Benchmarks

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Automated Method for Quantifying Fluorophore Colocalization in Fluorescence Double-Labeling Experiments

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Fluorescence double-labeling is an important method for investigating associations of molecules (1). For example, evidence of co-association of proteins often includes demonstrating that they are colocalized in the same compartment of the cell. Furthermore, colocalization studies are not limited to proteins but also include measuring the colocalization of separate lipid species (8), lipids with proteins (9), and nucleic acids (4,5). Thus, double-labeling experiments are performed to address a broad range of questions.

Quantification of fluorophore colocalization is an important feature of double-labeling experiments, and methods for accomplishing this have been described (12). One important method for quantifying fluorophore colocalization is that of the cross-correlation analysis (13). Several recent studies have provided important examples of using this type of analysis to quantitate pro-

tein associations in cells (6,7,10,11,13).

Cross-correlation analysis is a multi-step process that has been difficult to accomplish for large numbers of cells. To address this issue, a method for quantifying fluorophore colocalization in a facile manner using the cross-correlation analysis was written using scriptable imaging software. With this tool, quantitation of fluorophore colocalization is largely automated, thus allowing for fast and convenient quantitation of large numbers of cells.

In the cross-correlation analysis, fluorophore colocalization is measured by determining the correlation coefficient (ρ) for the separate fluorophores using the equation:

$$\rho = \frac{\frac{1}{N} \sum_i (x_i - \langle x \rangle)(y_i - \langle y \rangle)}{\sqrt{\frac{1}{N} \sum_i (x_i - \langle x \rangle)^2} \sqrt{\frac{1}{N} \sum_i (y_i - \langle y \rangle)^2}} \quad [\text{Eq. 1}]$$

where x_i and y_i represent the fluorescence intensity of each pixel in the respective images and $\langle x \rangle$ and $\langle y \rangle$ are the corresponding average pixel intensity values. N represents the total pixels

in each image. The ρ can assume a value from zero (no correlation) to -1.0 or +1.0 (perfect negative or positive correlation) (2).

Figure 1 shows two separate scripts for calculating ρ . Each script can be downloaded from our Web site (http://www.omrf.org/OMRF/Research/05/W._Rodgers_homepage.html). The script in Figure 1A was written for IPLab[®] Spectrum software (Scanalytics, Fairfax, VA, USA). However, the operations are such that they can be written into a macro using any scriptable imaging software, including NIH Image (Figure 1B). In both scripts, the calculation of ρ proceeds by the generation of a set of images corresponding to the operations:

$$(x_i - \langle x \rangle) \text{ and } (y_i - \langle y \rangle) \quad [\text{Eq. 2}]$$

These difference images are multiplied or squared to generate images corresponding to:

$$(x_i - \langle x \rangle)(y_i - \langle y \rangle), (x_i - \langle x \rangle)^2, (y_i - \langle y \rangle)^2 \quad [\text{Eq. 3}]$$

Figure 1. Script for determining the correlation coefficient in double-labeled cells. The scripts were written for IPLab Spectrum(A) or NIH Image (B) to determine ρ . Both scripts require the user to save each image of a double-labeled cell as a separate image termed "a" and "b". In part A, the first 11 lines of the script represent the measurement of the background in each image and subtracting this value from all pixels in the image. Next, the windows are made into a stack (line 12) named "Mean Measurement". This ensures that precisely the same region is measured in each image during the operation. Lines 13–16 represent the measurement of the average fluorescence intensity of the region of interest (ROI) in the top stack image corresponding to image "a", followed by the same measurement in the stack image corresponding to image "b" (lines 18 and 19). The measured values appear in a table, and these must be entered at lines 25 and 31 of the script. In lines 20–25, a window is created containing only the top image of the Mean Measurement stack. The mean fluorescence intensity measured in line 17 is subtracted from each point (Point Function in line 25). Lines 27–31 represent the same operation of the bottom image of the Mean Measurement stack to generate the difference image "b-mean". In line 32, the separate difference images are multiplied, and in lines 33 and 34 they are squared. Next, a new stack (termed "Composite") is created containing, from top to bottom, each image from the Mean Measurement stack, the product of the difference images, and the square of each difference image. In lines 43–46, the segment outlining the ROI is copied from Mean Measurement stack and pasted onto the top image of the Composite stack. Lines 48–55 represent the measurement of the sum of the fluorescence intensity in the ROI in the images: (a-mean)*(b-mean), (a-mean)², and (b-mean)². The sum and the area of the ROI of the latter images are placed in a table. The remaining operations to satisfy Equation 1, including dividing each sum by the area, square root, multiplication, and division, can be performed using a spreadsheet. The resulting value represents ρ . The script for NIH Image is composed of three separate macros. The first macro titled "Background Correction" subtracts the background from each image and generates a stack with the top and bottom windows being background-corrected "a" and "b", respectively. Note that for this script the ROI for measuring the background must be drawn before beginning the operation. At the end of the macro "Background Correction", the user is prompted to outline the ROI for the region to be measured and begin macro "Correlation Coefficient". The latter macro corresponds to lines 17–35 of part A, where the mean of the ROI is measured, subtracted from the corresponding image, and the multiplication operations are performed using the resulting difference images. Unlike part A, a final stack containing each of the images from the operation is not generated. This feature was originally written into the script, but it was removed after it was determined that this truncated the pixel intensity values because of conversion from a 16- to an 8-bit data format. Thus, with the NIH Image script, the ROI needs to be redrawn in each image generated in script "Correlation Coefficient", and the user is prompted to do so by the program. The sum of the ROI is measured in the final macro "Measure Sum" in the order: (a-mean)*(b-mean), (a-mean)², and (b-mean)².

Benchmarks

Figure 1

A

1. Alert: outline area of background in "a"
2. Measure ROI
3. Adjust Contrast
4. Apply Color Table
5. Change Data Type (Short Int.)
6. Alert: outline area of background in "b"
7. Measure ROI
8. Adjust Contrast
9. Apply Color Table
10. Change Data Type (Short Int)
11. Change Window (goto a)
12. Frames to Sequence (call stack "Mean Measurement")
13. Segment Line
14. Alert: Draw segment in "Mean Measurement"
15. ROI Wand
16. Alert: Select ROI
17. Measure ROI
18. Next Frame
19. Measure ROI
20. Next Frame
21. Select All
22. Copy
23. New Image (call window "a-mean")
24. Paste
25. Point Function [subtract mean of ROI (step 17) from image "a-mean"]
26. Change Window (go to Mean Measurement)
27. Select Frame
28. Copy
29. New Image (call window "b-mean")
30. Paste
31. Point Function [subtract mean of ROI (step 19) from image "b-mean"]
32. Image Arithmetic [perform (a-mean)*(b-mean)]
33. Image Arithmetic [perform (a-mean)^2]
34. Image Arithmetic [perform (b-mean)^2]
35. New Image (Create stack "Composite" containing five frames)
36. Transfer Frames (transfer frames 0 and 1 of "Mean Measurement" to frames 0 and 1 of "Composite")
37. Transfer Frames [transfer "(a-mean)*(b-mean)" to frame 2 of "Composite"]
38. Transfer Frames [transfer "(a-mean)^2" to frame 3 of "Composite"]
39. Transfer Frames [transfer "(b-mean)^2" to frame 4 of "Composite"]
40. Select Frame (frame 0)
41. Show Image
42. Segment Line
43. Change Window (goto Mean Measurement)
44. Segment Copy
45. Change Window (goto Composite)
46. Segment paste
47. ROI Wand
48. Alert: Select ROI
49. Next Frame
50. Next Frame
51. Measure ROI
52. Next Frame
53. Measure ROI
54. Next Frame
55. Measure ROI
56. Next Frame
57. Tile
58. Change Window
59. End

B

1. macro 'Background Correction'
2. var
3. bga:integer;
4. bgb:integer
5. negbg:integer;
6. pic:integer;
7. width,height:integer;
8. begin;
9. SetScale(1, 'pixel');
10. SelectWindow('a');
11. pic:=PicNumber;
12. Measure;
13. ShowResults;
14. bga:=GetNumber('neg of mean:', -5. 0);
15. SelectAll;
16. AddConstant(bga);
17. SelectWindow('b');
18. pic:=PicNumber;
19. Measure;
20. ShowResults;
21. bgb:=GetNumber('neg of mean:', -5. 0);
22. SelectAll;
23. AddConstant(bgb);
24. SelectWindow('a');
25. SelectAll;
26. Copy;
27. GetPicSize(width,height);
28. SetNewSize(width,height);
29. MakeNewStack('mean measurement');
30. Paste;
31. SelectWindow('b');
32. SelectAll;
33. Copy;
34. SelectWindow('mean measurement');
35. AddSlice;
36. Paste;
37. Selectslice(1);
38. SelectTool('freehand');
39. ShowMessage('Draw ROI and then go to Correlation Coefficient');
41. Beep;
42. End

17. SetNewSize(width,height);
18. mystack := picnumber;
19. for i:=1 to nslices do begin
20. SelectSlice(i);
21. SelectAll;
22. Copy;
23. MakeNewWindow(i);
24. Paste;
25. pid:=PidNumber;
26. value:=GetNumber('Neg Mean of ROI:', -50. 0);
27. AddConstant(value);
28. SelectPic(myStack);
29. end;
30. SelectWindow(0001);
31. sqa:=picnumber;
32. SelectWindow(0002);
33. sqb:=picnumber;
34. ImageMath('mul real', sqa, sqb, 1, 0, 'a-mean*b-mean');
35. ImageMath('mul real', sqa, sqa, 1, 0, 'a-mean^2');
36. ImageMath('mul real', sqb, sqb, 1, 0, 'b-mean^2');
37. SelectWindow('mean measurement');
38. Dispose;
39. SelectWindow(0001);
40. Dispose;
41. SelectWindow(0002);
42. Dispose;
43. SelectWindow('a');
44. Dispose;
45. SelectWindow('b');
46. Dispose;
47. ShowMessage('Draw ROI in each image and perform "Correlation Coefficient". rho = [Sum(a-mean*b-mean)/Area (in pixels)]/([Sum(a-mean^2)/Area)^0.5 * (Sum(b-mean^2)/Area)^0.5);
48. End

1. macro 'Correlation Coefficient '
2. begin
3. SetUserLabel('Mean*Area');
4. SelectWindow('a-mean*b-mean');
5. Measure;
6. rUser1[rCount]:=rMean[rCount]*rArea[rCount];
7. UpdateResults;
8. SelectWindow('a-mean^2');
9. Measure;
10. rUser1[rCount]:=rMean[rCount]*rArea[rCount];
11. UpdateResults;
12. SelectWindow('b-mean^2');
13. Measure;
14. rUser1[rCount]:=rMean[rCount]*rArea[rCount];
15. UpdateResults;
16. End

Benchmarks

The fluorescence intensity values in the latter images are summed and divided by the area (in pixels). Square root and division operations of the resulting integers are then performed as defined by Equation 1. This can be performed either manually or by importing the integers into a spreadsheet such as Microsoft® Excel®. With the scripts in Figure 1, determining ρ for each double-labeled cell takes less than 1 min. Thus, a population of 30–40 cells can be quantitated in less than 1 h.

Figure 2 shows an example where colocalization of two separate fluorophores was quantified using the script in Figure 1. Figure 2A shows a cell expressing a construct of the nuclear pro-

tein Ku70 that is fused at its C-terminus to GFP. The Ku70-GFP construct was generated using the GFP-cloning vector pWay20 (3). The cell was double-labeled using a monoclonal antibody that recognizes a heterodimer composed of Ku70 and Ku86. The cell in Figure 2A shows nearly perfect colocalization of Ku70-GFP and heterodimer staining, and this is reflected by a ρ of 0.92. However, a histogram of the ρ measured using approximately 30 separate cells that were double-labeled in this manner shows there is significant variance in the amount of fluorophore colocalization within the sample (Figure 2B). Importantly, the experiment illustrated in Figure 2 was performed with-

out pre-processing of the images. The variance observed in Figure 2B may be due to discrete pools of protein in some cells that do not form heterodimers. In any event, these results illustrate the utility of the scripts in Figure 1 in quantitating fluorophore colocalization in large sets of cell. For example, this approach minimizes the subjectivity in the analysis and provides a description of the range of fluorophore colocalizations that occurs within a population of double-labeled cells.

Colocalization studies using fluorescence imaging represent a powerful method for exploring putative associations between molecules and their targeting to discrete intracellular compartments. Described here is a convenient method for quantitating this colocalization using scriptable imaging software, thereby allowing facile measurement of populations of double-labeled cells.

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Figure 2. Quantitation of fluorophore colocalization immunostained cells. (A) Images of the nucleus of a HeLa cell expressing a GFP fusion protein of Ku70. The cells were transfected using cationic liposomes (SuperFect™; Qiagen, Valencia, CA, USA). Forty-eight hours after transfection, the sample was fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton® X-100 in PBS (pH 7.4), and labeled with a monoclonal antibody that recognizes the heterodimer Ku70 and Ku86. Staining by the monoclonal antibody was detected using a Texas Red®-conjugated secondary antibody. Images of the cell were collected using a Leica® TCS laser scanning confocal microscope (William K. Warren Medical Research Institute, Oklahoma City, OK, USA). GFP was excited at 488 nm, and Texas Red was excited at 568 nm. Emission wavelengths of 530–560 nm were collected for imaging GFP, and wavelengths of 600–660 nm were collected for Texas Red. The cell in panel A exhibits extensive colocalization of the two fluorophores, and the ρ has a value of 0.92. However, a histogram of the ρ measured in a population of 27 cells (B) shows that extensive variation of fluorophore colocalization occurs within the sample. The average value of ρ for the population is 0.54 ± 0.22 . The white bar in panel A represents 5 μ m.

Benchmarks

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Fluorescence Polarization Standards for High-Throughput Screening and Imaging

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Fluorescence polarization has been utilized for some time in the form of fluorescence polarization immunoassays for therapeutic drugs, drugs of abuse, and other small molecules (3, 11), and more recently for immunoassays for macromolecules (9). Polarization-based assays have been developed for high-throughput screening applications, particularly lead identification. These assays include protease assays (24), kinase assays (2,22,23), and DNA hybridization reactions (20,21,27), as

well as assays for free metal ions (26) and glucose (16). These assays exploit the unique advantages of fluorescence polarization-based assays in general: good sensitivity, rapid response, homogenous format (no separation required), simple instrumentation, and a robust format that minimizes artifacts. The robust nature of the polarization measurement arises from its ratiometric character, readily achieved in the classic T-format of Weber (28). The polarization of fluorescence can be expressed in terms of the anisotropy (r) or the polarization (p):

$$p = (I_{\parallel}/I_{\perp} - 1)/(I_{\parallel}/I_{\perp} + 1) \quad [\text{Eq. 1.1}]$$

$$r = (I_{\parallel}/I_{\perp} - 1)/(I_{\parallel}/I_{\perp} + 2) \quad [\text{Eq. 1.2}]$$

where $I_{\parallel}$ is the intensity of the fluorescence emission with the emission polarizer oriented parallel to the vertically oriented excitation polarizer, and $I_{\perp}$ the intensity with the emission polarizer oriented perpendicular (horizontal) to the excitation. Like other ratiometric fluorescence measurements, polarization measurements can thus be insensitive to fluctuations in excitation intensity, fluorophore concentration, sample thickness, and some inner filter effects. Therefore, it is well suited for quantitative assays and also imaging applications such as fluorescence polarization microscopy (1,5,6). Note that polarization measurements taken in the more common L-format are not strictly ratiometric since the intensities in Equation 1 are measured serially; however, most excitation sources vary only a few tenths of 1% over a few minutes, so the precision can be quite good. Although we will refer mainly to fluorescence polarization as opposed to fluorescence anisotropy since the former has come into wide use in the high-throughput screening community, it is nevertheless true that, although the terms are synonymous and algebraically easy to interconvert, anisotropy is much more tractable theoretically (see Equation 2).

The need for and utility of standards in all forms of chemical analysis are widely accepted, and the same is true for fluorescence polarization assays. The availability of convenient standards permits calibration of the response of the instrument in terms of the analyte amount or concentration; veri-

fies the correct functioning of the instrument, particularly following alignment or repair; and provides a basis for troubleshooting anomalous results. For some clinical assays, frequent recalibration is required by law. Ideally, a standard material should be stable, easy to formulate, transportable, readily transferable among laboratories, traceable to some primary standard, and inexpensive. The standard should be usable under conditions that closely mimic (if they are not identical to) the measurement of "real" samples.

Although fluorescence polarization measurements are easy to make and relatively free from artifact, some agents and influences may perturb a polarization measurement, especially in 96-well plates and similar formats. In general, the extent of fluorescence polarization from a particular sample depends primarily on the ratio of the fluorescence lifetime to the rotational correlation time, according to the Perrin equation:

$$(1/p - 1/3) = (1/p_0 - 1/3)(1 + \tau/\theta_c) \quad [\text{Eq. 2.1}]$$

$$r/r_0 = 1/(1 + \tau/\theta_c) \quad [\text{Eq. 2.2}]$$

where τ is the lifetime, θ_c is the rotational correlation time, p is the measured polarization, and p_0 is the polarization measured in the absence of rotational diffusion, ordinarily 0.5. Among these influences is temperature, which can affect both rotational diffusion and fluorescence lifetime. However, temperature can readily be controlled. Similarly, the viscosity of the solvent can be controlled by its composition and temperature. Also of potential concern is scattered light (Rayleigh or Raman), which, because of its high polarization (1.0), can readily corrupt measurements even when it represents only a small fraction of the light hitting the detector. Again, this problem can be avoided by careful optical filtration of the emission. Another potential source of error is collisional quenching of the fluorophore by various solutes because it affects the lifetime. However, unless the fluorescence lifetime is long, it will be little affected by this phenomenon unless the quenching solutes are present at nearly millimolar concentrations, or in certain rare cases. Again, avoidance of such solutes at high levels minimizes the problem. The perturba-