and how much discrimination you can expect IDEAS to find for you. If you don't see enough differences to define truth, then you probably want to revisit the protocol…



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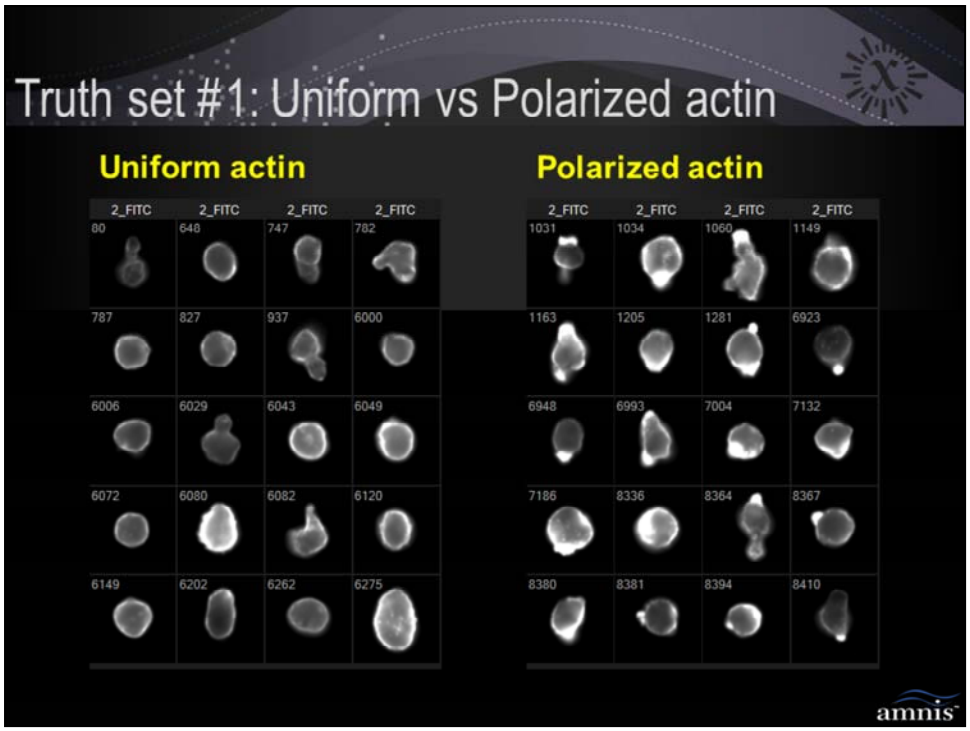

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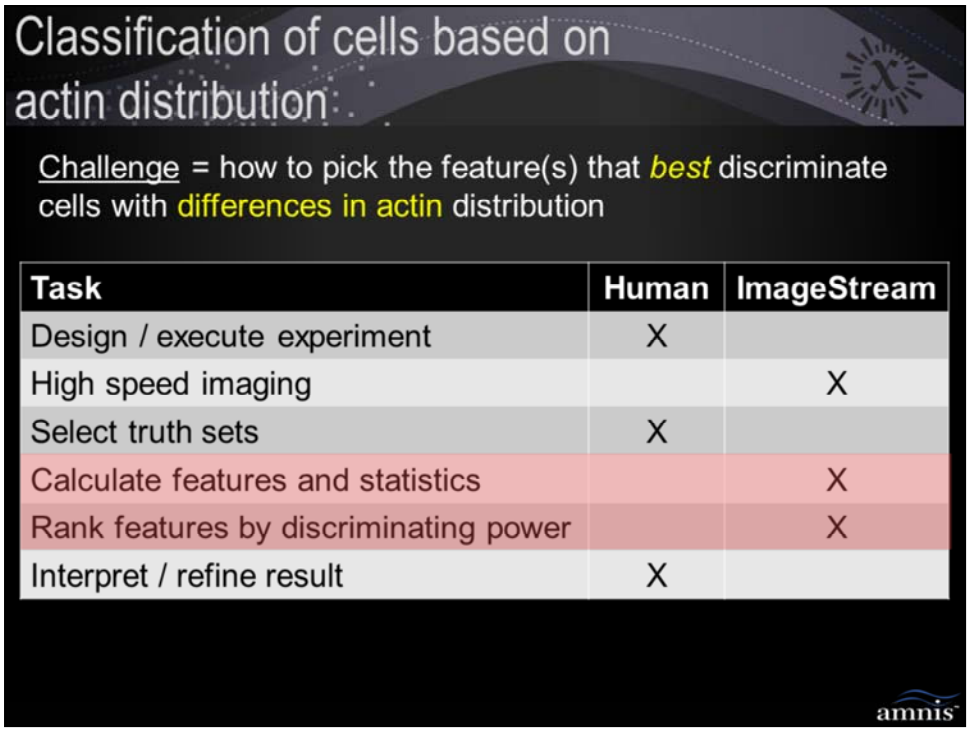
<u>The job = classify cells based on their actin polarity</u>

Tasks:

3 – identify phenotypes and select truth sets = <u>HUMAN</u>; Cells with uniform actin – the cells chosen 'span the range' of examples with uniform actin, including dim and bright, round and elongated, small and large. Same holds for the polarized truth. This method of choosing cells that 'span the range' ensure that the feature that wins is indeed a measure of polarity (and not size, shape, etc)



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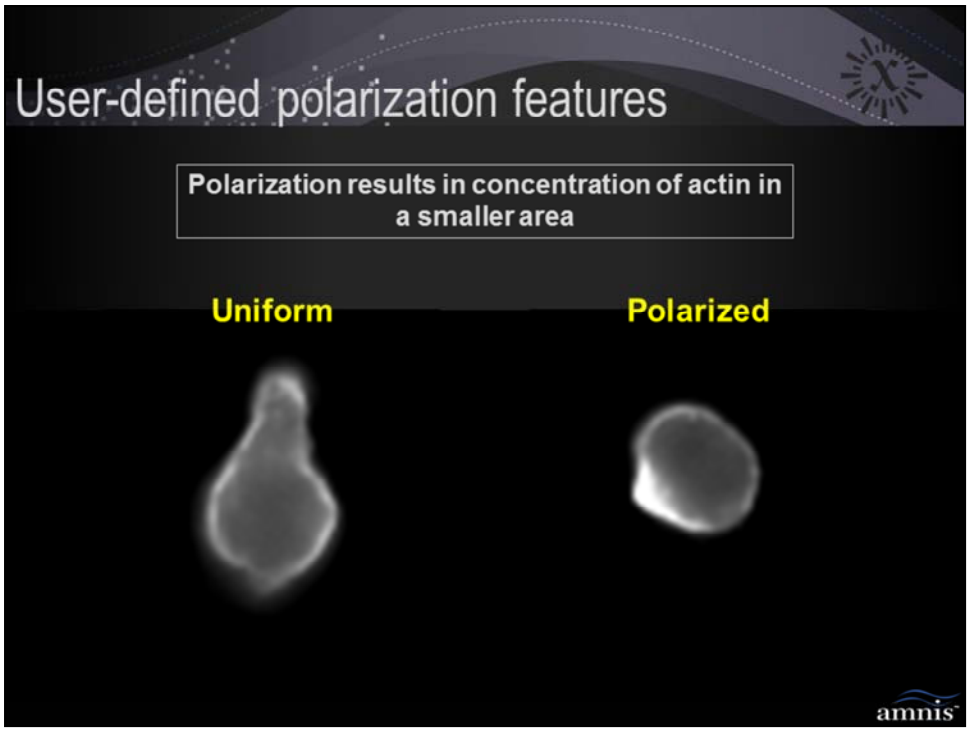
4 – Quantify imagery = IMAGESTREAM; humans are horrible at quantifying the differences they see. Here we will show how to get IDEAS to calculate multiple features and to export the Rd statistic for ranking in Excel

5 – Quantify statistical discrimination power of the features, and rank to choose best one = IMAGESTREAM; Use the Rd statistic in IDEAS to measure the discriminating power of each feature; use excel to rank order to choose the best one



Example number 2: Treatment-induced change in actin distribution

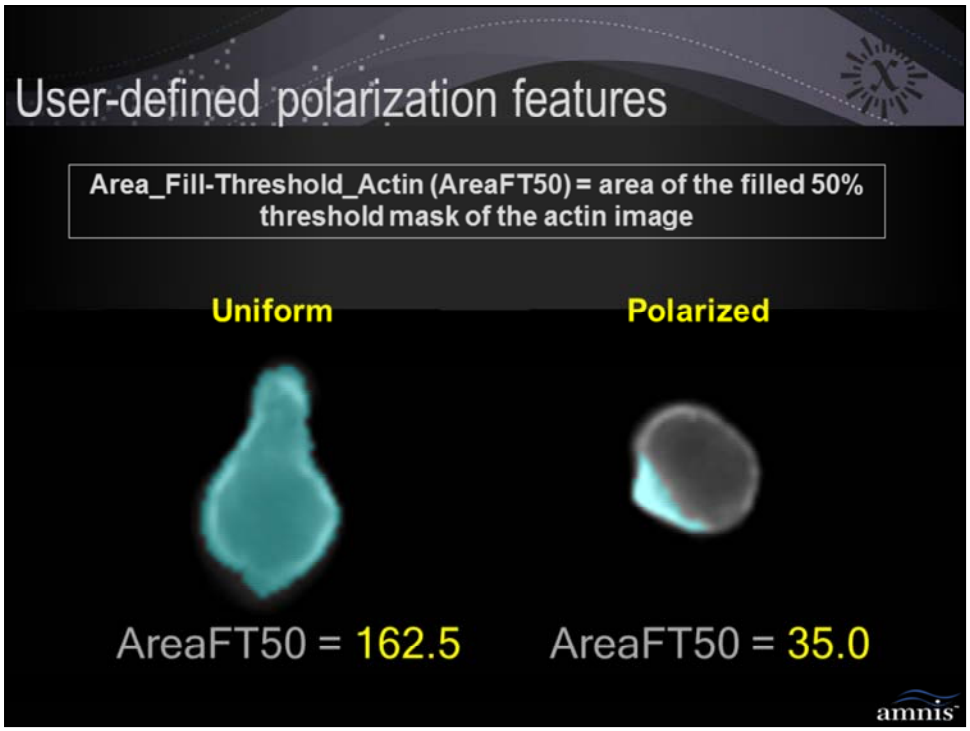
Cell line incubated with compound to induce changes in actin, then stained with FITC phalloidin (actin, green) and DAPI (nuclear dye, purple).

The job = classify cells based on their actin polarity

Tasks:

4 – Quantify imagery = IMAGESTREAM; humans are horrible at quantifying the differences they see, but lets try to do it anyway:

To do this, we try to imagine a way to numerically score the difference in actin polarity between these two images. With a bit of training, we know that a threshold mask can be generated that will just mask the brightest portion of the image. Because the image on the righ has concentrated actin in a small area, the are of the threshold mask is expected to be lower than the image on the left.



Example number 2: Treatment-induced change in actin distribution

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The job = classify cells based on their actin polarity

Tasks:

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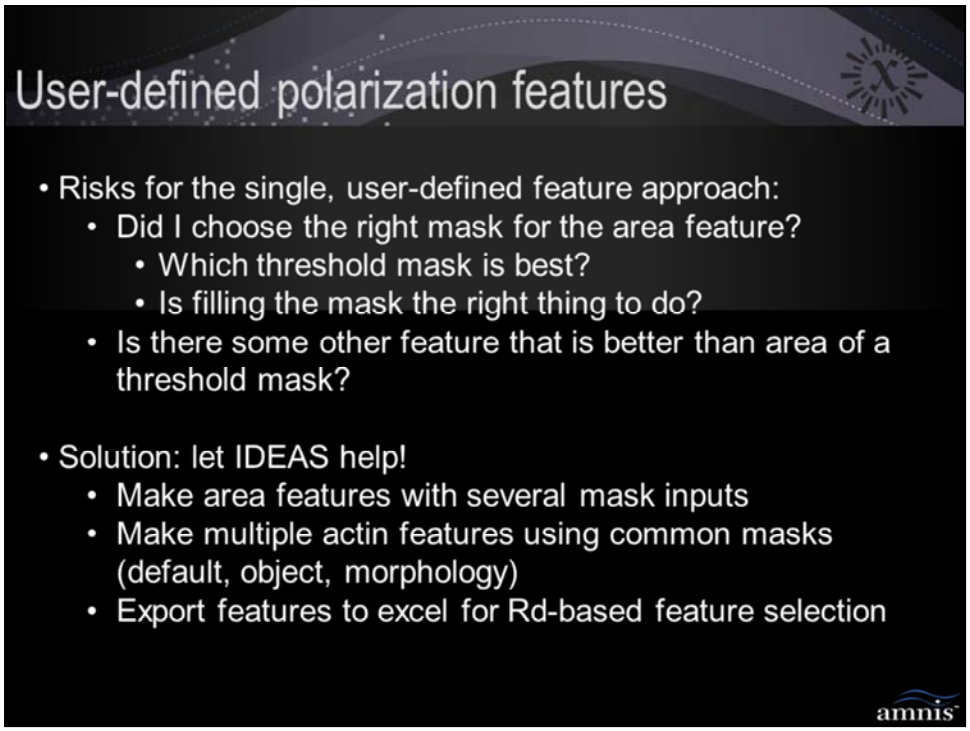
To do this, we try to imagine a way to numerically score the difference in actin polarity between these two images. With a bit of training, we know that a threshold mask can be generated that will just mask the brightest portion of the image. Because the image on the righ has concentrated actin in a small area, the are of the threshold mask is expected to be lower than the image on the left.

Here we show the area of the Filled 50% actin threshold mask for both cells. So this feature provides good separation FOR THESE TWO CELLS. At this point we would need a way to evaluate this feature on more cells to see if the thresholding we did was best…



Example number 2: Treatment-induced change in actin distribution

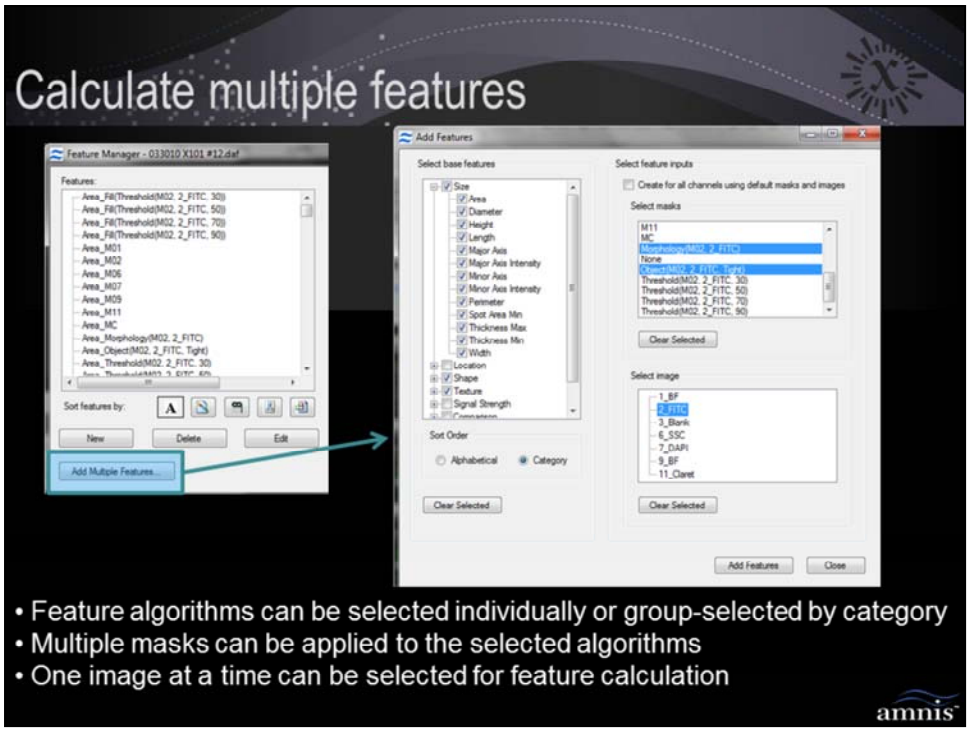
Cell line incubated with compound to induce changes in actin, then stained with FITC phalloidin (actin, green) and DAPI (nuclear dye, purple).

The job = classify cells based on their actin polarity

Tasks:

4 – Quantify imagery = IMAGESTREAM;

By trying to quantify the imagery all by ourselves, we are trying to do what the ImageStream is best at (namely, calculating tons of image-based features for all cells and measuring statistical separation), and by doing so, we are looking for a needle in a haystack. At this point, a little teamwork is in order. You can help create some smarter features by applying all masks that you think will help, like the threshold mask (since you don't know what threshold %, make several threshold masks, each with a different %), and the simple cellular masks (default, object, morphology) to the actin channel. Then let IDEAS calculate all the features and statistics necessary to find the one that best separates you truth sets.



Example number 2: Treatment-induced change in actin distribution

Cell line incubated with compound to induce changes in actin, then stained with FITC phalloidin (actin, green) and DAPI (nuclear dye, purple).

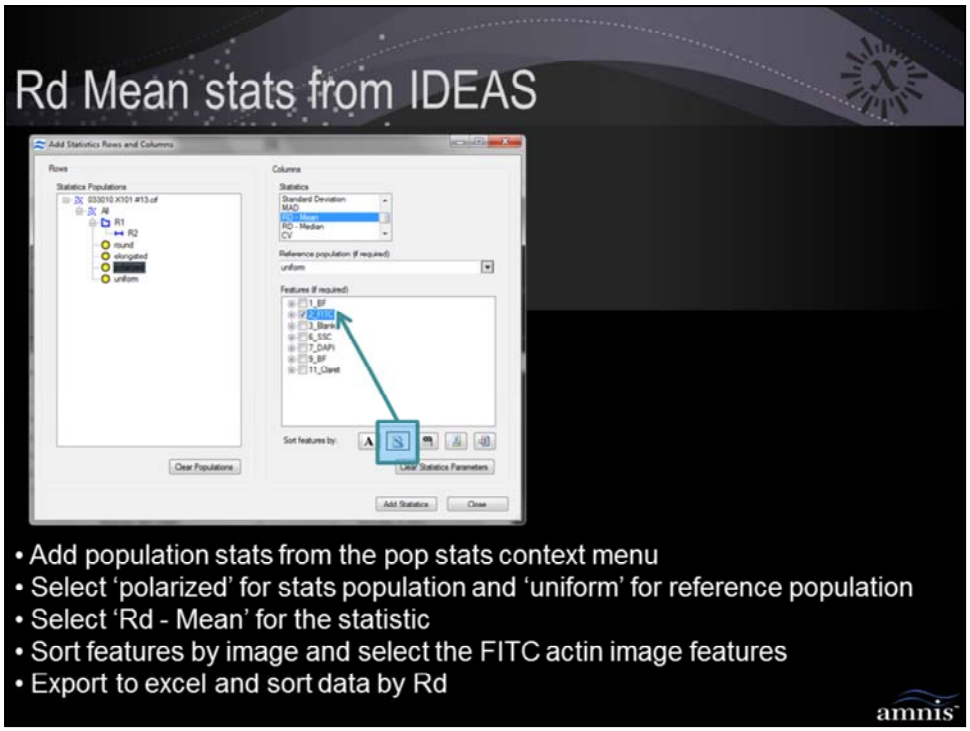
The job = classify cells based on their actin polarity

Tasks:

4 – Quantify imagery = IMAGESTREAM;

This slide shows the interface for rapidly instructing IDEAS to calculate multiple featrues…Apply all masks that you think will help, like the threshold mask (since you don't know what threshold %, make several threshold masks, each with a different %), and the simple cellular masks (default, object, morphology) to the actin channel. Then let IDEAS calculate all the features and statistics necessary to find the one that best separates you truth sets.

Note, you can also limit the features to calculate by category (ie maybe you just want a shape feature), by mask, by channel, by base feature…



Example number 2: Treatment-induced change in actin distribution

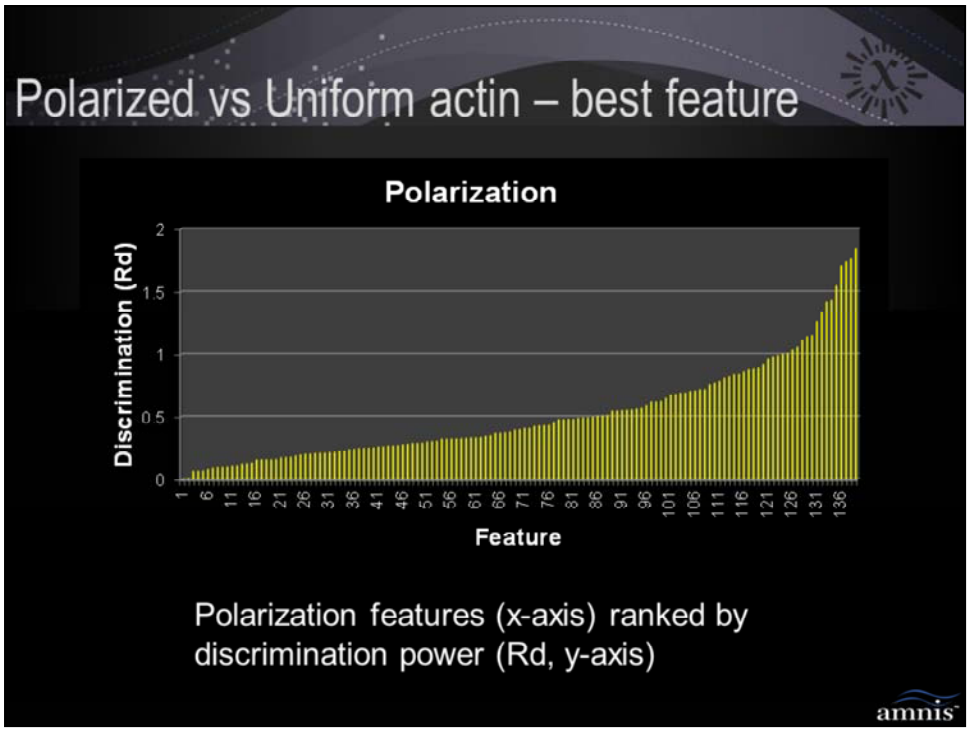
Cell line incubated with compound to induce changes in actin, then stained with FITC phalloidin (actin, green) and DAPI (nuclear dye, purple).

<u>The job = classify cells based on their actin polarity</u>

Tasks:

5 – Quantify statistical discrimination power of the features, and rank to choose best one = <u>IMAGESTREAM</u>; Use the Rd statistic in IDEAS to measure the discriminating power of each feature; use excel to rank order to choose the best one

This slide shows how to export the Rd-mean statistic for the actin features (polarized vs uniform)



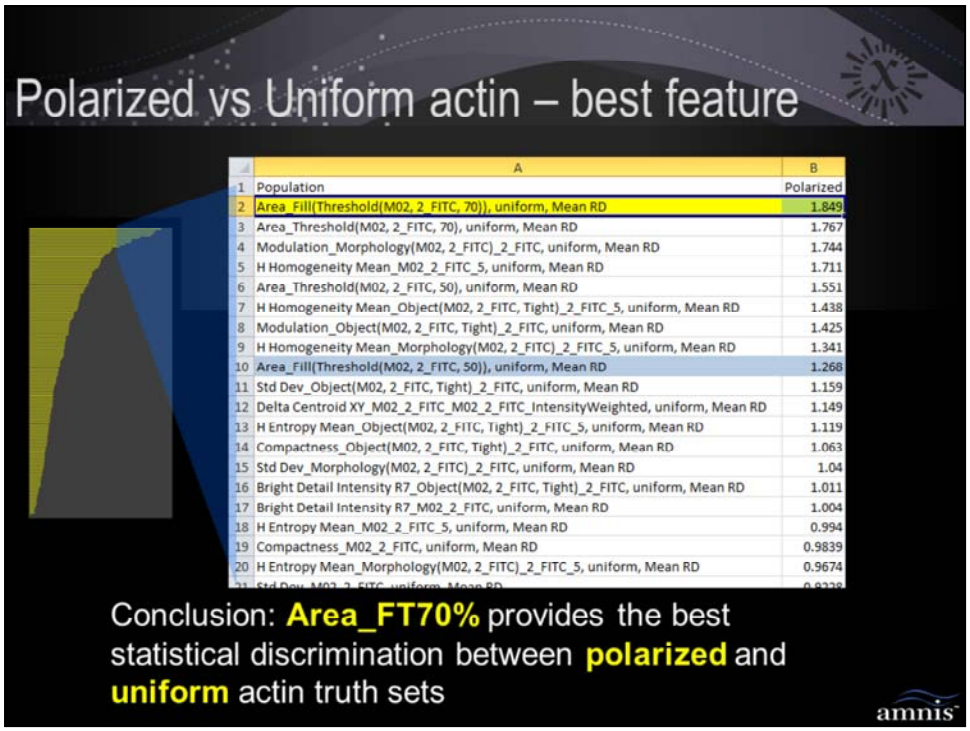
Example number 2: Treatment-induced change in actin distribution

Cell line incubated with compound to induce changes in actin, then stained with FITC phalloidin (actin, green) and DAPI (nuclear dye, purple).

The job = classify cells based on their actin polarity

Tasks:

5 – Quantify statistical discrimination power of the features, and rank to choose best one = IMAGESTREAM; This is a plot of IDEAS actin polarization features (X-axis) vs. Discrimination power (Rd, Y-axis). Data for ~140 features are shown. The feature with the highest Rd will be chosen…



	A	B
1	Population	Polarized
2	Area_Fill(Threshold(M02, 2_FITC, 70)), uniform, Mean RD	1.849
3	Area_Threshold(M02, 2_FITC, 70), uniform, Mean RD	1.767
4	Modulation_Morphology(M02, 2_FITC)_2_FITC, uniform, Mean RD	1.744
5	H Homogeneity Mean_M02_2_FITC_5, uniform, Mean RD	1.711
6	Area_Threshold(M02, 2_FITC, 50), uniform, Mean RD	1.551
7	H Homogeneity Mean_Object(M02, 2_FITC, Tight)_2_FITC_5, uniform, Mean RD	1.438
8	Modulation_Object(M02, 2_FITC, Tight)_2_FITC, uniform, Mean RD	1.425
9	H Homogeneity Mean_Morphology(M02, 2_FITC)_2_FITC_5, uniform, Mean RD	1.341
10	Area_Fill(Threshold(M02, 2_FITC, 50)), uniform, Mean RD	1.268
11	Std Dev_Object(M02, 2_FITC, Tight)_2_FITC, uniform, Mean RD	1.159
12	Delta Centroid XY_M02_2_FITC_M02_2_FITC_IntensityWeighted, uniform, Mean RD	1.149
13	H Entropy Mean_Object(M02, 2_FITC, Tight)_2_FITC_5, uniform, Mean RD	1.119
14	Compactness_Object(M02, 2_FITC, Tight)_2_FITC, uniform, Mean RD	1.063
15	Std Dev_Morphology(M02, 2_FITC)_2_FITC, uniform, Mean RD	1.04
16	Bright Detail Intensity R7_Object(M02, 2_FITC, Tight)_2_FITC, uniform, Mean RD	1.011
17	Bright Detail Intensity R7_M02_2_FITC, uniform, Mean RD	1.004
18	H Entropy Mean_M02_2_FITC_5, uniform, Mean RD	0.994
19	Compactness_M02_2_FITC, uniform, Mean RD	0.9839
20	H Entropy Mean_Morphology(M02, 2_FITC)_2_FITC_5, uniform, Mean RD	0.9674

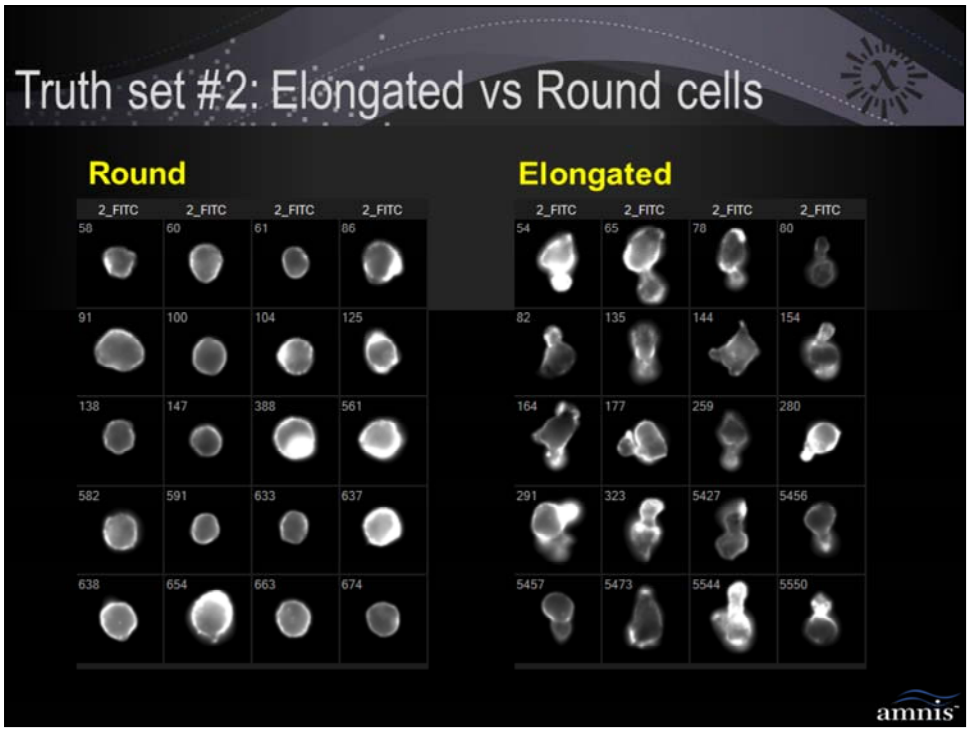
Example number 2: Treatment-induced change in actin distribution

Cell line incubated with compound to induce changes in actin, then stained with FITC phalloidin (actin, green) and DAPI (nuclear dye, purple).

The job = classify cells based on their actin polarity

Tasks:

5 – Quantify statistical discrimination power of the features, and rank to choose best one = IMAGESTREAM; Top-20 actin polarization features are shown. The feature with the highest Rd is **Area_FT70%** and represents the feature that provides the best statistically discrimination between the uniform and polarized **TRUTH** sets. Note, the feature that wins is critically dependent on the truth sets, and if upon evaluation of the feature on the larger file it is found that the feature does not perform well, you may need to refine the truth sets and repeat the process…



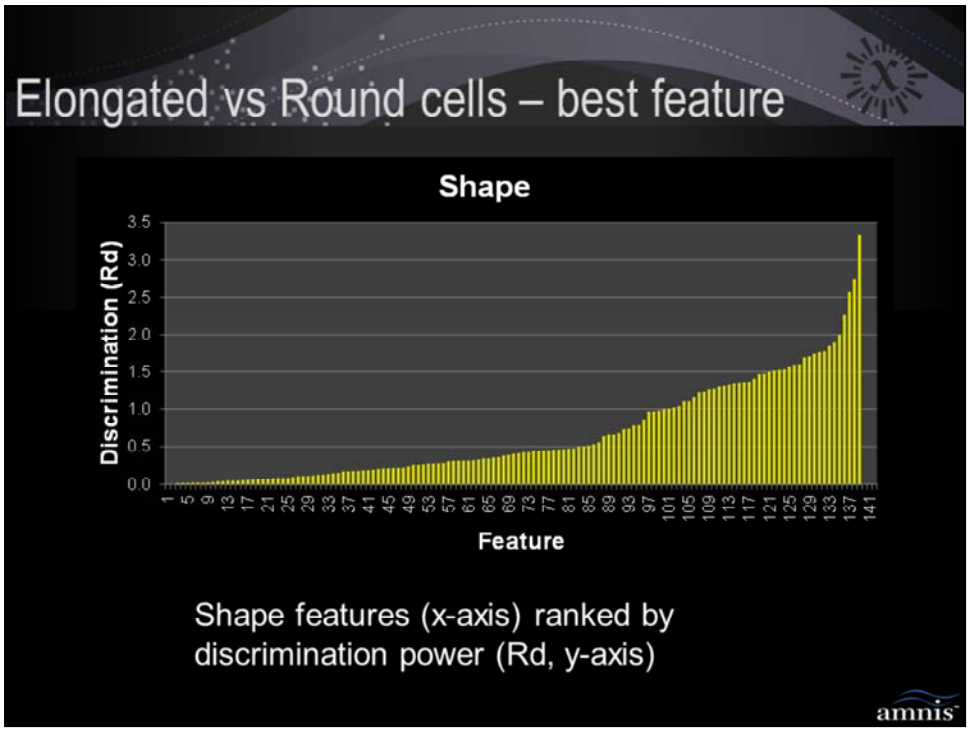
Example number 2: Treatment-induced change in actin distribution

Cell line incubated with compound to induce changes in actin, then stained with FITC phalloidin (actin, green) and DAPI (nuclear dye, purple).

The job = classify cells based on their shape

Tasks:

3 – identify phenotypes and select truth sets = HUMAN; Round vs Elongated truth – the cells chosen 'span the range' of examples, including dim and bright, small and large, uniform and polarized. This method of choosing cells that 'span the range' ensure that the feature that wins is indeed a measure of polarity (and not size, polarity, etc)



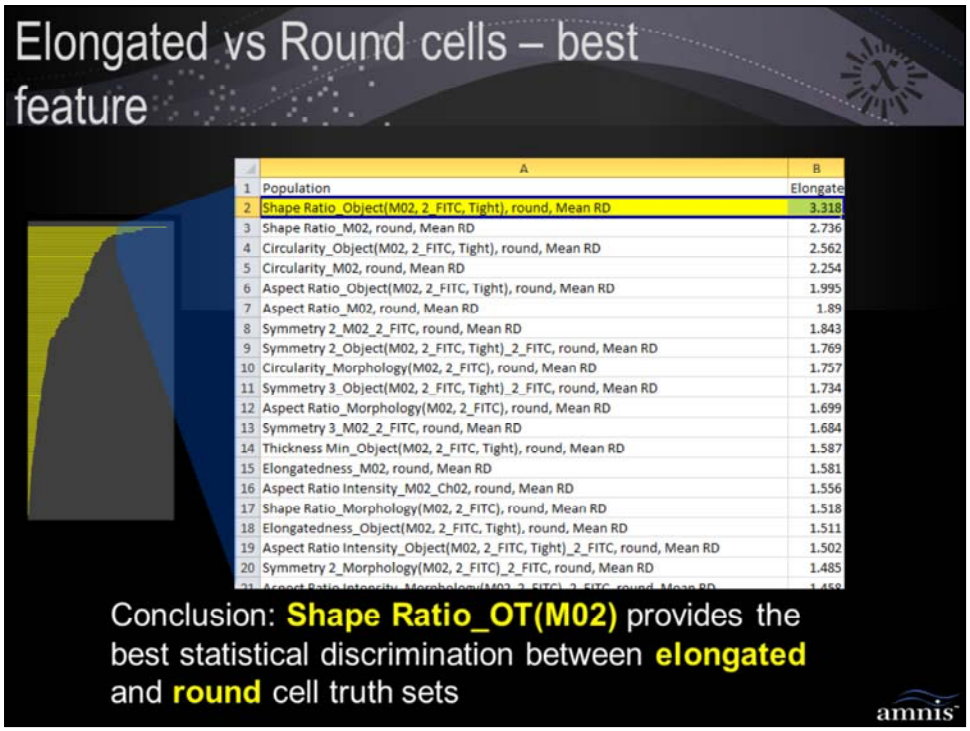
Example number 2: Treatment-induced change in actin distribution

Cell line incubated with compound to induce changes in actin, then stained with FITC phalloidin (actin, green) and DAPI (nuclear dye, purple).

The job = classify cells based on their shape

Tasks:

5 – Quantify statistical discrimination power of the features, and rank to choose best one = IMAGESTREAM; This is a plot of IDEAS actin shape features (X-axis) vs. Discrimination power (Rd, Y-axis). Data for ~140 features are shown. The feature with the highest Rd will be chosen…



	A	B
1	Population	Elongate
2	Shape Ratio_Object(M02, 2_FITC, Tight), round, Mean RD	3.318
3	Shape Ratio_M02, round, Mean RD	2.736
4	Circularity_Object(M02, 2_FITC, Tight), round, Mean RD	2.562
5	Circularity_M02, round, Mean RD	2.254
6	Aspect Ratio_Object(M02, 2_FITC, Tight), round, Mean RD	1.995
7	Aspect Ratio_M02, round, Mean RD	1.89
8	Symmetry 2_M02_2_FITC, round, Mean RD	1.843
9	Symmetry 2_Object(M02, 2_FITC, Tight)_2_FITC, round, Mean RD	1.769
10	Circularity_Morphology(M02, 2_FITC), round, Mean RD	1.757
11	Symmetry 3_Object(M02, 2_FITC, Tight)_2_FITC, round, Mean RD	1.734
12	Aspect Ratio_Morphology(M02, 2_FITC), round, Mean RD	1.699
13	Symmetry 3_M02_2_FITC, round, Mean RD	1.684
14	Thickness Min_Object(M02, 2_FITC, Tight), round, Mean RD	1.587
15	Elongatedness_M02, round, Mean RD	1.581
16	Aspect Ratio Intensity_M02_Ch02, round, Mean RD	1.556
17	Shape Ratio_Morphology(M02, 2_FITC), round, Mean RD	1.518
18	Elongatedness_Object(M02, 2_FITC, Tight), round, Mean RD	1.511
19	Aspect Ratio Intensity_Object(M02, 2_FITC, Tight)_2_FITC, round, Mean RD	1.502
20	Symmetry 2_Morphology(M02, 2_FITC)_2_FITC, round, Mean RD	1.485

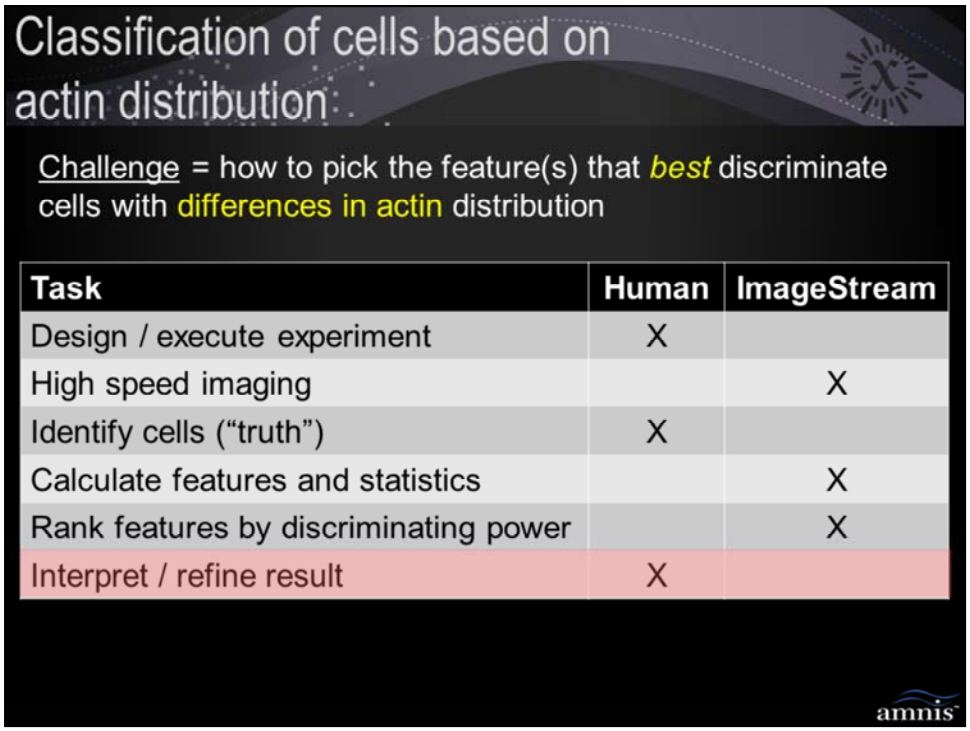
Example number 2: Treatment-induced change in actin distribution

Cell line incubated with compound to induce changes in actin, then stained with FITC phalloidin (actin, green) and DAPI (nuclear dye, purple).

The job = classify cells based on their shape

Tasks:

5 – Quantify statistical discrimination power of the features, and rank to choose best one = IMAGESTREAM; Top-20 actin shape features are shown. The feature with the highest Rd is **Shape Ratio_OT(M02)** and represents the feature that provides the best statistically discrimination between the round and elongated **TRUTH** sets. Note, the feature that wins is critically dependent on the truth sets, and if upon evaluation of the feature on the larger file it is found that the feature does not perform well, you may need to refine the truth sets and repeat the process…



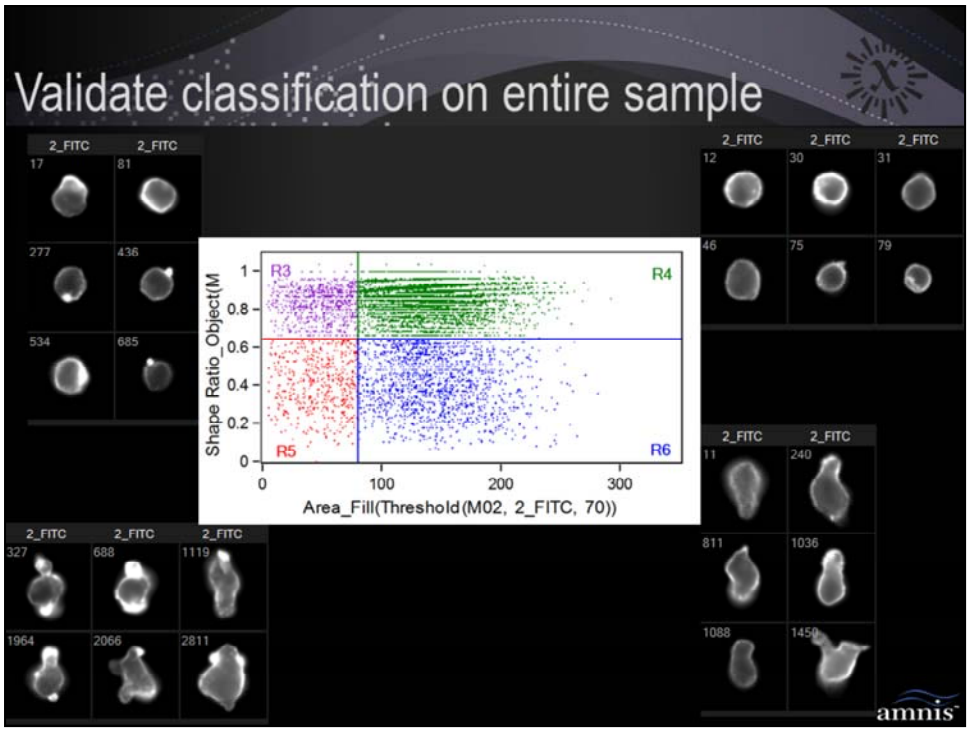
Example number 2: Treatment-induced change in actin distribution

Cell line incubated with compound to induce changes in actin, then stained with FITC phalloidin (actin, green) and DAPI (nuclear dye, purple).

The job = classify cells based on their actin morphology

Tasks:

6 – Evaluate / refine the result = HUMAN; apply the **Shape Ratio_OT(M02) and Area_FT70%** features to the whole sample and look for trends in any false pos/neg that appear; repick truth if necessary; add advanced features (as you learn the system); protocol development if necessary - NOTE THAT YOU DO NOT NEED TO OBTAIN PERFECTION – REMEMBER THAT YOU HAVE STATISTICALLY LARGE NUMBER OF EVENTS TO COUNTER…;



Example number 2: Treatment-induced change in actin distribution

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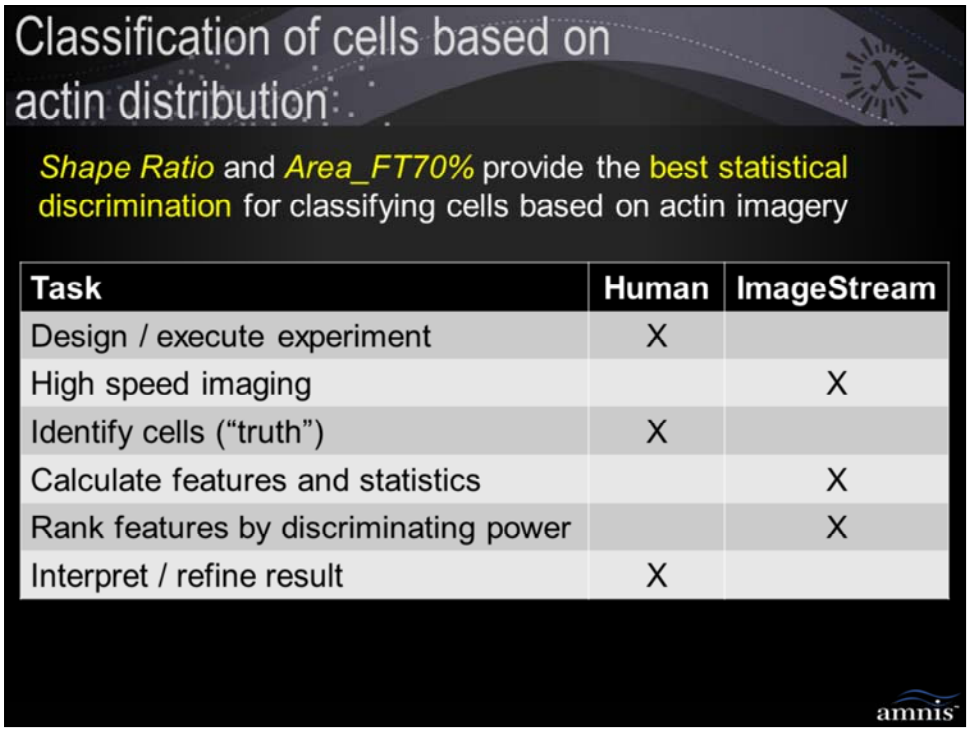
<u>The job = classify cells based on their actin morphology</u>

Tasks:

6 – Evaluate / refine the result = <u>HUMAN</u>; plot the **Shape Ratio_OT(M02) and Area_FT70%** features for the whole sample and look for trends in any false pos/neg that appear; repick truth if necessary; add advanced features (as you learn the system); protocol development if necessary - NOTE THAT YOU DO NOT NEED TO OBTAIN PERFECTION – REMEMBER THAT YOU HAVE STATISTICALLY LARGE NUMBER OF EVENTS TO COUNTER…;

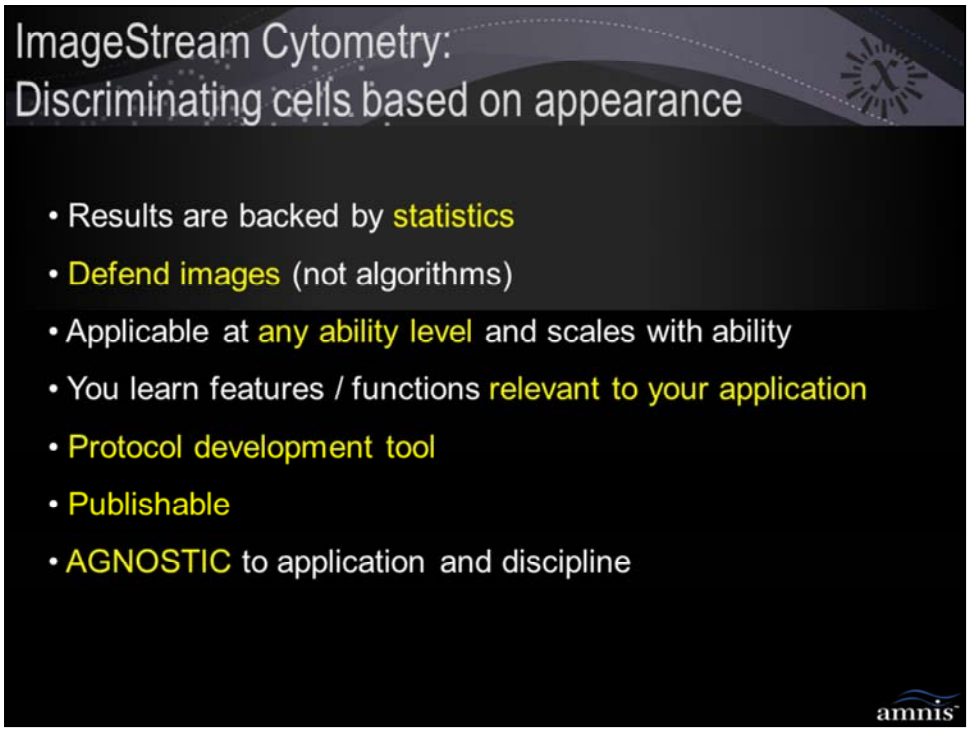


Example number 2: Treatment-induced change in actin distribution

Cell line incubated with compound to induce changes in actin, then stained with FITC phalloidin (actin, green) and DAPI (nuclear dye, purple).

The job = classify cells based on their actin morphology

Conclusion: Shape Ratio_OT(M02) and Area_FT70% discriminate cells based on actin polarity and shape



Results backed by statistics:

feature with best statistical discrimination power selected from a large list of competitors)

Defend images not algorithms:

you don't need to know what the math behind the algorithm to defend it in front of peers – just defend the truth populations. This places the debate within the arena where humans work best, ie qualitatively discriminating cells that represent the expected biology, as opposed to arguing about complicated math problems…)

Applicable by all ability levels and scales with ability:

With this process, you can get statistically defensible (and publishable) results before you learn any of the algorithms. As you learn more advanced operations, you can add new functions/features to the list for Rd ranking, potentially improving the results…

You learn features/functions relevant to your application:

Instead of trying to learn all 85 features and 16 masking functions at once, this

process points you to the features that work best in your system – you learn these first. As you refine the truth, you learn advanced operations that improve your top features…

Protocol development tool:

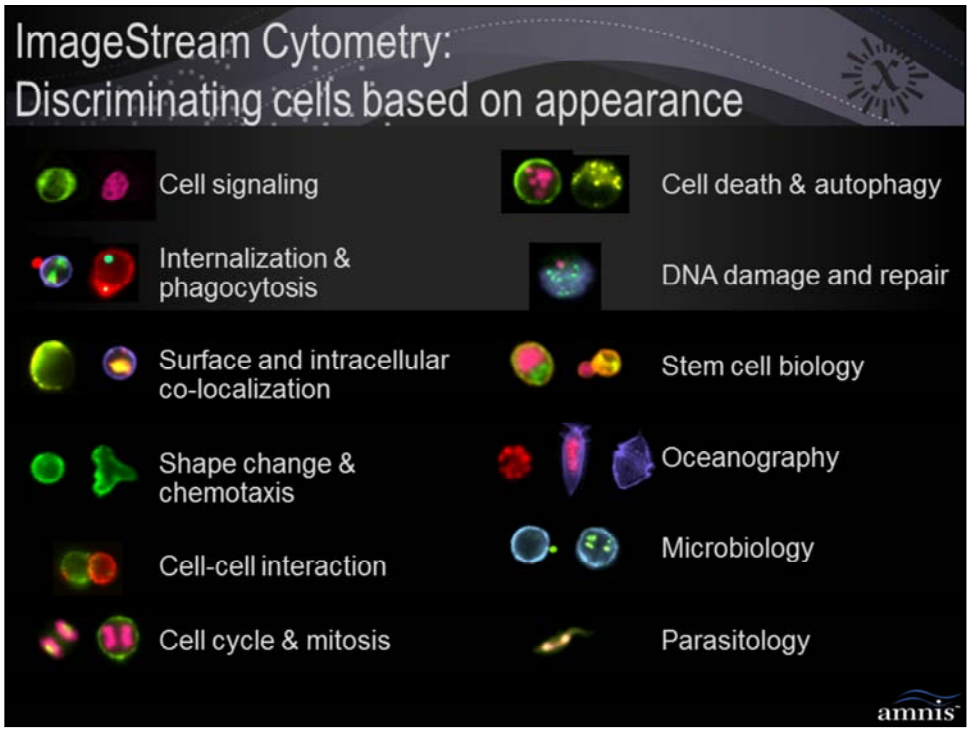
Protocol development is made easy using this sytem – two major reasons: First, you can see your cells for quick qualitative assessment of success/failure of experimental design (negative and positive biologic controls, stains, fix/perm, etc). Second, the discriminant ratio can be employed to quantify the effect of any protocol change. For example, if you compare triton perm vs saponin perm for nuc trans assay, you choose the perm that provides the highest Rd Similarity between the untreated and treated samples.

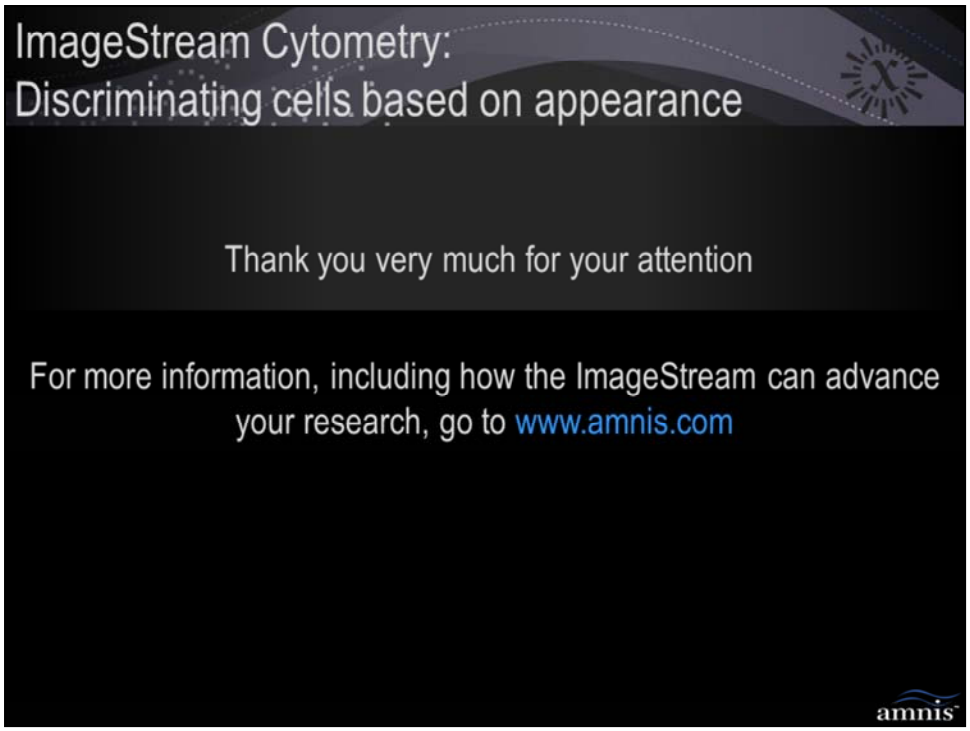
Publishable:

ImageStream technology is relatively new – many (if not most) new protocols you develop with the system will be novel and publishable.

Agnostic to application and discipline:

As long as your experiment results in populations of cells that can be differentiated by the ImageStream, this process will find features that discriminate the populations


ImageStream Cytometry:
Discriminating cells based on appearance
Cell signaling
Internalization & phagocytosis
Surface and intracellular co-localization
Shape change & chemotaxis
Cell-cell interaction
Cell cycle & mitosis
Cell death & autophagy
DNA damage and repair
Stem cell biology
Oceanography
Microbiology
Parasitology
amnis


ImageStream Cytometry:
Discriminating cells based on appearance
Thank you very much for your attention
For more information, including how the ImageStream can advance your research, go to www.amnis.com
amnis

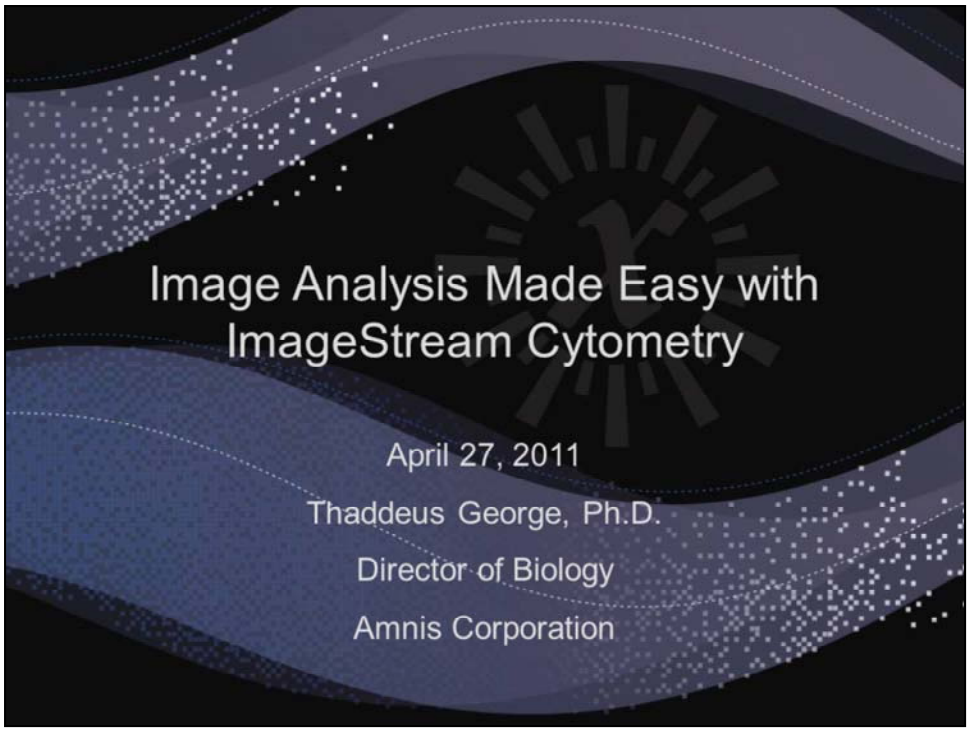


Image Analysis Made Easy with ImageStream Cytometry
April 27, 2011
Thaddeus George, Ph.D.
Director of Biology
Amnis Corporation