Loading Image Stacks and Movies

In this context, the term *image sequence* is used to refer to a collection of images which can be from a time-lapse assay, a three-dimensional (3D) Z-stack assay, or both. This tutorial will instruct you how to load these collections in order to properly represent your data for processing.

Example #1: Sequences of individual files

For some microscopes, the simplest method of capturing image sequences is to simply acquire them as a series of individual images, where each image represents a single timepoint or Z-slice (for simplicity, we will refer to *timepoints* in the rest of this example). Typically, the image filename reflects the timepoint, such that the alphabetical image listing corresponds to the proper sequence, e.g., *img000.png*, *img001.png*, *img002.png*, etc. It is also not uncommon to store the movie such that one movie's worth of files is stored in a single folder.

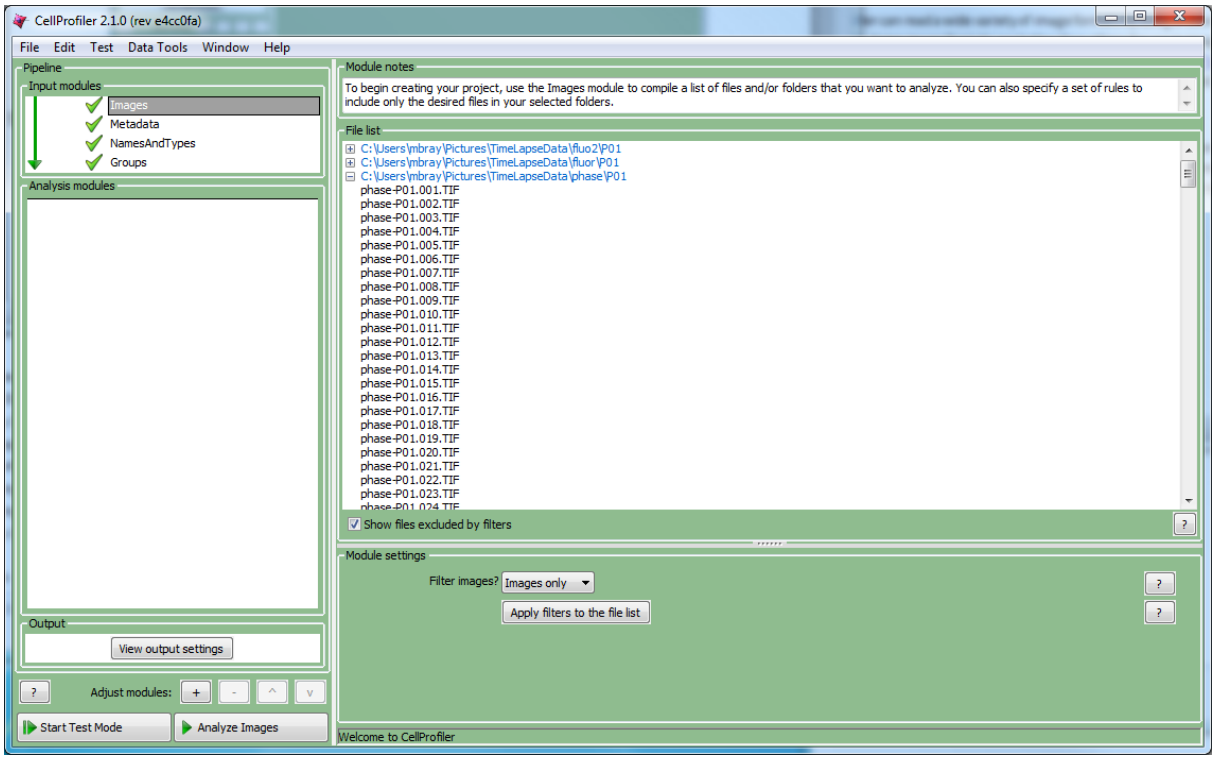
For example, let's say you have a time-lapse movie of individual files set up as follows:

- Three folders, one for each image channel, named *fluo2*, *fluor* and *phase*.
- In each folder, the files are named for the imaging modality used as follows:
  - *fluo2*: calibrate2-P01.001.TIF, calibrate2-P01.002.TIF,..., calibrate2-P01.287.TIF
  - *fluor*: calibrated-P01.001.TIF, calibrated-P01.002.TIF,..., calibrated-P01.287.TIF
  - phase: phase-P01.001.TIF, phase-P01.002.TIF,..., phase-P01.287.TIF

  where the file names are in the format <*Stain*>-<*Well*>.<*Timepoint*>.*TIF*.
- There are 287 timepoints per movie, and a movie of the 3 channels above is acquired from each well.

In this case, the first step to set up the input modules to handle these files would be to select the **Images** module and drag-and-drop your folders of images into the File list panel. If necessary, set your filtering rules accordingly in order to omit any files that are not part of a movie sequence.

In the above example, you would drag-and-drop the *fluo2*, *fluor* and *phase* folders into the File list panel. In this case, the default "Images only" filter is sufficient to capture the necessary files.



In the **Metadata** module, check the box to enable metadata extraction. The key step here is to obtain the necessary metadata tags to do two things:

- Distinguish the movies from each other. This information is typically encapsulated in the filename and/or the folder name.
- For each movie, distinguish the timepoints from each other, ensuring their proper ordering. This information is often contained in the filename.

To accomplish this, do the following:

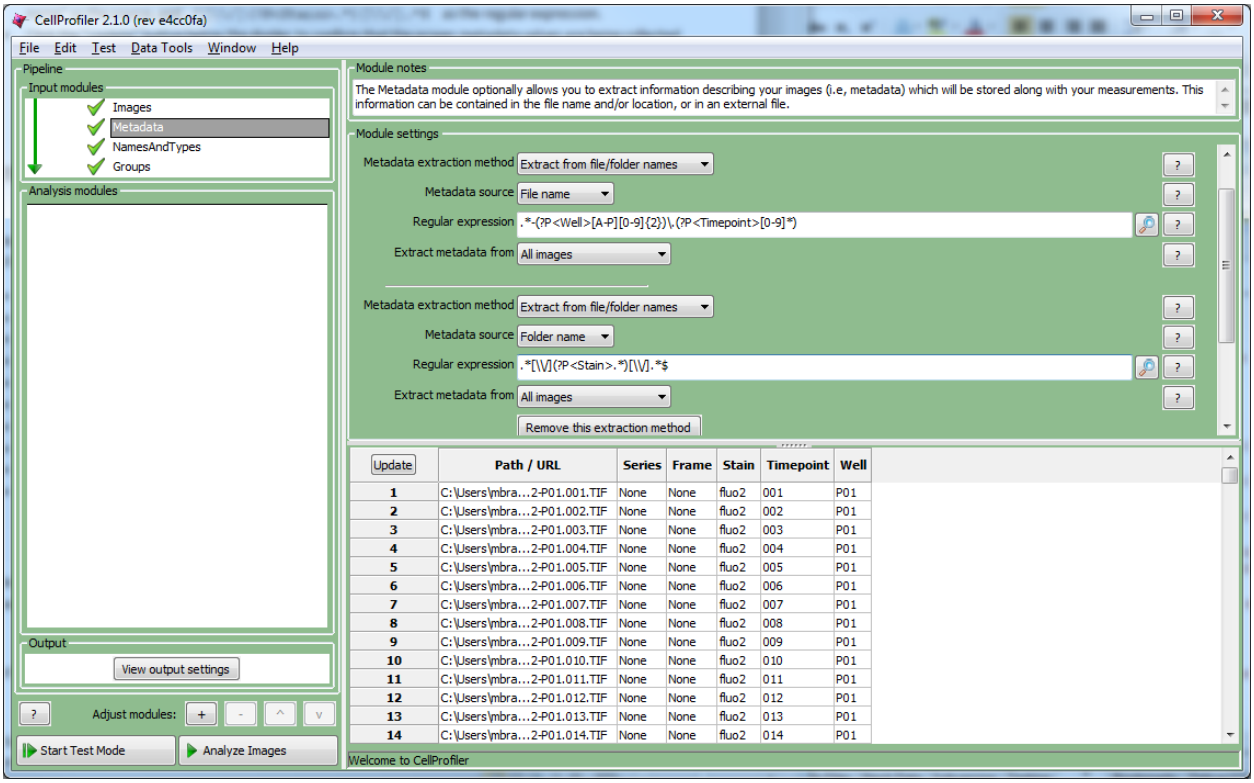
- Select "Extract from file/folder names" or "Import from file" as the metadata extraction method. You will use these to extract the movie and timepoint tags from the images. Use "Extract from file/folder names" to create a regular expression to extract the metadata from the filename and/or path name.
- Or, use "Import from file" if you have a comma-delimited file (CSV) of the necessary metadata columns (including the movie and timepoint tags) for each image.

If there are multiple channels for each movie, this step may need to be performed for each channel.

In this example, you could do the following:

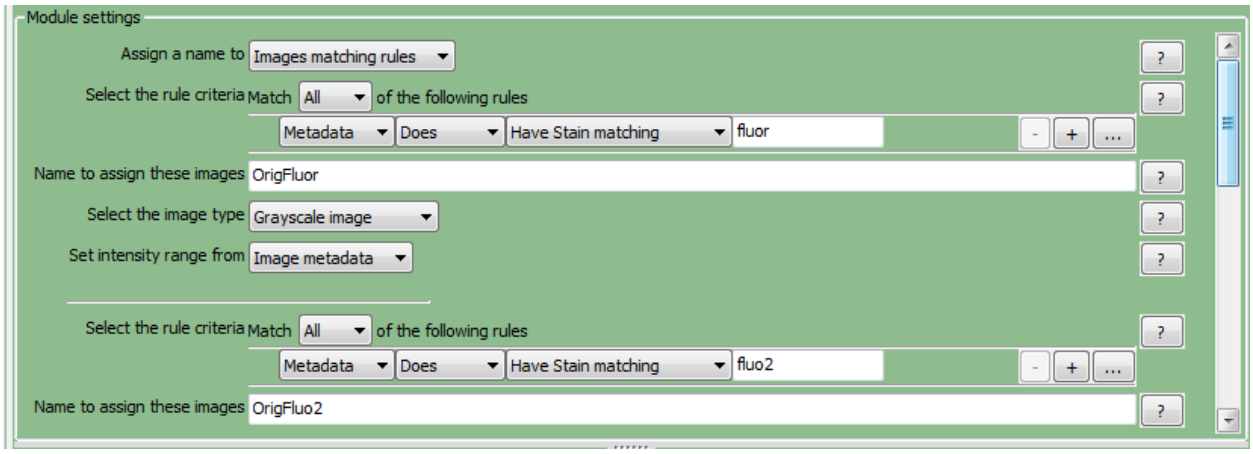
- Select "Extract from file/folder names" as the method, "From file name" as the source, and specify
  `.*-(?P<Well>[A-P][0-9]{2})\.(?P<Timepoint>[0-9]{3})`
  as the regular expression. This step will extract the well ID and timepoint from each filename.
- Click the "Add" button to add another extraction method.
- In the new group of extraction settings, select "Extract from file/folder names" as the method, "From folder name" as the source, and
  `.*[\\/](?P<Stain>.*)[\\/].*$`
  as the regular expression. This step will extract the stain name from each folder name.

- Click the "Update" button below the divider and check the output in the table to confirm that the proper metadata values are being collected from each image.



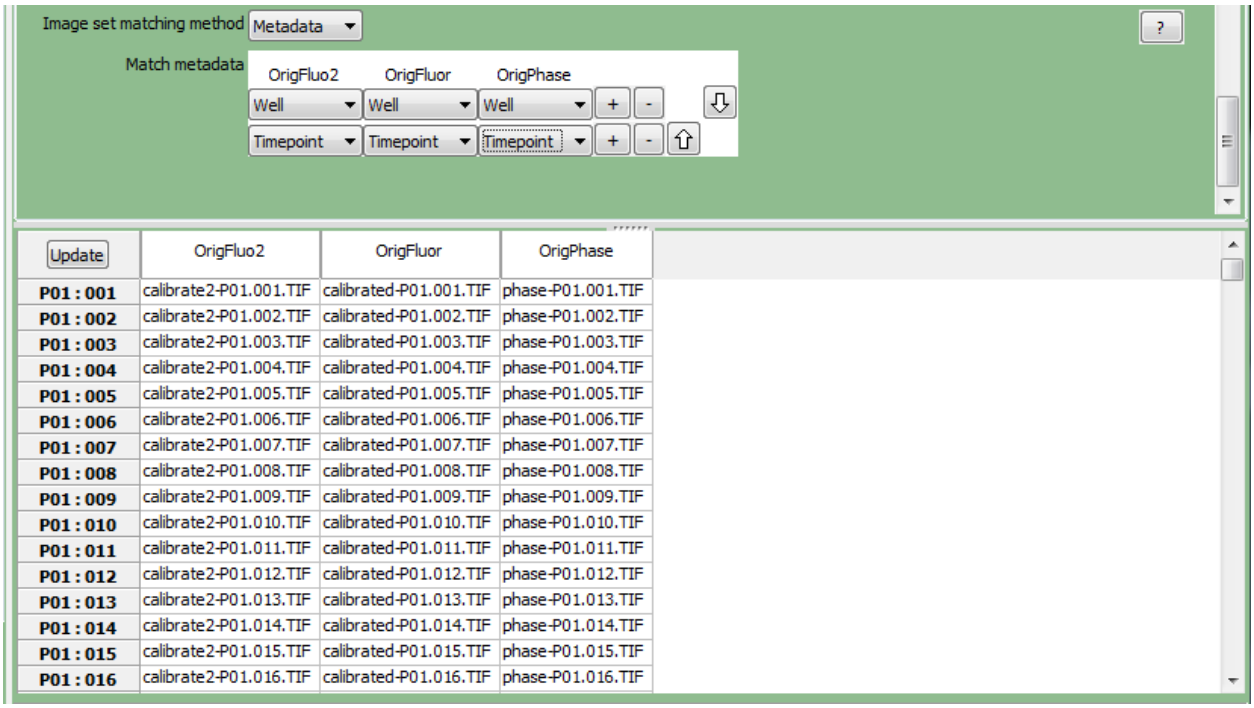
In the **NamesAndTypes** module, assign the channel(s) to a name of your choice. If there are multiple channels, this would need to be done for each channel. For this example, you could do the following:

- Select "Assign images matching rules".
- Make a new rule `[Metadata][Does][Have Stain matching][fluor]` and name it *OrigFluor*.
- Click the "Add another image" button to define another image with a rule.
- Make a new rule `[Metadata][Does][Have Stain matching][fluo2]` and name it *OrigFluo2*.
- Click the "Add another image" button define another image with a rule.



- Make a new rule `[Metadata][Does][Have Stain matching][phase]` and name it *OrigPhase*.
- In the "Assign channel by" setting, select "Metadata".
- Select "Well" for the OrigFluor, OrigFluo2, and OrigPhase channels.
- Click the + button to the right to add another row, and select "Timepoint" for each channel.

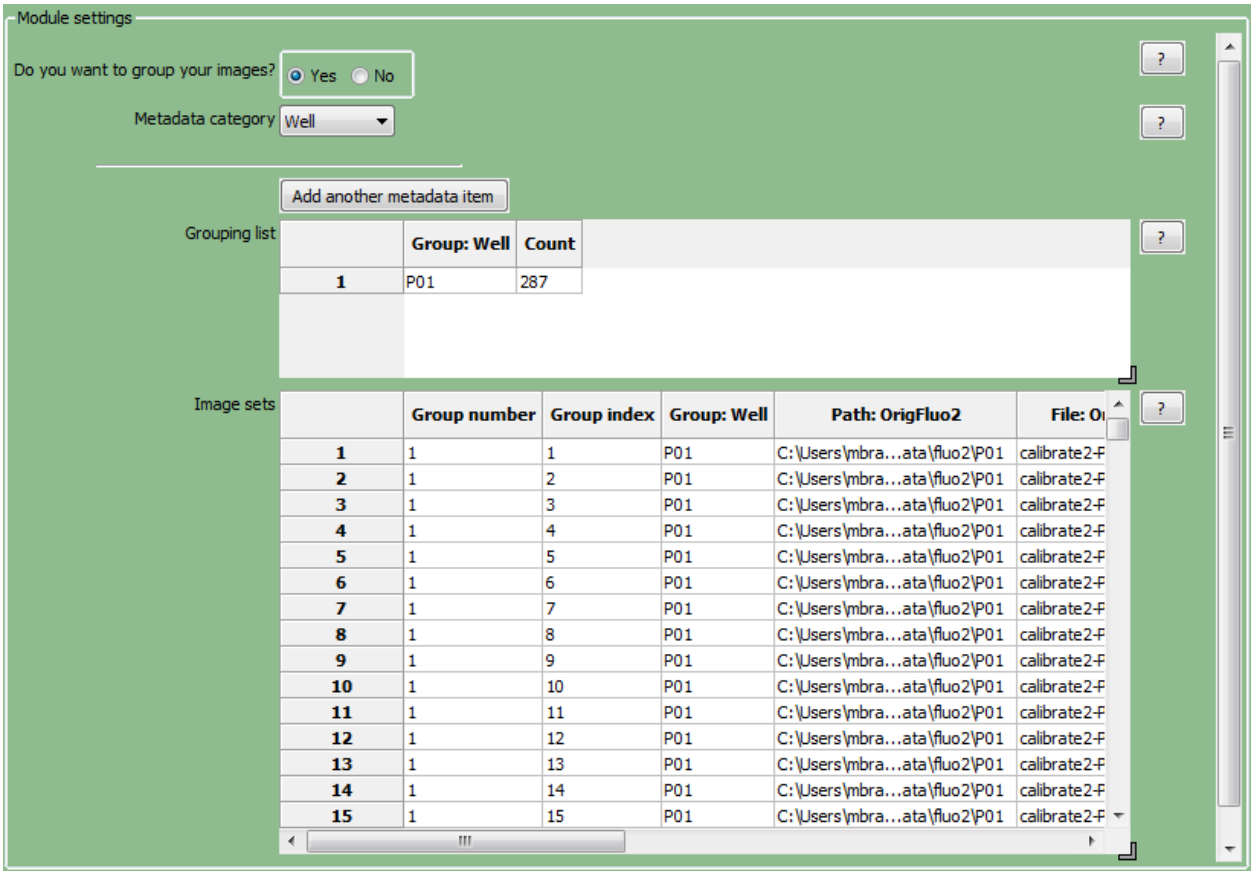
- Click the "Update" button below the divider to view the resulting table and confirm that the proper files are listed and matched across the channels. The corresponding well and frame for each channel should now be matched to each other.



	OrigFluo2	OrigFluor	OrigPhase
P01 : 001	calibrate2-P01.001.TIF	calibrated-P01.001.TIF	phase-P01.001.TIF
P01 : 002	calibrate2-P01.002.TIF	calibrated-P01.002.TIF	phase-P01.002.TIF
P01 : 003	calibrate2-P01.003.TIF	calibrated-P01.003.TIF	phase-P01.003.TIF
P01 : 004	calibrate2-P01.004.TIF	calibrated-P01.004.TIF	phase-P01.004.TIF
P01 : 005	calibrate2-P01.005.TIF	calibrated-P01.005.TIF	phase-P01.005.TIF
P01 : 006	calibrate2-P01.006.TIF	calibrated-P01.006.TIF	phase-P01.006.TIF
P01 : 007	calibrate2-P01.007.TIF	calibrated-P01.007.TIF	phase-P01.007.TIF
P01 : 008	calibrate2-P01.008.TIF	calibrated-P01.008.TIF	phase-P01.008.TIF
P01 : 009	calibrate2-P01.009.TIF	calibrated-P01.009.TIF	phase-P01.009.TIF
P01 : 010	calibrate2-P01.010.TIF	calibrated-P01.010.TIF	phase-P01.010.TIF
P01 : 011	calibrate2-P01.011.TIF	calibrated-P01.011.TIF	phase-P01.011.TIF
P01 : 012	calibrate2-P01.012.TIF	calibrated-P01.012.TIF	phase-P01.012.TIF
P01 : 013	calibrate2-P01.013.TIF	calibrated-P01.013.TIF	phase-P01.013.TIF
P01 : 014	calibrate2-P01.014.TIF	calibrated-P01.014.TIF	phase-P01.014.TIF
P01 : 015	calibrate2-P01.015.TIF	calibrated-P01.015.TIF	phase-P01.015.TIF
P01 : 016	calibrate2-P01.016.TIF	calibrated-P01.016.TIF	phase-P01.016.TIF

In the **Groups** module, enable image grouping for these images in order to select the metadata that defines a distinct movie of data. For this example, do the following:

- Select "Well" as the metadata category.
- The tables below this setting will update themselves, and you should be able to visually confirm that each well is defined as a group, each with 287 frames' worth of images.



Without this step, CellProfiler would not know where one movie ends and the next one begins, and would process the images in all movies together as if they were constituents of only one movie.

Example #2: Basic image sequences consisting of a single file

Another common means of storing time-lapse/Z-stack data is as a single file containing the entire movie. Examples of this approach include image formats such as:

- Multi-frame TIF
- Metamorph stack: STK
- Evotec/PerkinElmer Opera Flex
- Zeiss ZVI, LSM
- Standard movie formats: AVI, Quicktime MOV, etc

CellProfiler uses the Bio-Formats library for reading various image formats. For more details on supported files, see http://www.openmicroscopy.org/site/support/bio-formats4/supported-formats.html. In general, we recommend saving stacks and movies in a format such as .TIF.

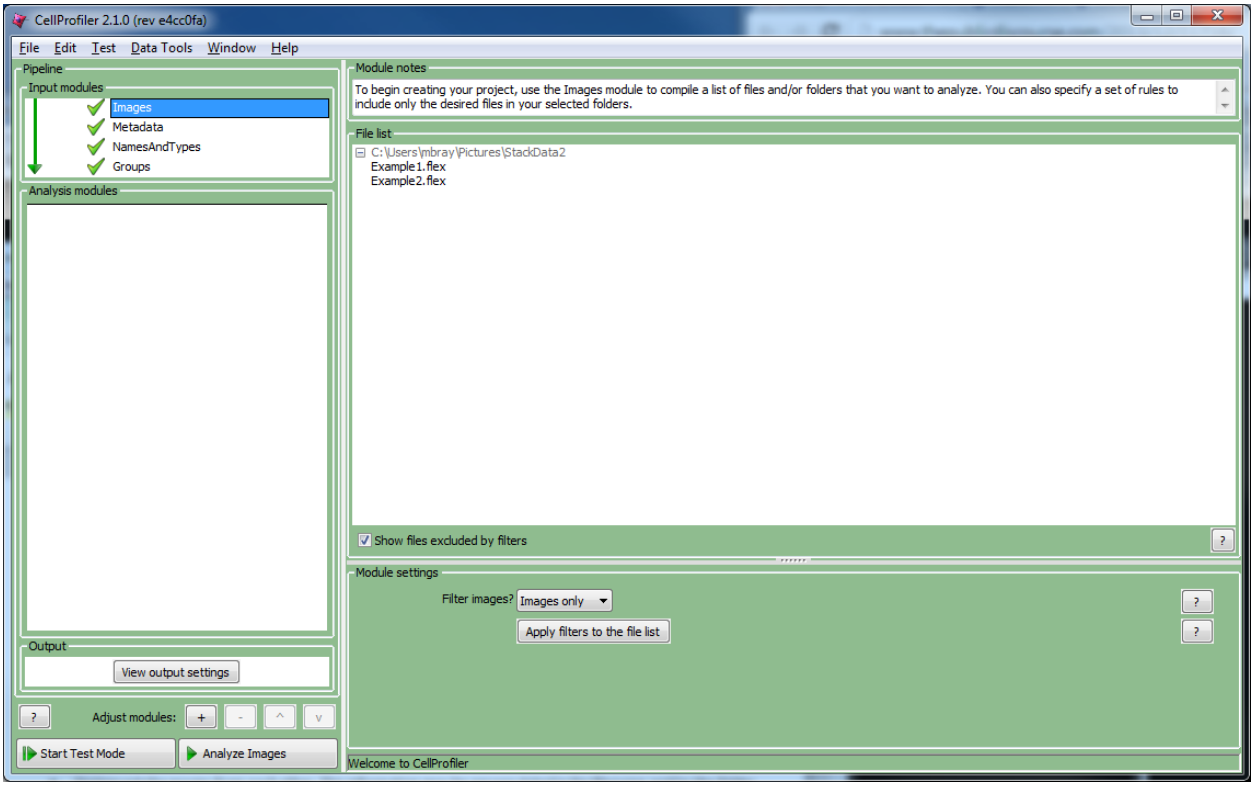
For our first example, let's say you have two image stacks in the following format:

- The stacks are Opera's FLEX format.
- Each FLEX file contains 8 fields of view, with 3 channels at each site (DAPI, GFP, Texas Red).
- Each channel is in grayscale format.

In this case, the procedure to set up the input modules to handle these files is as follows (please note that this procedure is basically identical whether the file is for a time-lapse assay or a Z-stack assay):

In the **Images** module, drag-and-drop your folders of images into the File list panel. If necessary, set your rules accordingly in order to filter out any files that are not images to be processed.

In the above example, you would drag-and-drop the FLEX files into the file list panel.



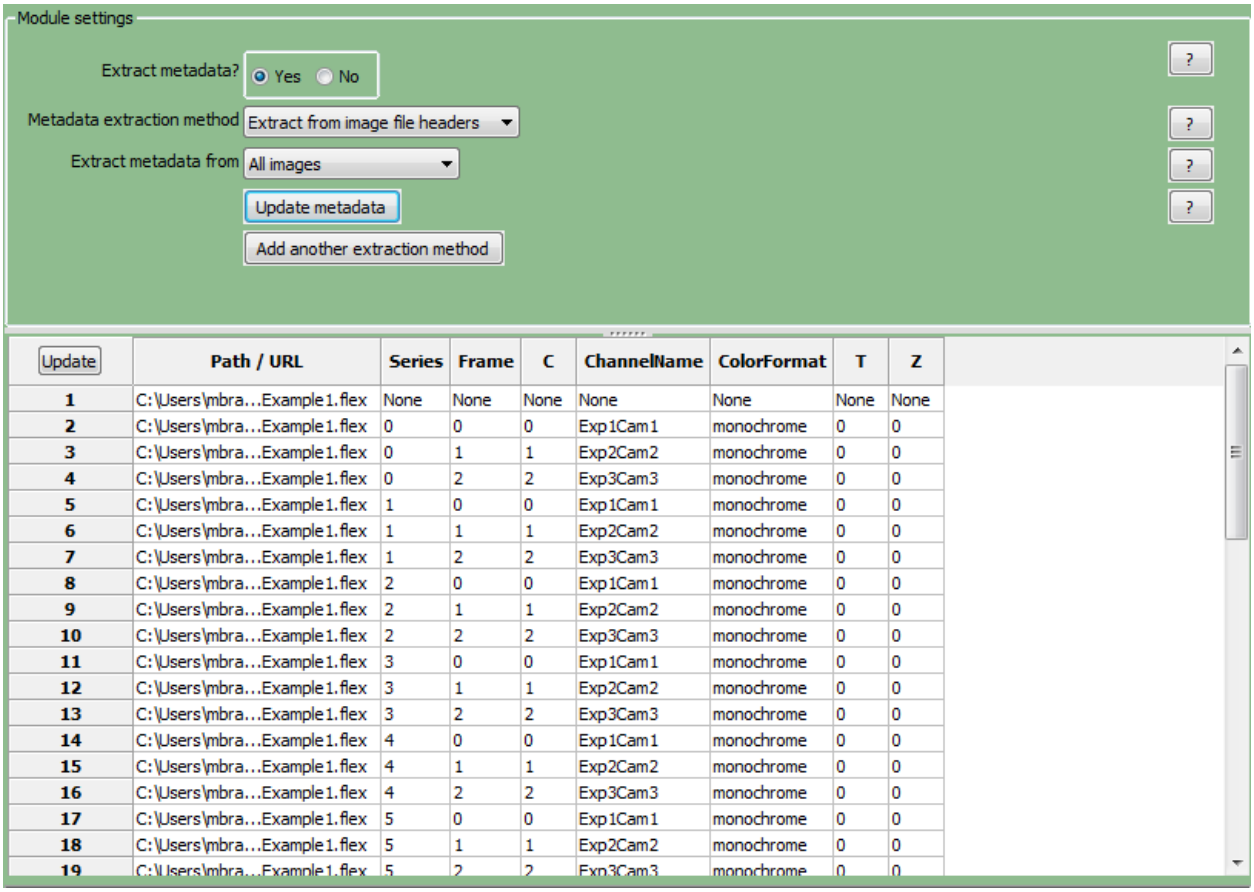
In the **Metadata** module, enable metadata extraction in order to obtain metadata from these files. The key step here is to obtain the metadata tags necessary to do two things:

- Distinguish the stacks from each other. This information is contained as the file itself, that is, each file represents a different stack.
- For each stack, distinguish the slices from each other, ensuring proper ordering. This information is usually contained in the image file's internal metadata, in contrast to Example #1 described above.

To accomplish this, do the following:

- Select "Extract from image file headers" as the metadata extraction method. In this case, CellProfiler will extract the requisite information from the metadata stored in the image headers.
- Click the "Update metadata" button. A progress bar will appear showing the time elapsed; depending on the number of files present, this step may take a while to complete.
- Click the "Update" button below the divider.

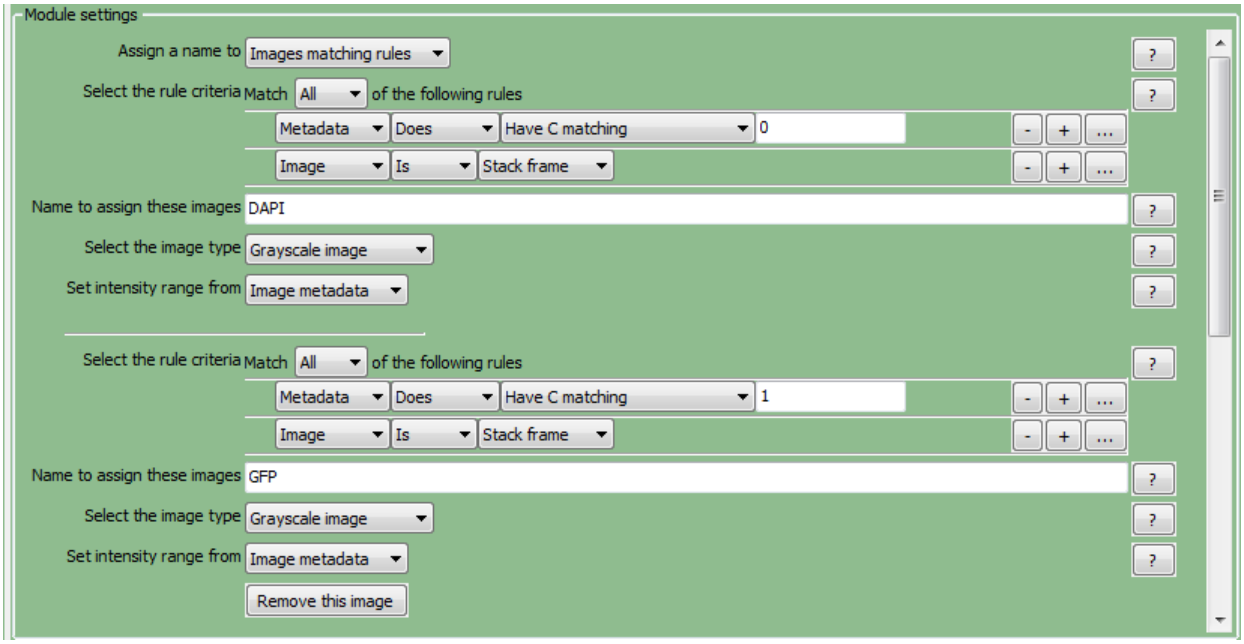
The resulting table should show the various metadata contained in the file. In this case, the relevant information is contained in the *C* and *Series* columns. In the figure shown, the *C* column shows three unique values for the channels represented, numbered from 0 to 2. The *Series* column shows 8 values for the slices collected in each stack, numbered from 0 to 7.



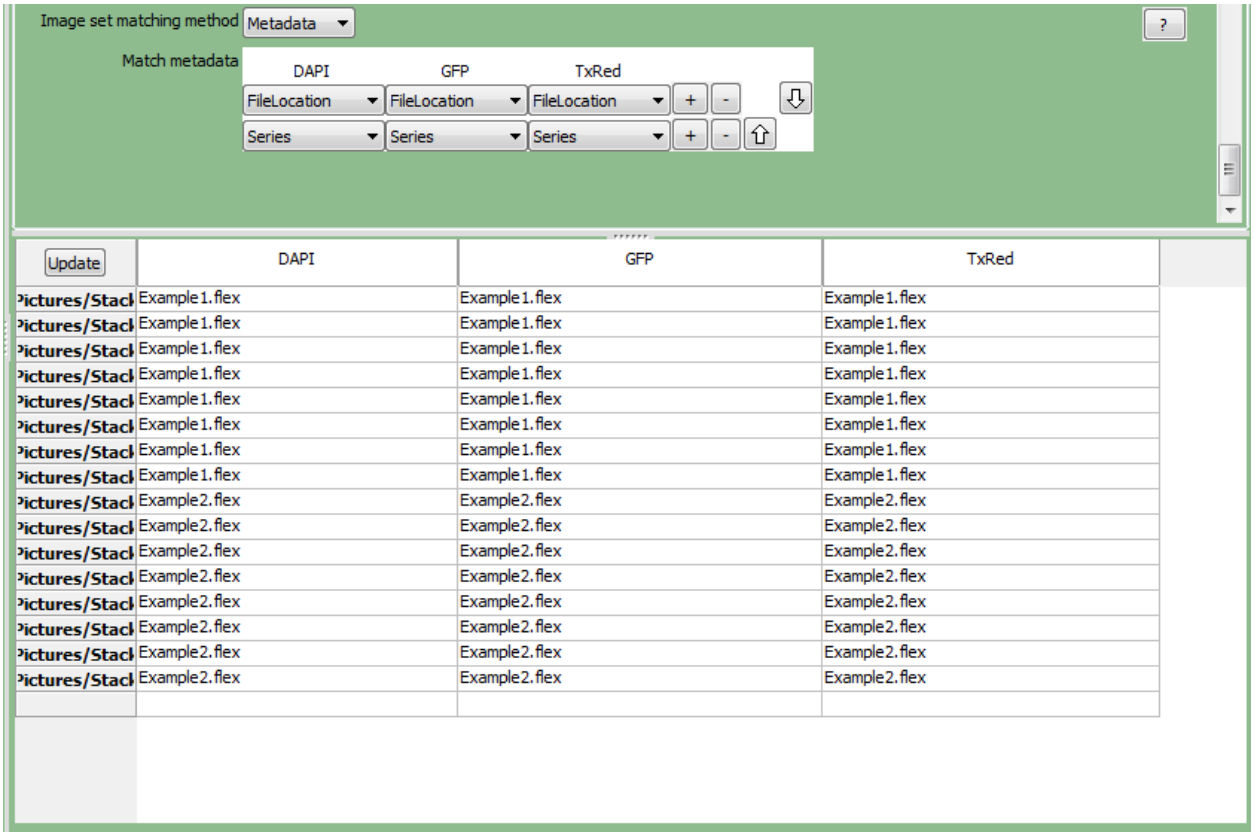
	Path / URL	Series	Frame	C	ChannelName	ColorFormat	T	Z
1	C:\Users\mbra...Example1.flex	None	None	None	None	None	None	None
2	C:\Users\mbra...Example1.flex	0	0	0	Exp1Cam1	monochrome	0	0
3	C:\Users\mbra...Example1.flex	0	1	1	Exp2Cam2	monochrome	0	0
4	C:\Users\mbra...Example1.flex	0	2	2	Exp3Cam3	monochrome	0	0
5	C:\Users\mbra...Example1.flex	1	0	0	Exp1Cam1	monochrome	0	0
6	C:\Users\mbra...Example1.flex	1	1	1	Exp2Cam2	monochrome	0	0
7	C:\Users\mbra...Example1.flex	1	2	2	Exp3Cam3	monochrome	0	0
8	C:\Users\mbra...Example1.flex	2	0	0	Exp1Cam1	monochrome	0	0
9	C:\Users\mbra...Example1.flex	2	1	1	Exp2Cam2	monochrome	0	0
10	C:\Users\mbra...Example1.flex	2	2	2	Exp3Cam3	monochrome	0	0
11	C:\Users\mbra...Example1.flex	3	0	0	Exp1Cam1	monochrome	0	0
12	C:\Users\mbra...Example1.flex	3	1	1	Exp2Cam2	monochrome	0	0
13	C:\Users\mbra...Example1.flex	3	2	2	Exp3Cam3	monochrome	0	0
14	C:\Users\mbra...Example1.flex	4	0	0	Exp1Cam1	monochrome	0	0
15	C:\Users\mbra...Example1.flex	4	1	1	Exp2Cam2	monochrome	0	0
16	C:\Users\mbra...Example1.flex	4	2	2	Exp3Cam3	monochrome	0	0
17	C:\Users\mbra...Example1.flex	5	0	0	Exp1Cam1	monochrome	0	0
18	C:\Users\mbra...Example1.flex	5	1	1	Exp2Cam2	monochrome	0	0
19	C:\Users\mbra...Example1.flex	5	2	2	Exp3Cam3	monochrome	0	0

In the **NamesAndTypes** module, assign the channel(s) to a name of your choice. If there are multiple channels, you will need to do this for each channel. For this above example, you could do the following:

- Select "Assign images matching rules".
- Make a new rule `[Metadata][Does][Have C matching][0]`.
- Click the + button to the right of the rule to add another set of rules underneath.
- Add the rule `[Image][Is][Stack frame]`. This combination tells CellProfiler not to treat the image as a single file, but rather as a series of frames.
- Name the image *DAPI*.
- Click the "Add another image" button to define a second image with a set of rules.
- Make a new rule `[Metadata][Does][Have C matching][1]`
- Click the + button to the right of the rule to add another set of rules underneath.
- Add the rule `[Image][Is][Stack frame].`
- Name the second image *GFP*.

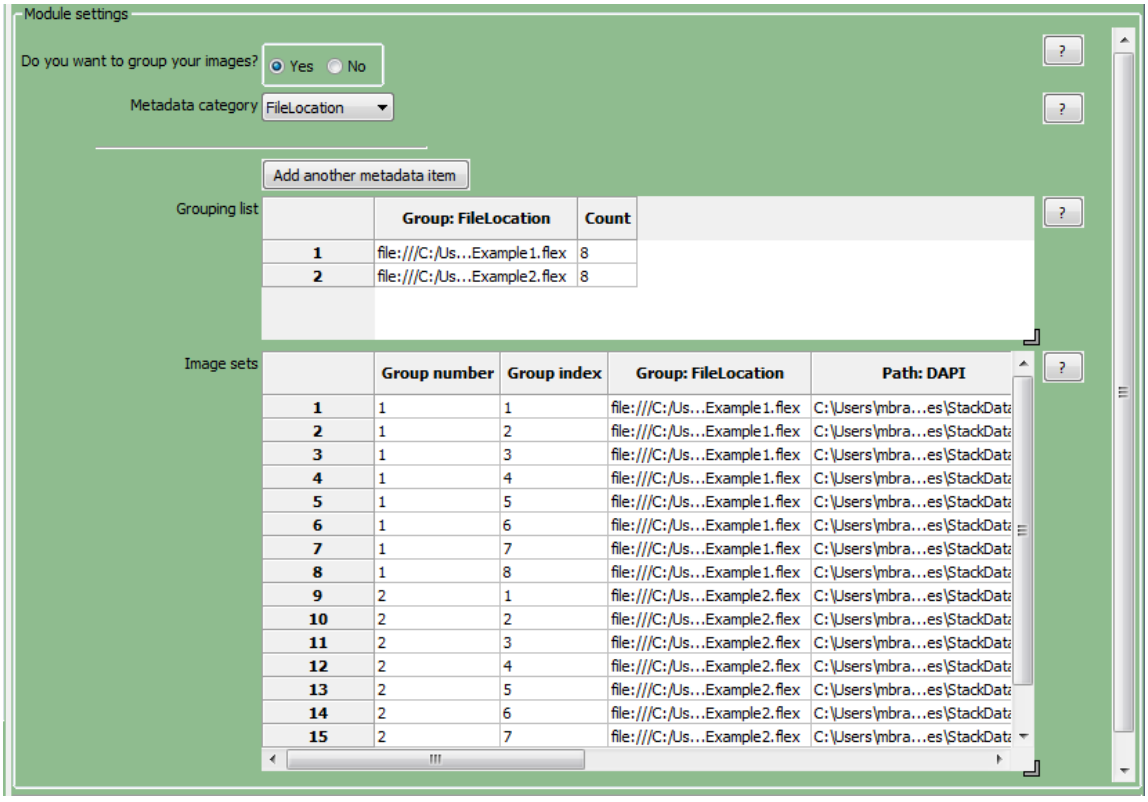


- Click the "Add another image" button to define a third image with a set of rules.
- Make a new rule `[Metadata][Does][Have C matching][2]`
- Click the + button to the right of the rule to add another set of rule options.
- Add the rule `[Image][Is][Stack frame].`
- Name the third image *TxRed*.
- In the "Image set matching method" setting, select "Metadata".
- Select "FileLocation" for the *DAPI*, *GFP,* and *TxRed* channels. The *FileLocation* metadata tag identifies the individual stack, and selecting this parameter ensures that the channels are first matched within each stack, rather than across stacks.
- Click the + button to the right to add another row, and select "Series" for each channel.
- Click the "Update" button below the divider to confirm that the proper image slices are listed and matched across the channels. The corresponding *FileLocation* and *Series* for each channel should now be matched to each other.



In the **Groups** module, select the metadata that defines a distinct image stack. For the example above, do the following:

- Select "FileLocation" as the metadata category.
- The tables below this setting will update themselves, and you should be able to visually confirm that each of the two image stacks are defined as a group, with 8 slices' worth of images.



Example #3: More advanced image sequences consisting of a single file

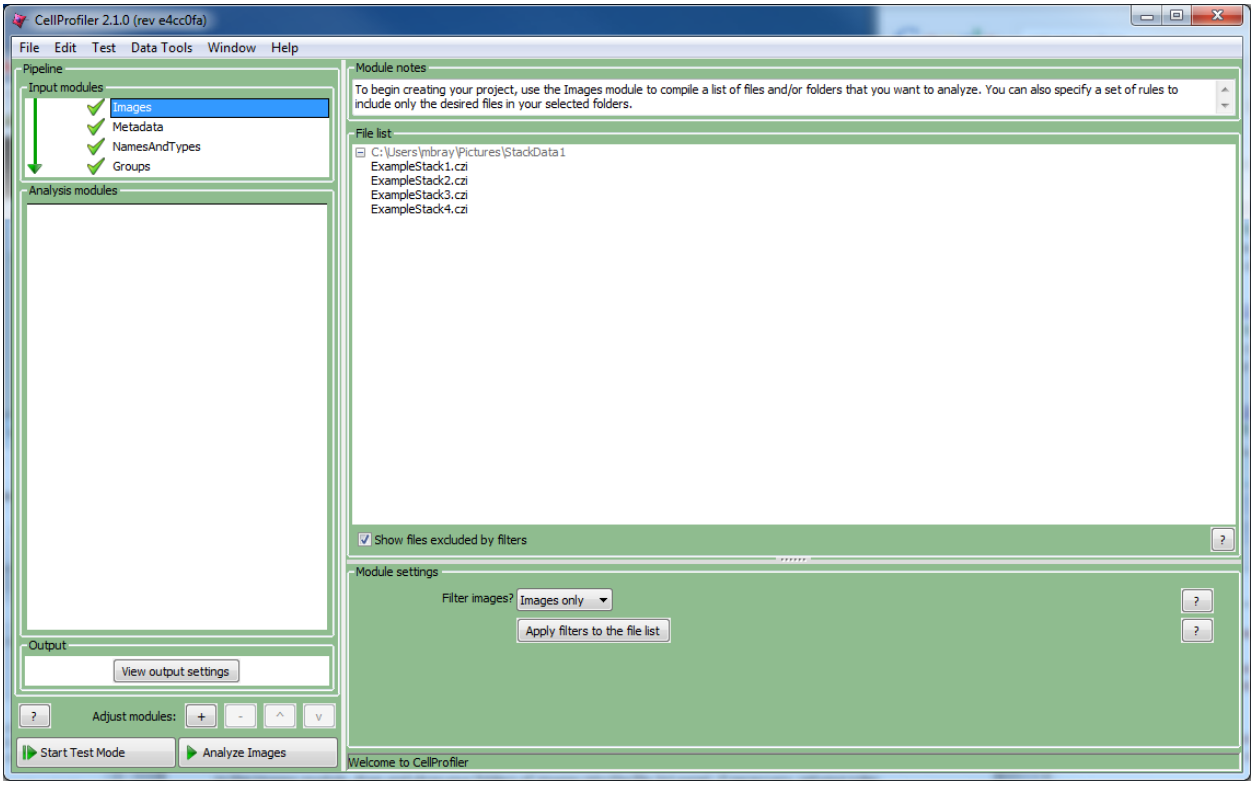
As another example, you have a set of Z-stacks in the following format:

- The stacks are in Zeiss' CZI format.
- Each stack consists of a number of slices with 4 channels (DAPI, GFP, Texas Red and Cy3) at each slice.
- One stack has 9 slices, two stacks have 7 slices and the fourth has 12 slices. Even though the stacks were collected with differing numbers of slices, the pipeline to be constructed is intended to analyze all stacks in the same manner.
- Each slice is in grayscale format.

In this case, the procedure to set up the input modules to handle these files is as follows (please note that this procedure is basically identical whether the file is for a time-lapse assay or a Z-stack assay):

In the **Images** module, drag-and-drop your folders of images into the File list panel. If necessary, set your rules accordingly in order to filter out any files that are not part of a Z-stack sequence.

In the above example, you would drag-and-drop the CZI files into the file list panel. In this case, the default “Images only” filter is sufficient to capture the necessary files.

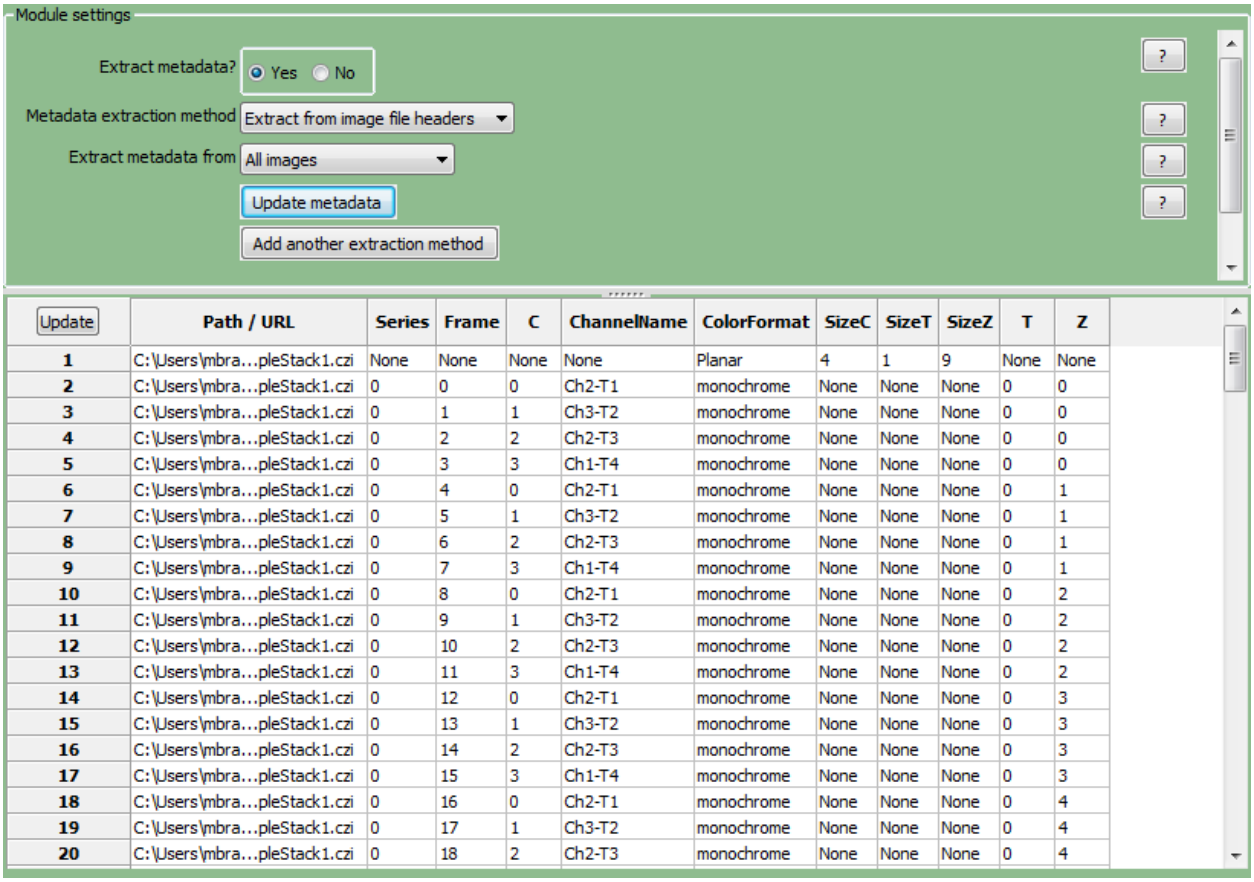


In the **Metadata** module, enable metadata extraction in order to obtain metadata from these files. The key step here is to obtain the metadata tags necessary to do two things:

- Distinguish the stacks from each other. This information is contained as the file itself, that is, each file represents a different stack.
- For each stack, distinguish the slices from each other. This information is usually contained in the image's internal metadata.

To accomplish this, do the following:

- Select "Extract from image file headers" as the metadata extraction method. In this case, CellProfiler will extract the requisite information from the metadata stored in the image headers.
- Click the "Update metadata" button. A progress bar will appear showing the time elapsed; depending on the number of files present, this step may take a while.
- Click the "Update" button below the divider.



Update	Path / URL	Series	Frame	C	ChannelName	ColorFormat	SizeC	SizeT	SizeZ	T	Z
1	C:\Users\mbra...pleStack1.czi	None	None	None	None	Planar	4	1	9	None	None
2	C:\Users\mbra...pleStack1.czi	0	0	0	Ch2-T1	monochrome	None	None	None	0	0
3	C:\Users\mbra...pleStack1.czi	0	1	1	Ch3-T2	monochrome	None	None	None	0	0
4	C:\Users\mbra...pleStack1.czi	0	2	2	Ch2-T3	monochrome	None	None	None	0	0
5	C:\Users\mbra...pleStack1.czi	0	3	3	Ch1-T4	monochrome	None	None	None	0	0
6	C:\Users\mbra...pleStack1.czi	0	4	0	Ch2-T1	monochrome	None	None	None	0	1
7	C:\Users\mbra...pleStack1.czi	0	5	1	Ch3-T2	monochrome	None	None	None	0	1
8	C:\Users\mbra...pleStack1.czi	0	6	2	Ch2-T3	monochrome	None	None	None	0	1
9	C:\Users\mbra...pleStack1.czi	0	7	3	Ch1-T4	monochrome	None	None	None	0	1
10	C:\Users\mbra...pleStack1.czi	0	8	0	Ch2-T1	monochrome	None	None	None	0	2
11	C:\Users\mbra...pleStack1.czi	0	9	1	Ch3-T2	monochrome	None	None	None	0	2
12	C:\Users\mbra...pleStack1.czi	0	10	2	Ch2-T3	monochrome	None	None	None	0	2
13	C:\Users\mbra...pleStack1.czi	0	11	3	Ch1-T4	monochrome	None	None	None	0	2
14	C:\Users\mbra...pleStack1.czi	0	12	0	Ch2-T1	monochrome	None	None	None	0	3
15	C:\Users\mbra...pleStack1.czi	0	13	1	Ch3-T2	monochrome	None	None	None	0	3
16	C:\Users\mbra...pleStack1.czi	0	14	2	Ch2-T3	monochrome	None	None	None	0	3
17	C:\Users\mbra...pleStack1.czi	0	15	3	Ch1-T4	monochrome	None	None	None	0	3
18	C:\Users\mbra...pleStack1.czi	0	16	0	Ch2-T1	monochrome	None	None	None	0	4
19	C:\Users\mbra...pleStack1.czi	0	17	1	Ch3-T2	monochrome	None	None	None	0	4
20	C:\Users\mbra...pleStack1.czi	0	18	2	Ch2-T3	monochrome	None	None	None	0	4

The resulting table should show the various metadata contained in the file. In this case, the relevant information is contained in the *C* and *Z* columns. In the figure shown, the *C* column shows four unique values for the channels represented, numbered from 0 to 3. The *Z* column shows nine values for the slices represented from the first stack, numbered from 0 to 8, followed by the slices for other stacks.

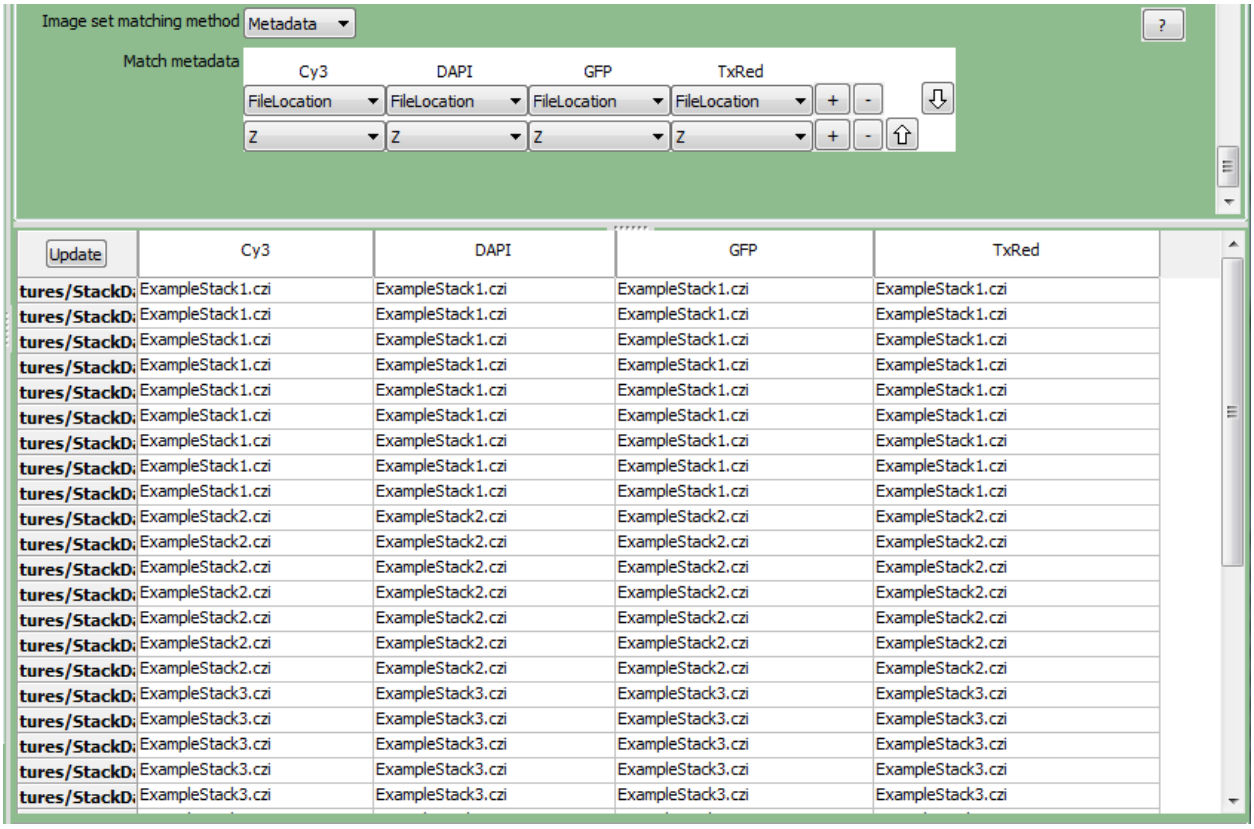
Of note in this case, for each file there is a single row summarizing this information. For the first stack shown, the *sizeC* column reports a value of 4 and *sizeZ* column shows a value of 9. You may need to scroll down the table to see this summary for the other stacks.

In the **NamesAndTypes** module, assign the channel(s) to a name of your choice. If there are multiple channels, you will need to do this for each channel. For this example, you could do the following:

- Select "Assign images matching rules".
- Make a new rule `[Metadata][Does][Have C matching][0]`.
- Click the + button to the right of the rule to add another set of rule options.
- Add the rule `[Image][Is][Stack frame].`
- Name the image *DAPI*.
- Click the "Add another image" button to define a second image with a set of rules.
- Make a new rule `[Metadata][Does][Have C matching][1]`

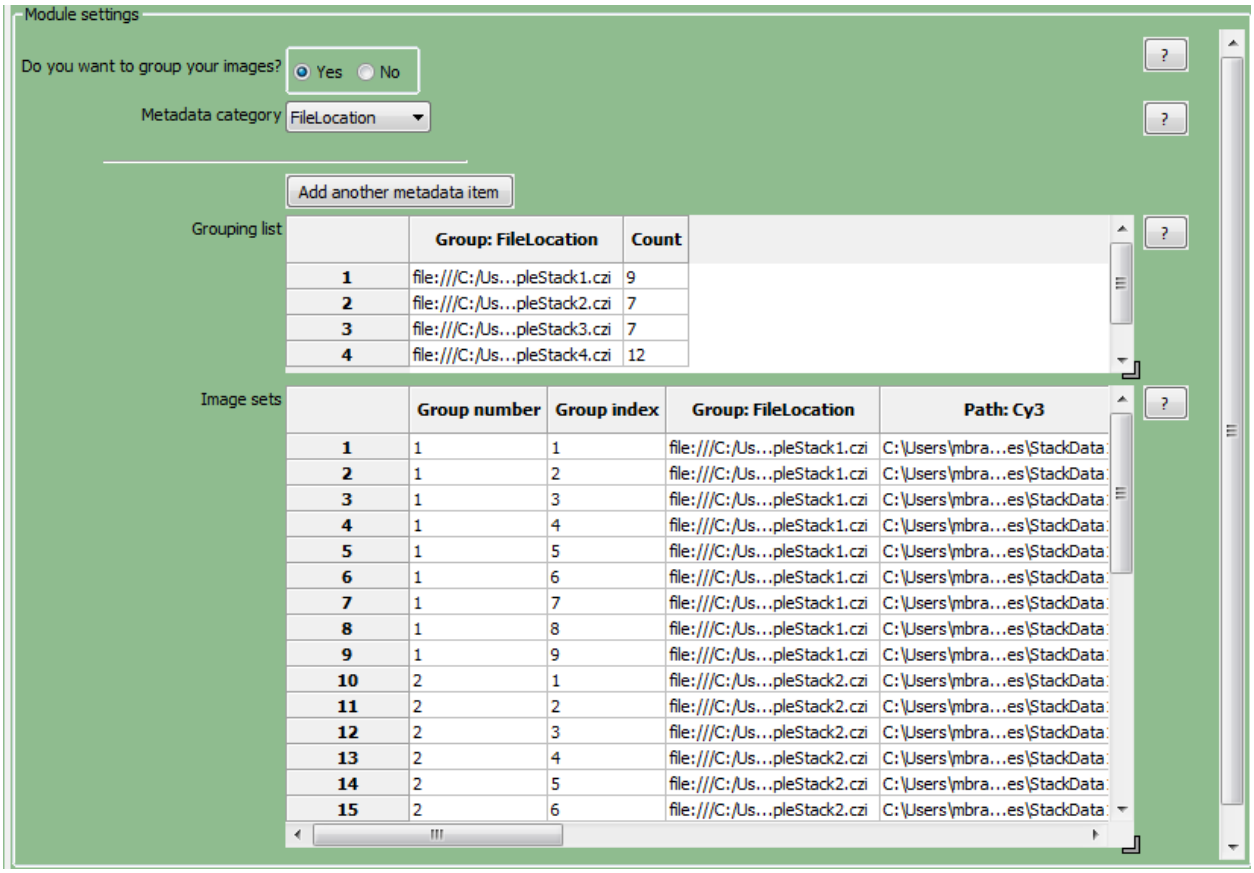
- Click the + button to the right of the rule to add another set of rule options.
- Add the rule `[Image][Is][Stack frame].`
- Name the second image *GFP*.

- Click the "Add another image" button to define a third image with a set of rules.
- Make a new rule `[Metadata][Does][Have C matching][2]`
- Click the + button to the right of the rule to add another set of rule options.
- Add the rule `[Image][Is][Stack frame].`
- Name the third image *TxRed*.
- Click the "Add another image" button to define a fourth image with set of rules.
- Make a new rule `[Metadata][Does][Have C matching][3]`.
- Click the + button to the right of the rule to add another set of rule options.
- Add the rule `[Image][Is][Stack frame].`
- Name the fourth image *Cy3*.
- In the "Image set matching method" setting, select "Metadata".
- Select "FileLocation" for the *DAPI*, *GFP*, *TxRed* and *Cy3* channels. The *FileLocation* metadata tag identifies the individual stack, and selecting this parameter insures that the channels are first matched within each stack, rather than across stacks.
- Click the + button to the right to add another row, and select "Z" for each channel.
- Click the "Update" button below the divider to confirm the channel matching. The corresponding *FileLocation* and *Z* for each channel should now be matched to each other.



In the **Groups** module, select the metadata that defines a distinct image stack. For the example above, do the following:

- Select "FileLocation" as the metadata category.
- The tables below this setting will update themselves, and you should be able to visually confirm that each of the four image stacks are defined as a group, with 9, 7, 7 and 12 slices' worth of images.



Without this step, CellProfiler would not know where one stack ends and the next one begins, and would process the slices in all stacks together as if they were constituents of only one stack.