

CellProfiler is a free, open-source software for image analysis, developed and supported by Anne E. Carpenter at the BROAD Institute. More information and software available at <a href="https://cellprofiler.org">https://cellprofiler.org</a> For in depth tutorials and training, check videos published by the Center for Open Bioimage Analysis (COBA): <a href="https://www.youtube.com/c/COBACenterforOpenBioimageAnalysis">https://www.youtube.com/c/COBACenterforOpenBioimageAnalysis</a>

IMPORTANT NOTE: For proper image analysis and coherent measurement of features, images must be supplied as original files, free of value modification (brightness/contrast adjustment), filtering and other deconvolution calculations. Image values must be absolute and comparable inside a same data set (same bit depth, same acquisition parameters for instance)

# 1) CellProfiler user interface overview

	CellProfiler 4.2.1	
C Images C Metadata C NamesAndTypes C Groups	To begin creating your project, use the Images module to compile a list of files and/or folders that you want to analyze. You can also specify a set of rules to inclus only the desired files in your selected folders.	de
b	d	
Drop a pipeline file here (.cppipe or .cpproj) or double-click to add modules	Drop files and folders here	
Output Settings View Workspace           ?         Adjust modules: + - ^ v           Ib Start Test Mode         Analyze Images	<ul> <li>✓ Show files excluded by filters</li> <li>Filter images? Images only</li> <li>✓</li> <li>Apply filters to the file list</li> <li>Found 0 rows</li> </ul>	?

a- Input modules, for image selection, metadata retrieval and integration

b- Image analysis pipeline window. Add modules to build your image analysis pipeline

**c**- Brief description of the module in use, either provided by the software or the user. Very helpful to review past pipelines, indispensable when sharing/publishing pipelines.

d- Settings windows, specific to each module

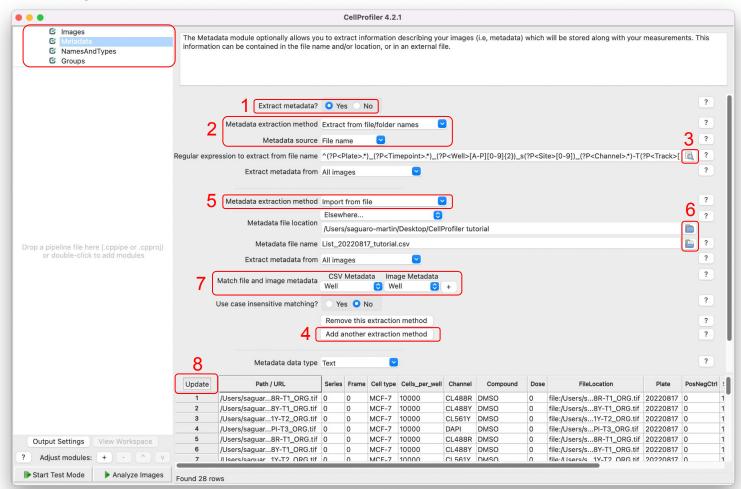
**?-** "Help" buttons throughout the user interface. The "Help" button on the left provides global help for the selected module. To the right, a button is available for each setting, providing a more specific description of the corresponding setting. Help is never far, and always available!

## 2) Adding image files/folders

	CellProfiler 4.2.1
<ul> <li>Images</li> <li>Metadata</li> <li>NamesAndTypes</li> <li>Groups</li> </ul>	To begin creating your project, use the Images module to compile a list of files and/or folders that you want to analyze. You can also specify a set of rules to include only the desired files in your selected folders.
1 Drop a pipeline file here (.cppipe or .cpproj) or double-click to add modules	V/Users/isaguaro-martin/Desktop/CellProfiler turnal/images 20220817_6h_B01_st_CL488R-T1_ORG.tif 20220817_6h_B01_st_CL488R-T1_ORG.tif 20220817_6h_B01_st_CL488R-T1_ORG.tif 20220817_6h_B02_st_CL488R-T1_ORG.tif 20220817_6h_B02_st_CL488R-T1_ORG.tif 20220817_6h_C02_st_CL488R-T1_ORG.tif 20220817_6h_C01_st_CL488R-T1_ORG.tif 20220817_6h_C01_st_CL488R-T1_ORG.tif 20220817_6h_C01_st_CL488R-T1_ORG.tif 20220817_6h_C01_st_CL488R-T1_ORG.tif 20220817_6h_C01_st_CL488R-T1_ORG.tif 20220817_6h_C01_st_CL488R-T1_ORG.tif 20220817_6h_C01_st_CL488R-T1_ORG.tif 20220817_6h_C02_st_CL488R-T1_ORG.tif 20220817_6h_C02_st_CL488R-T1_ORG.tif 20220817_6h_C02_st_CL488R-T1_ORG.tif 20220817_6h_C02_st_CL488R-T1_ORG.tif 20220817_6h_C02_st_CL488R-T1_ORG.tif 20220817_6h_C01_st_CL488R-T1_ORG.tif
	Show files excluded by filters
Output Settings View Workspace	Filter images? Images only ?
? Adjust modules: + - ^ 2/	Apply filters to the file list Apply filters to the file list ?
Start Test Mode Analyze Images	Apply inters to the file list Apply inters to the file list ?

Add files and/or folders by simply dragging and dropping (1). Here the "Images" folder was selected. Additional files/folders can further be added to the list. Right-clicking on elements of the file list allows you to select or delete them (from the list). By default, only image files are selected. Custom filters can further be added to select specific files (2).

# 3) Extracting metadata



In this example, metadata (more specifically, information regarding the plate, well and channel) is extracted (1) from the filename(2) via a regular expression (3) (see Note at the end of this section). Additionally (4), a .csv file (5) containing further information has been provided (6) and matched well to well (7). This provides metadata for each image, which will help direct the right analysis modules to the right images, and complement the extracted measurements. The extracted metadata can be previewed at anytime by updating the bottom table (8).

NOTE: Extracting metadata from the filename requires the use of a regular expression. More information can be found on this subject, but here is the regular expression for this particular example:

Filename: 20220817\_6h\_B01\_s1\_CL488R-T1\_ORG.tif Regular expression: ^(?P<Plate>.\*)\_(?P<Timepoint>.\*)\_(?P<Well>[A-P][0-9]{2})\_s(?P<Site>[0-9]) \_(?P<Channel>.\*)-T(?P<Track>[0-9])\_(?P<other>.\*)

# 4) Assigning Names and Types

				CellP	rofiler 4.2.1				
Output Settings       Verw Workspace       9         Output Settings       Verw Workspace       9	<ul><li>Metadata</li><li>NamesAndTypes</li></ul>	The Name	esAndTypes module	allows you to assign a m	eaningful name to each image by v	which other moo	dules will refer to it.		
Output Settings       View Workspace         Output Settings       View Workspace		2 2	Process as 3D?	Yes O No	owing rules	S DAPI		- +	?
Output Settings       View Workspace       Output Settings       ?         Output Settings       View Workspace       6       20220817_6h_C01_s1_CL4888-T1_OR6.iff       20220817_6h_C02_s1_CL561Y-T2_OR6.iff       20220817_6h_C01_s1_CL4888-T1_OR6.iff         Output Settings       View Workspace       6       20220817_6h_C01_s1_CL4888-T1_OR6.iff       20220817_6h_C02_s1_CL4887-T1_OR6.iff       20220817_6h_C02_s1_CL561Y-T2_OR6.iff       20220817_6h_C02_s1_CL681Y-T1_OR6.iff	3	Name to assi	ign these images D/	API					?
Output Settings       View Workspace       4       Duplicate this image       ?         Output Settings       View Workspace       6       20220817.6h.C01_s1_CL488R-T1_ORG.iff       20220817.6h.C01_s1_CL488R-T1_ORG.iff       ?		Selec	ct the image type Gr	rayscale image 🛛 💽					?
Drop a pipeline file here (coppipe or coppro) or double-click to add modules       Match All © of the following rules Metadata © Does © Have Channel matching © CL488Y       ?         Name to assign these images       ChromaLive488_Yellow       ?         Select the rule criteria       Match All © of the following rules       ?         Duplicate this image       ?         Betlet the rule criteria       Match All © of the following rules       ?         Emmove this image       ?         Duplicate this image       ?         Duplicate this image       ?         Select the rule criteria       Match All © of the following rules       ?         Update       ChromaLive488_Yellow       ?         1       2020817_6h_B02_s1_CL488R-T1_ORG.tif       20220817_6h_B02_s1_CL488R-T1_ORG.tif       20220817_6h_B02_		Set inte	ensity range from Im	age metadata 🛛 😒					?
Drop a pipeline file here (coppipe or .cpproj) or double-click to add modules       Select the rule criteria       Metadata @ Does @ Have Channel matching @ CL488Y       - +         Name to assign these images       ChromaLive488.Yellow       ?         Select the image type       Grayscale image       ?         Duplicate this image       ?         Remove this image       ?         Select the rule criteria       Match. All @ of the following rules       ?         5       Select the rule criteria       Match. All @ of the following rules       ?         1       20220817_6h_B01_s1_CL488R-T1_0RG.tif       20220817_6h_B01_s1_CL488R-T1_0RG.tif       20220817_6h_B01_s1_CL488R-T1_0RG.tif       20220817_6h_B01_s1_CL488R-T1_0RG.tif       20220817_6h_B01_s1_CL488R-T1_0RG.tif       20220817_6h_B01_s1_CL561Y-T2_ORG.tif       20220817_6h_B01_s1_CL488R-T1_0RG.tif       20220817_6h_B01_s1_CL561Y-T2_ORG.tif       20220817_6h_B01_s1_CL68Y-T1_ORG.tif       20220817_6h_B01_s1_CL561Y-T2_ORG.tif       20220817_6h_B01_s1_CL68Y-T1_ORG.tif       20220817_6h_B01_s1_CL68Y-T1_ORG.tif       20220817_6h_D01_s1_CL68Y-T1_ORG.tif       <			4 🖸	Duplicate this image					?
Select the image type         Grayscale image         ?           Set intensity range from         Image metadata         ?           Duplicate this image         ?           Remove this image         ?           Set intensity range from         Match All e of the following rules         ?           Set intensity         ?           Match All e of the following rules         ?           Select the rule criteria         Metadata of Does of Have Channel matching et CL488R         ?           Update         ChromaLive488_Red         ChromaLive488_Vellow         ChromaLive561_Yellow           Update         ChromaLive488_Red         ChromaLive488_Vellow         ChromaLive561_Yellow           1         20220817_6h_B01_s1_CL488R-T1_ORG.tif         20220817_6h_B01_s1_CL561Y-T2_ORG.tif         20220817_6h_B02_s1_CL488Y-T1_ORG.tif           2         20220817_6h_D01_s1_CL488R-T1_ORG.tif         20220817_6h_C01_s1_CL488Y-T1_ORG.tif         20220817_6h_C01_s1_CL561Y-T2_ORG.tif         20220817_6h_C01_s1_CL488Y-T1_ORG.tif           3         20220817_6h_C01_s1_CL488R-T1_ORG.tif         20220817_6h_C01_s1_CL561Y-T2_ORG.tif         20220817_6h_C01_s1_CL488Y-T1_ORG.tif           4         20220817_6h_C01_s1_CL488R-T1_ORG.tif         20220817_6h_C01_s1_CL561Y-T2_ORG.tif         20220817_6h_C01_s1           5         20220817_6h_C01_s1_CL488R-T1_ORG.tif		Selec:	Ma t the rule criteria			CL488Y		- +	?
Output Settings         View Workspace         Output Chromative ABBR-T1_ORG.tif         20220817_6h_D01_s1_CL488R-T1_ORG.tif         20220817_6h_D01_s1_CL661Y-T2_ORG.tif         20220817_6h_D01_s1_CL488R-T1_ORG.tif           Output Settings         View Workspace         6         20220817_6h_D01_s1_CL488R-T1_ORG.tif         20220817_6h_D01_s1_CL561Y-T2_ORG.tif         20220817_6h_D01_s1_CL488R-T1_ORG.tif         20220817_6h_D01_s1_CL561Y-T2_ORG.tif         20220817_6h_D01_s1_CL488R-T1_ORG.tif		Name to assi	ign these images Ch	nromaLive488_Yellow					?
Output Settings         View Workspace         Chromative 488         Chroma		Selec	ct the image type Gr	rayscale image 🛛 💽					?
Remove this image         ?           Match All © of the following rules         ?           5         Select the rule criteria         Match All © of the following rules         ?           Match All © of the following rules         ?           Match All © of the following rules         ?           1         20220817_6h_B01_s1_CL488R-T1_ORG.tif         20220817_6h_B01_s1_CL561Y-T2_ORG.tif         20220817_6h_B02_s1_CL488R-T1_ORG.tif           2         20220817_6h_B02_s1_CL488R-T1_ORG.tif         20220817_6h_B02_s1_CL488R-T1_ORG.tif         20220817_6h_B02_s1_CL561Y-T2_ORG.tif         20220817_6h_B02_s1_CL488R-T1_ORG.tif           3         20220817_6h_O0_s1_s1_CL488R-T1_ORG.tif         20220817_6h_C01_s1_CL488R-T1_ORG.tif         20220817_6h_C01_s1_CL561Y-T2_ORG.tif         20220817_6h_C02_s1_CL488Y-T1_ORG.tif           4         20220817_6h_C01_s1_CL488R-T1_ORG.tif         20220817_6h_C01_s1_CL561Y-T2_ORG.tif         20220817_6h_C02_s1_CL561Y-T2_ORG.tif         20220817_6h_C02_s1_CL561Y-T2_ORG.tif         20220817_6h_C01_s1           Mutput Settings         View Workspace         5         20220817_6h_C01_s1_CL488R-T1_ORG.tif         20220817_6h_C01_s1_CL561Y-T2_ORG.tif         20220817_6h_C01_s1           6         20220817_6h_C01_s1_CL488R-T1_ORG.tif         20220817_6h_C01_s1_CL561Y-T2_ORG.tif         20220817_6h_C01_s1         20220817_6h_C01_s1         20220817_6h_C01_s1		Set inte	ensity range from Im	age metadata 🛛 😒					?
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2         20220817_6h_B02_s1_CL488R-T1_ORG.tif         20220817_6h_B02_s1_CL488Y-T1_ORG.tif         20220817_6h_B02_s1_CL561Y-T2_ORG.tif         20220817_6h_B02_s1_CL561Y-T2_ORG.tif         20220817_6h_B02_s1_CL561Y-T2_ORG.tif         20220817_6h_B02_s1_CL561Y-T2_ORG.tif         20220817_6h_C01_s1_CL561Y-T2_ORG.tif         202208		Update	Chroma	Live488_Red	ChromaLive488_Yellow	, I	ChromaLive561_Yellow		DAP
3         20220817_6h_C01_s1_CL488R-T1_ORG.tif         20220817_6h_C01_s1_CL488Y-T1_ORG.tif         20220817_6h_C01_s1_CL561Y-T2_ORG.tif         20220817_6h_C01_s1_CL488Y-T1_ORG.tif           4         20220817_6h_C01_s1_CL488R-T1_ORG.tif         20220817_6h_C02_s1_CL488Y-T1_ORG.tif         20220817_6h_C02_s1_CL561Y-T2_ORG.tif         20220817_6h_C02_s1_CL561Y-T2_ORG.tif         20220817_6h_C02_s1_CL561Y-T2_ORG.tif         20220817_6h_C01_s1_CL561Y-T2_ORG.tif         20220817_6h_C01_s1_CL5		1	20220817_6h_B01	_s1_CL488R-T1_ORG.tif	20220817_6h_B01_s1_CL488Y-T	1_ORG.tif 2022	20817_6h_B01_s1_CL561Y-T2_ORG.tif	20220817_6h_E	301_s1
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Output Settings         View Workspace         5         20220817_6h_D01_s1_CL488R-T1_ORG.tif         20220817_6h_D01_s1_CL488R-T1_ORG.tif         20220817_6h_D01_s1_CL561Y-T2_ORG.tif         20220817_6h_D01_s1_CL488Y-T1_ORG.tif         20220817_6h_E01_s1_CL561Y-T2_ORG.tif									
Output settings         View Workspace           6         20220817_6h_E01_s1_CL488R-T1_ORG.tif         20220817_6h_E01_s1_CL488Y-T1_ORG.tif         20220817_6h_E01_s1_CL561Y-T2_ORG.tif         20220817_6h_E01_s1_CL561Y-T2_ORG.tif		4							
		-			2022001/ 01 DUI SI CL4001-1	2022	2001/_01_001_51_0L5011-12_0RG.tll	2022031/_011_L	201_51
	Output Settings View Workspace					1 ORG tif 2023	20817 6h E01 s1 CI 561Y-T2 ORG tif	20220817 6h F	-01 c1

This module allows you to assign names to specific images (1). For example, the previously extracted metadata (2) assign a desired name (3) to the selected images. By duplicating this filtering (4), image sets can be created containing multiple images. Again, the "Update" button (5) shows a preview of these image sets.

# 5) Adding modules to the analysis pipeline

Modules can be added to the analysis pipeline by simply clicking the "+" button by "Adjust modules:", at the lower left of the screen. The popup window provides a search field to find modules, or they can be found in their corresponding modules category. Double-clicking a module adds it to the pipeline. Module can also be dragged inside the pipeline to reorder them. Again, help is also available to learn about each module. It can be necessary to start an analysis pipeline with preliminary modules such as "Align" in case of shifted images, or "CorrectIllumination" in case of vignetting for example.

Find Modules:		Search Help
Module Categories	Align	
File Processing	ColorToGray	
Image Processing	CorrectIllumination	Apply
Object Processing	CorrectIllumination	Calculate
Measurement	Crop	
Data Tools	EnhanceEdges	
Advanced	EnhanceOrSuppres	sFeatures
Worm Toolbox	FlipAndRotate	
Other	GrayToColor	
All	ImageMath	
	InvertForPrinting	
	MakeProjection	
	MaskImage	
	Morph	
	OverlayObjects	
	OverlayOutlines	
	RescaleIntensity	
+ Add to Pipeline	Resize	
	Smooth	
? Module Help	Threshold	
Getting Started	Tile	
Done	UnmixColors	

# 6) Identify primary objects

In our case, our first module of interest is the "IdentifyPrimaryObjects", located in the "Object Processing" category. In this module, we segment the cell nuclei with the help of nuclear staining. This is done by selecting the proper image (1), as identified in the NamesAndTypes section, and naming the resulting object (2). It can be useful to discard objects outside the diameter range or touching the image border as they can generate false non-relevant objects (3). Advanced settings are also available in order to obtain more precise and thorough image segmentation and object identification (4).

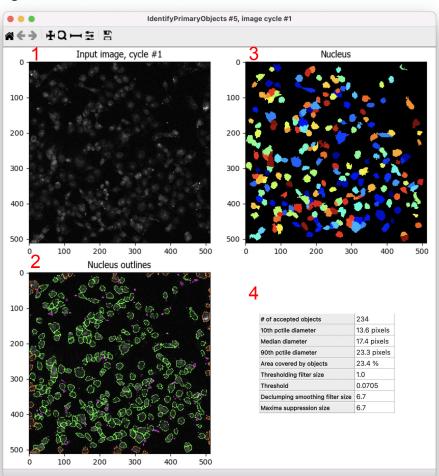
• • •	CellProfiler 4.2.1
<ul> <li>☑ Images</li> <li>☑ Metadata</li> <li>☑ NamesAndTypes</li> <li>☑ Groups</li> <li>☑ Groups</li> <li>☑ IdentifyPrimaryObjects</li> </ul>	4       Use advanced settings?       Yes       No       ?         1       Select the input image       DAPI       Ø (from NamesAndTypes)       ?         2       Name the primary objects to be identified       Nucleus       ?         Typical diameter of objects, in pixel units (Min,Max)       10       40       ?         Discard objects outside the diameter range?       Yes       No       ?         Discard objects touching the border of the image?       Yes       No       ?
Output Settings View Workspace ? Adjust modules: + - ^ v	
Start Test Mode Analyze Images	Found 7 image sets

# 7) Test mode, to preview segmentation settings

Once preliminary settings are set in the module, it can be useful to use the "Start Test Mode" feature of CellProfiler, located at the bottom left. CellProfiler will then run through each module step by step (by clicking successively the "Next Step" button), and display the results of the ones selected (eye icon next to the module).

In the case of the "IdentifyPrimary Object" module, this will present the input image (1), as well as an overlay of the object outlines (2) and a final color image with the different objects represented (3). Finally a table presents some statistics of the segmentation (4).

"Next Image Set" moves through the image sets defined in the "Names AndTypes" module previously. "Exit Test Mode" allows you to end the test run and return to the pipeline.



# 8) Identify secondary objects

Building upon our primary object (Nucleus), CellProfiler can then identify whole cells. This is done by selecting the input image (1), here ChromaLive561 shows the best whole cell staining, and selecting the object to build from (2) (here: Nucleus). Other settings can be adjusted for optimal cell segmentation. Again, perform test runs with the "Test Mode" feature optimise relevant settings.

	CellProfil	er 4.2.1	
© Images © Metadata © NamesAndTypes © Groups © © IdentifyPrimaryObjects © © IdentifySecondaryObjects			
	Select the input image	ChromaLive561_Yellow (from NamesAndTypes)	?
	2 Select the input objects	Nucleus 💟 (from IdentifyPrimaryObjects #05)	?
	Name the objects to be identified	Cell	?
	Select the method to identify the secondary objects	Propagation 💙	?
	Threshold strategy	Global	?
	Thresholding method	Minimum Cross-Entropy	?
	Threshold smoothing scale	0.0	?
	Threshold correction factor	1.0	?
	Lower and upper bounds on threshold	0.0 1.0	?
	Log transform before thresholding?	Ves O No	?
	Regularization factor	0.05	?
Output Settings View Workspace	Fill holes in identified objects?	• Yes No	?
? Adjust modules: + - ^ V	Discard secondary objects touching the border of the image?	Yes O No	?
Start Test Mode Analyze Images	Found 7 image sets		

#### 9) Adding measurement modules

Once all the objects of interest have been selected and identified, it is time to add measurement modules. The following screenshot shows a list of modules from the "Measurement" category. The guiding principle here is to gather as many measurements as possible for further data analysis. In the case of ChromaLive dyes, we recommend measuring fluorescence intensity as well as shapes and sizes of objects as a bare minimum. Object texture (influenced for example by the presence of highly fluorescent vesicles in the cytoplasm, or condensing DNA in the nucleus) can also be a relevant measurement.

ind Modules:	Search Help
Module Categories File Processing Image Processing Object Processing Measurement Data Tools Advanced Worm Toolbox Other All	MeasureColocalization MeasureGranularity MeasureImageAreaOccupied MeasureImageOverlap MeasureImageQuality MeasureImageSkeleton MeasureObjectIntensity MeasureObjectIntensity MeasureObjectNeighbors MeasureObjectOverlap MeasureObjectSkeleton MeasureObjectSkeleton MeasureTexture
+ Add to Pipeline	
? Module Help	
Getting Started	
Done	

# 10) Calculating some math

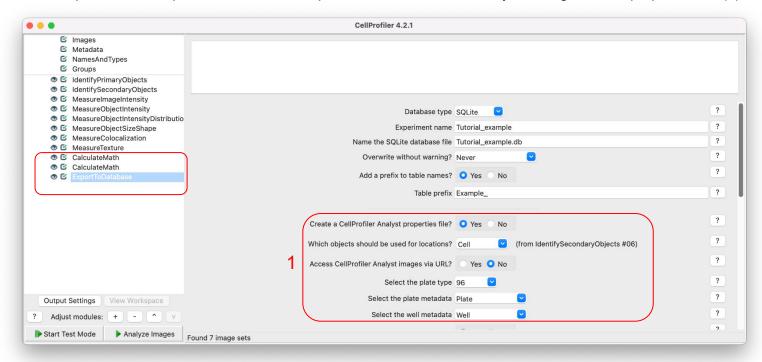
Depending on the phenotype, and the ChromaLive dye used, it can also be relevant to include some extrapolated measurements. These measurements can be obtained via the "CalculateMath" module, in the "Data Tools" category. Here we are normalising fluorescence measurements, by dividing the intensity from one acquisition (ChromaLive561) by another (ChromaLive488\_Yellow).

Name and define the desired calculation (1). From the previously performed measurements, select the type of measurement for the numerator (2) and similarly for the denominator (3). Add additional modules if other calculations are needed.

•••	CellProfiler 4.2.1	
<ul> <li>☑ Images</li> <li>☑ Metadata</li> <li>☑ NamesAndTypes</li> <li>☑ Groups</li> <li>☑ IdentifyPrimaryObjects</li> <li>☑ ☑ IdentifySecondaryObjects</li> </ul>		
<ul> <li>G MeasureImageIntensity</li> <li>G MeasureObjectIntensity</li> <li>G MeasureObjectIntensityDistributio</li> <li>G MeasureObjectSizeShape</li> <li>G MeasureObjectSizeShape</li> </ul>	Name the output measurement     Ratio_CL561_CL488Y       Operation     Divide	?
<ul> <li>♥ MeasureTexture</li> <li>♥ CalculateMath</li> </ul>	Select the numerator measurement type Object Select the numerator objects Cell (from IdentifySecondaryObjects #06)	?
	Category: Intensity  Category: Category: Intensity  Category: Intensity  Category: Intensity  Category: ChromaLive561_Yelle	?
	Multiply the above operand by 1.0 Raise the power of above operand by 1.0	?
	Select the denominator measurement type Object 💟	?
Output Settings View Workspace  Adjust modules: + - ^ v	Select the denominator objects Cell  (from IdentifySecondaryObjects #06) Category: Intensity	?
Start Test Mode Analyze Images	Found 7 image sets	

#### 11) Exporting measurements and saving files

By default, CellProfiler does not save any, it only offers to save the designed analysis pipeline. To export measurements or save images, additional modules have to be added to the pipeline. These can be found in the "File Processing" module category. Here is the example of the "ExportToDatabase" module which exports data compatible with the subsequent use of CellProfiler Analyst via a generated properties file (1).



## 12) Masking modules and running pipeline

Once the pipeline is designed and tested, it is time to run it on the entire image set. To avoid being saturated with popup modules, it is recommend to hide all the module (greyed eye icon next to each one). This can be achieved by the "Windows">"Hide all windows on run" menu. Then click the "Analyse images" button in the lower left. Processing time will depend on the pipeline, the available computing power as well as the number and size of the images to analyse.