This Document is biased towards confocal microscopes with Photo Multiplier Tube (PMT) detectors but similar tips apply to digital cameras.

1) Make sure you have no saturated pixels or zero value pixels. Use the range indicator Look Up Table (LUT) to be sure. Look at what you think will be the brightest sample first and optimize the settings with that image. You will be reusing these exact settings for all your images. You never want a saturated pixel because that has no information and will mess up your analyses and comparisons.

2) Collect images at a bit depth of at least 12 or 16. This is very important. Using 8-bit images means you lose a lot of sensitivity when your labeling intensity varies a great deal.

3) I would make sure that my frame size is at least 1024 x 1024 but you probably don't need more than 2048 x 2048. Make sure your structure pretty much fills the field of view. You could use the confocal scanning mirrors to zoom in but see Problem 1 below. It's best to use a higher power objective to make your structure fit the field of view but you want some area around it that is unlabeled for control measurements.

4) Use the region of interest (ROI) tool to select an area that just includes your label and another ROI in an unlabeled area for your background. Make sure that the ROI is always the same size and small enough to just have your labeled cells or intracellular structure. Now you could do a ratio of labeled to unlabeled regions for each experimental condition, for your comparisons and statistics but if your unlabeled areas are always the same you should be safe to just compare your labeled ROIs.

5) Measure lots of different ROIs across many different embryos, tissues or cells for all your conditions. Calculate the variation for each experimental condition and if the distribution is normal than you can do a simple T-test to compare the conditions if you have only 2 you want to compare. If the distribution is not normal than you'll have to use a non-parametric version of the T-test which all the statistical software have (I'm being particularly picky here, most people don't bother with determining normal distributions).

6) Measure the power of the laser you are using to collect your images before you begin and check it every week or so to make sure it hasn't changed as you collect your data (see Problem 2 below). You can do this by using the transmitted light detector and a line scan. Take out all the filters from your transmitted light path and place the condenser in the BF position. Save the file and reuse those settings when you do your checks.

Problem 1: How much does your fluorescent signal fade after repeated scans? If it fades in intensity after repeated measurements, you'll have to make sure to just use the first image collected for all comparisons. Zooming in with the confocal can cause more bleaching. You might need to turn the laser power down but then your signal to noise could get worse. The degree that your fluorescent signal bleaches will also impact how much you can slow down your scan or average your image to minimize background noise. You'll have to test this by saving images after repeated scans and seeing if the ROI values go down.

Problem 2: Lasers can get less intense over time though solid-state lasers are less subject to this. This is usually over a time scale of months but depending on how long you are doing your measurements this can be a problem. If you follow step (6) above you should be fine. This is more of a problem if you are using the Argon laser (488 line for example). If you want to be super picky you can measure the laser intensity every day to see if it fluctuates. There is a power meter available in the Biological Imaging Facility for precise measurement of laser power at the objective image plane.