ACADEMIC PRESS

Testing effects of signal transduction pathways on cadherin junctional complex assembly using quantitative image analysis

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Abstract

Cadherin adhesion molecules function in numerous cell biological processes that influence embryo development, normal cell physiology, and pathophysiology of many disease processes. Cadherins nucleate the assembly of the adherens junction, a cell-to-cell adhesion plaque that is prominent in simple epithelial cells and found in many cell types. Numerous cell biological approaches have been used to study this interesting class of molecules. Here, we outline methodology used in our studies of junctional complexes to examine effects of signaling molecules on assembly mechanisms. This is a quantitative method that allows the investigator to test the combined effect of two different signaling processes to determine whether these two signals act in concert within the same pathway. We discuss how this method could be generalized to other studies to examine consequences of various experimental manipulations on the assembly of cellular structures.
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1. Introduction

Cadherins are a large family of cell adhesion molecules that control cell biological processes [1]. These homotypic cell adhesion molecules form strong adhesion complexes that are required to maintain tissue integrity. Cadherins are type I membrane proteins with an adhesive extracellular domain, a transmembrane domain and a cytoplasmic domain, that is conserved among different family members. Cadherins have repeating sequence motifs within their extracellular domains. The cadherin family of proteins can be subdivided into different groups that include the adherens junction cadherins (or classic cadherins) which are discussed in this article. The other two groups, desmosomal cadherins and protocadherins, perform similar adhesion functions. In contrast to adherens junction cadherins, desmosomal cadherins form complexes with a distinct set of proteins [2]. Protein interactions with protocadherins are poorly defined, but several of this class of cadherins have been shown to interact with the tyrosine kinase fyn [3].

Adherens junction cadherin function is dependent on the association of several proteins with the cadherin cytoplasmic domain [4,5]. The best known of the cadherin-associated proteins are the catenins. These proteins were first identified as proteins that co-immunoprecipitate with cadherins [6,7]. Now, it is known that these proteins regulate the association of the cadherin/catenin complex with the actin cytoskeleton. In addition, β-catenin is an important developmental signaling protein that translocates to the nucleus and participates in transcriptional regulation [8]. The cadherin/catenin complex also associates with signal transduction proteins, including growth factor receptors and protein kinases [9–11]. The adherens junction produces a juxtaposition of protein complexes that regulate cell adhesion, cytoskeleton dynamics, and signal transduction. Therefore, the adherens junction integrates cellular signals and forces to control cell biological and developmental processes.

Cadherin function is regulated by intracellular signals, and cadherin adhesion activates normal physiological and pathophysiological signaling processes. Levels of adherens junction assembly can be manipulated by intracellular signals, for example, Rho-family GTPase signaling, tyrosine kinases, and tyrosine phosphatases

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[5,10]. Cadherin adhesion also stimulates intracellular signal transduction pathways, including Rho-GTPase signaling and tyrosine kinases [5,9,10,12–16]. Here we describe quantitative microscopic methodology that allows the investigator to determine whether a specific treatment or combination of treatments affects cadherin assembly mechanisms. Quantitative microscopy allows one to compare measured effects under various experimental conditions and provides the basis for statistical analysis of effects that may be subtle or subject to variation between different cells.

2. Methodology: measuring changes in junctional complex assembly in response to signaling factors

We have developed a method to measure combined effects of signal transduction pathways or pathophysiological treatments that uses high-resolution laser scanning confocal microscopy and detailed image analysis. The ability to examine effects that result from a combination of treatments on the junctional complex assembly state permits the investigator to examine whether two factors may function in the same signal transduction pathway that impinges on junctional complex assembly mechanisms. This method is highly robust, providing an effective statistical test of a hypothesis. This method also can be used to measure the relative effect of one signaling molecule that is expressed by transfection or by microinjection in cells because it uses the untransfected or uninjected cells within the same microscopic fields as internal controls for comparison.

For example, we have used this method to examine whether Rho-family GTPase signaling affects junctional complex disassembly during epithelial cell injury [17,18]. ATP depletion of cultured renal epithelial cell lines is a useful model for effects of epithelial cell injury that occur during ischemic injury to the kidney [19]. Various disease processes, surgical procedures, renal transplantation and trauma result in reduced perfusion of the kidney and subsequent injury to specific cell types within the organ [20]. Ischemic injury is a leading cause of acute renal failure, which is a significant factor in morbidity and mortality in hospitalized patients [20]. Ischemia causes a rapid drop in ATP levels in kidney epithelial cells, particularly proximal tubule epithelial cells, and ATP depletion of cultured epithelial cells is a very useful model of this process [21]. Ischemic injury to renal epithelium and ATP depletion of cultured cells lead to disruption of actin cytoskeleton and junctional complex assembly [19,21].

We have found that Rho-family GTPase signaling can protect renal epithelial cell junctional complexes from disassembly during ATP depletion [17,18]. Loss of junctional complex integrity contributes to defects in cell polarity and paracellular permeability that lead to organ dysfunction during acute renal failure [19]. Actin cytoskeleton structures in renal epithelial cells are also protected from disassembly by Rho-family GTPase signaling [22]. Protecting epithelial cell architecture or stimulating its recovery from injury may help the kidney recover from ischemic injury and restore normal organ function. The image processing methods described below were used to study the combined effects of Rho-family GTPase signaling and ATP depletion.

The first important feature of any image analysis method is to take care in the way that the data are collected. A standard immunofluorescence staining protocol is performed to label a junctional complex component and the epitope tag for the transfected or microinjected signal transduction molecules. Instead of an epitope tag, GFP or co-injection of a fluorescent molecule like FITC-dextran could be used. Transient transfection and microinjection are used in this method because these expression methods allow the investigator to compare the expressing cells with nonexpressing cells (untransfected or uninjected cells) within the same microscopic field.

A microscope fields is selected that contains groups of cells expressing the transfected or microinjected molecule and are adjacent to one another. Junctions between these pairs of cells that express the molecule are designated as transfected or microinjected cell junctions. Junctional complexes between pairs of cells that do not express the molecule are designated as untransfected or uninjected junctions.

Using laser scanning confocal microscopy, we collect a series of x–y image planes in a z series through the entire volume (0.5-μm steps) of the polarized epithelial cell monolayer for the junctional complex molecule. This ensures that the entire vertical extent of the junctional complex protein that is being analyzed is imaged and represented in the data set. A single image plane is usually sufficient to determine which cells express the epitope tag for the signal transduction molecule. We also collect z series of x–y image planes through epithelial cell volumes and corresponding epitope tag images following a treatment, which is, ATP depletion in our studies, but could be a drug, toxin, or other treatment. The combination of factors and treatments is obviously determined by the investigator and the goal of the studies.

To ensure that all staining information is contained within the image data set, gain levels are set to avoid saturation of the photomultiplier tube. Confocal microscope interface software will contain an indicator for photomultiplier tube saturation; photomultiplier gain settings are adjusted so that there is no saturation through the most intensely staining portion of the cell volume. Once an image stack that represents the complete staining profile for the cells within the field is collected, other fields are collected using the same

procedure. Different gain levels are used for each data collection, but as described below, data from one field are not directly compared with those from another field. Rather, a ratio of fluorescence intensity per unit length for junctions between two transfected or microinjected cells to fluorescence intensity per unit length for junctions between untransfected or uninjected cells from junctions within the same microscopic field is produced, and ratios from several microscopic fields are averaged (this is defined below as the *fluorescence intensity ratio*). This ratio measurement provides an internal control that normalizes for different signal levels.

When all data for an experiment are collected, image processing is performed using the MetaMorph image analysis software package (Universal Imaging Corporation, Downingtown, PA, USA). The steps described are for MetaMorph Version 4.6. Some details may change for older or future versions of the software package used in the investigator's laboratory. In Meta-Morph, a specific series of image processing functions are performed on each image stack that quantifies the fluorescence intensity per unit length within individual junctions. We describe the image processing steps below, and the steps are outlined in Tables 1 and 2. Use the MetaMorph help function to find any command described here if you have trouble finding it.

Open image stack from the **File** menu on the toolbar, and use the **Open** command to open the file that

Table 1
Constructing a journal file for junctional complex image stack (Sum-Filter-Subtract)

Step	Menu-Command	Dialog Box	Action
1	**File-Open**		**Open image stack**
2	**Journal-Start Recording**		**Begin recording journal file**
3	**Process-Stack Arithmetic**		**Sum stack of images into one image**
4		**Stack Arithmetic:** **Operation: Sum of** **-Apply**	
5	**Process-Low Pass...**		**Smooth data**
6		**Low Pass:** **Horizontal size=3** **Vertical size=3** **Source: Sum** **Dest: Low Pass** **-Apply**	
7	**Process-Median Filter...**		**Create a median image**
8		**Median Filter:** **Horizontal size=24** **Vertical size=24** **Sub-sample Ratio=1** **Source: Low Pass** **Dest: Median** **-Apply**	
9	**Process-Arithmetic...**		**Subtract median image from filtered image**
10		**Arithmetic:** **Operation: Subtract** **Source 1: Low Pass** **Source 2: Median** **Dest: Subtract** **Second Source: Image** **Result Depth: 16** **Value: 0** **-Apply**	
11	**Journal-Stop Recording**		**Stop recording journal file**
12		**Save As:** **Sumsubtract.jnl**	**Save journal file with name Subsubtract.jnl**

Steps of preparation of the journal file (a macro file) are outlined. In Menu-Command column, the menu from the menu bar and the command to be used are shown. When a dialog box is opened, the Dialog Box column describes the settings, followed by the final button to select (for example, Apply). The Action column describes the function of the commands that are used. See text for details.

Table 2
Steps for junctional complex quantitative image analysis

Step	Menu-Command	Dialog Box	Tool	Action
1	File-Open			Open image stack
2	Journal-Run Journal…			Select journal file
3		/Run Journal/ Subsubtract.jnl		
4	File-Save As			Save Subtract file as a TIFF file
5	File-Open			Open image file showing epitope tagged cells
6			Image Window Tools-Pseudocolor	Changes greyscale image to Pseudocolor
7			Image Window Tools-Magnification tool	Magnifies image 400-800%
8			Region Tools-Trace Region Tool	Traces region containing the junctional complex area to be quantified
9	Measure-Show Region Statistics…			To export pixel intensity of junctional complex
10		/Show Region Statistics…/ select Configure Log… Parameter Configuration: Image Name and Integrated Logging Options: select Log column titles -OK		
11		/Show Region Statistics…/ select Open Log		
12		/Open Data Log/ Log Measurements to: Dynamic Data Exchange (DDE) -OK		
13		/Export Log Data/ Application: e.g. Microsoft Excel 2000		
14		/Show Region Statistics…/ click F9: Log Data button		
15			Region Tools-Traced Line Tool	Traces length of line to be measured
16	Measure-Region Measurements…			
17		/Region Measurements/ Include (pull down menu) select Active Region select Configure tab and select Active Region; Image Name and uncheck Region Label box		
18		/Region Measurements/ click F9: Log Data button		

Steps of image analysis are outlined. In Menu-Command column, the menu from the menu bar and the command to be used are shown. When a dialog box is opened, the Dialog Box column describes the settings, followed by the final button to select (for example, OK). The Tool column outlines the tool bar functions used to analyze the junctions. The Action column describes the function of the commands that are used. See text for details.

contains the complete image volume of junctional complex antibody staining that was collected on the confocal microscope as described above. With this im-age window selected, create a journal. This journal is a small Macro file that will perform a set of commands the same way for each data set. The journal in Table 1 sums

all the images into one image and performs background subtraction.

The set of commands used repeatedly during image analysis can be recorded and repeated using the journal function. To begin recording the set of commands and writing a journal of the commands that we used to analyze junctional complex assembly, select the **Journal** menu from the menu bar, and select the **Start Recording** command. Any commands used until you stop recording become part of the journal.

The first command in the journal sums all the images in a stack to produce a single image with the summed data for analysis. Select the **Process** menu from the menu bar, and select the **Stack Arithmetic** command. This opens the **Stack Arithmetic** dialog box. In the **Stack Arithmetic** dialog box, make sure that **Sum of** is selected under **Operation** choices and then select the **Apply** button.

The second command in the journal applies a low-pass filter to smoothe the data and improve your ability to highlight the junctions later in the image analysis. Under the **Process** menu on the menu bar, select the **Low Pass...** command. The **Low Pass** dialog box opens. In the **Low Pass** dialog box, enter 3 for **Horizontal Size** and 3 for **Vertical Size**. This filter takes one pixel and assigns an average value for that pixel relative to the pixels in a 3 × 3-pixel area surrounding that pixel. Make sure that the **Source** is **Sum** and the **Dest** (Destination) is **Low Pass**. This means that the image that the low-pass filter is applied to is the **Sum** image from the previous step, and that the filtered image will be in a new file called **Low Pass**. Finally, click the **Apply** button.

The next step is to create a median background image that will be subtracted from the **Low Pass** image. Select the **Process** menu from the menu bar, and select the **Median Filter...** command. This opens the **Median Filter** dialog box. In this dialog box, enter 24 for **Horizontal Size**; enter 24 for **Vertical Size**; and enter 1 for **Sub-sample Ratio**. This function identifies the median background for each pixel in the image from a 24 × 24-pixel area surrounding that pixel and generates an image that contains that background that will be subtracted in the next step. As long as the structure to be analyzed occupies less than half the size of the neighborhood from which the median is measured, the median provides an accurate estimate of the local background fluorescence. A 24 × 24-pixel area is good for images that show junctional complexes, but for larger structures, a larger area may be more appropriate. Setting the **Sub-sample Ratio** at 1 means that the median background process is performed for every pixel; if another value like 2 or 3 is selected, then this process is performed for every other or every third pixel. Subsampling provides a less accurate local median. Make sure that the **Source** is **Low Pass** and the **Dest**

(Destination) is **Median**. Again, this means that the image that the median is calculated for is the **Low Pass** image from the previous step, and that the median filtered image is in a new file called **Median**. Then click the **Apply** button.

The final step is to subtract the median image from the filtered image. Select the **Process** menu from the menu bar, and select the **Arithmetic...** command. This opens the **Arithmetic** dialog box. In the **Arithmetic** dialog box, make sure that **Subtract** is selected under the **Operation** selections. Also, make sure that **Source 1** is **Low Pass**; that **Source 2** is **Median**; and that **Dest** is **Subtract**. Under the **Second Source** choices, **Image** should be selected, and under **Result Depth** choices, **16** should be selected to make a 16-bit image that contains sufficient information for each pixel in a summed image. The **Value** setting is left on 0. **Value** is used in some cases when a value needs to be added to each pixel, which is not necessary in this operation. Click the **Apply** button, and the subtracted image is generated.

At this point, stop recording the journal. Select the **Journal** menu from the menu bar, and select the **Stop Recording** command. A dialog box will open to allow you to save the journal as a file with a *.jnl suffix. In this example, we have used the "Sumsubtract.jnl" file name.

If you examine the journal "Sumsubtract.jnl" it would read as follows.

New Sum = StackSum([Current At Start])
New Low Pass = LowPass([Image?], 3, 3)
New Median = MedianFilter(Image?], 24, 24, 1)
New Subtract = [Image?] − [Image?] + 0

To use your journal to process image stacks (Table 2), open the image stack from the **File** menu on the toolbar. Use the **Open** command to open the file that contains the complete image volume collected on confocal microscope. With this image window selected, select the **Journal** menu from the menu bar, and select the **Run Journal...** command. In the **Run Journal** dialog box, select the journal file that was created above: select the "Sumsubtract.jnl" file. The sequence of processes begins, which sums the images and performs background subtraction.

After the journal has run, the summed and subtracted image that is currently named **Subtract** is saved by selecting **File** from the menu bar and selecting the **Save As** command; replace the name **Subtract** with a new file name as a TIFF file. We usually use the same file name as the *.pic stack file (which is the format of the image generated using our Bio-Rad 1024 confocal microscope; Bio-Rad, Hercules, CA, USA) file to avoid confusion.

Open the individual plane that is stained for the epitope tag to find transfected or microinjected cells. By comparing the summed and subtracted image

with the epitope-tagged image (Fig. 1A), individual junctions are selected and quantified in the following way.

On the **Image Window Tools** toolbar on the image window, select the color lookup table (the circle with a gradient of color) and select **Pseudocolor**. This makes it
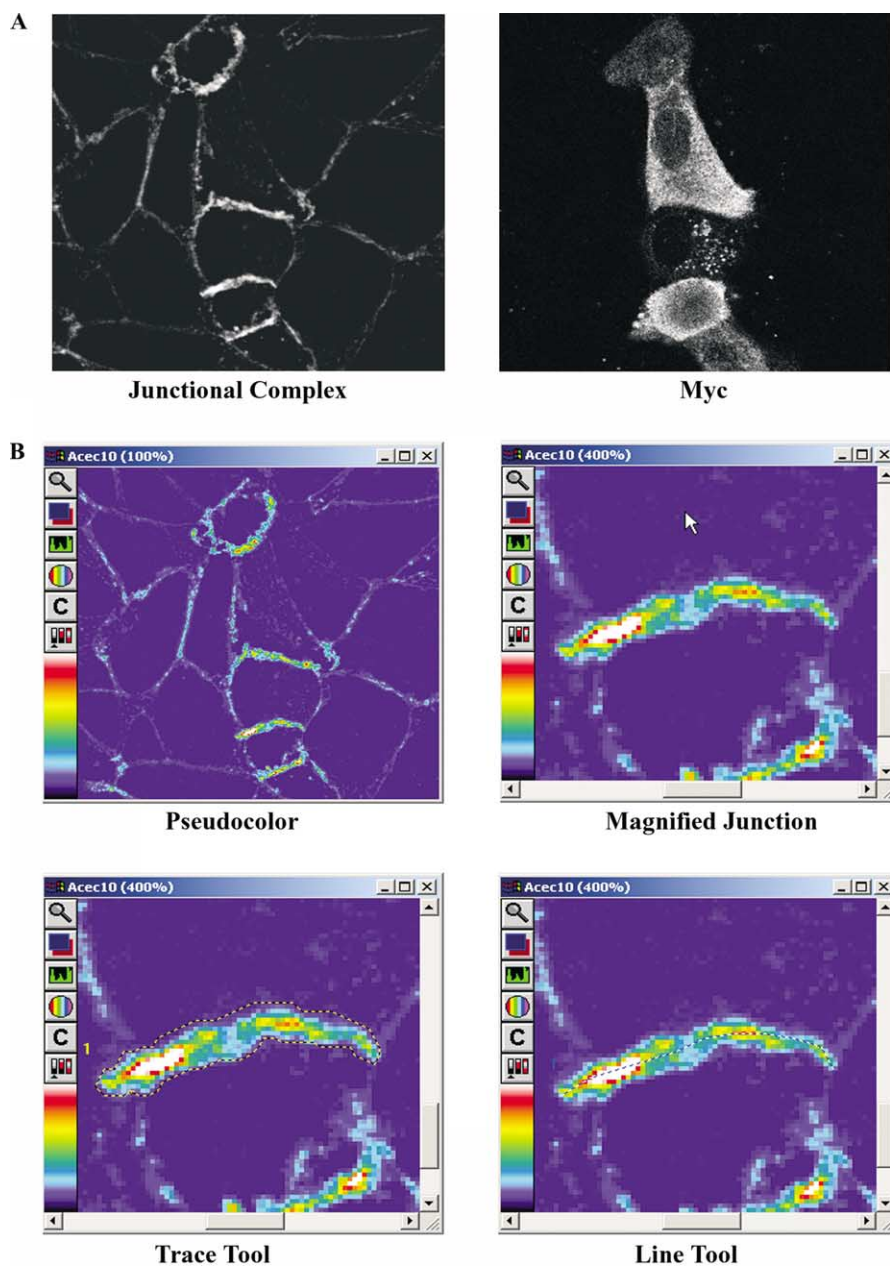
$$\text{Fluorescence intensity ratio} = \frac{\text{Fluorescence intensity per unit length (transfected)}}{\text{Fluorescence intensity per unit length (untransfected)}}$$

Fig. 1. Quantitation of junctional complex fluorescence intensity and junctional complex length. (A) An image showing a summed 16-bit image of junctional complex fluorescence from a stack of images through the entire volume of the epithelial monolayer (left: Junctional Complex), and a single confocal section through the epithelial cell monolayer showing epitope tag staining used to identify transfected cells (right: Myc). By comparing these two images, one can see that junctional complex protein has accumulated between cells that express the transfected protein. (B) Screen shot images from MetaMorph showing sequential steps in the image analysis of a junction from the image in (A). Pseudocolor image of field shown in (A) is shown (top left image: Pseudocolor). A magnified image of a junction is shown (top right image: Magnified Junction). Highlighting of junctional fluorescence intensity by freehand tracing of junction is shown by dotted traced line (bottom left image: Trace Tool). Highlighting of the length of a junction is shown by the dotted line (bottom right: Line Tool). Ratio measurement described in text is generated from numerous junctions quantified using this technique. A general formula for the *fluorescence intensity ratio* is shown at the bottom. See text for details.

easier to see and outline junctions with the freehand tool (Fig. 1B).

On the **Image Window Tools** toolbar on the image window, use the magnification tool (which looks like a magnifying glass) to magnify the junction (usually 400–800% is good) (Fig. 1B).

Select the **Trace Region Tool** on the **Region Tools** toolbar; left-click and hold the mouse button down, and trace the entire junction. Then, release the mouse button, and double left-click on the region selected. The region selected will show a streaming dotted line to indicate that the selection was correctly made. If you want to start again, then double left-click and press **Backspace** on the keyboard; the selection disappears. Repeat the above process until you are satisfied that you have selected the entire junction complex staining area (Fig. 1B).

To measure the total intensity of the pixels selected, go to the **Measure** tool bar menu and select the **Show Region Statistics**... command. A dialog box appears with many statistics. Select the **Configure Log**... button in this dialog box. Select **Image Name** and **Integrated** from the **Parameter Configuration** list (under **Logging Options**, be sure **Log column titles** is selected), and left-click the **OK** button. Next, while the **Show Region Statistics**... dialog box is open, select the **Open Log** button. The **Open Data Log** dialog box appears; under **Log Measurements to** make sure the check mark selects **Dynamic Data Exchange** (**DDE**), and select **OK**. The **Export Log Data** dialog box opens. Select the spreadsheet program that you use under the **Application** pulldown menu (we use Microsoft Excel 2000). Use default settings for other boxes. This opens the spreadsheet program, but the data are not entered yet. Go back to MetaMorph and click the **F9: Log Data** button within the **Show Region Statistics**... dialog box that is still open (this button has replaced the **Open Log** button, and the words above this button have changed from **Data Log Not Open** to **Data Log: DDE App**). When the **F9: Log Data** button is selected, then the words above this button change to **Logged to DDE App**. Now, when you check your spreadsheet, the data should have been entered (if it hat not, then click the **F9: Log Data** button again).

Now, measure the length of the junction. Select the **Traced Line Tool** on the **Region Tools** toolbar. Left-click and hold the mouse button down while pointing at the beginning point of the junction to be measured, and stretch the line over the length of the junction and release the mouse button at the end of the junction to be measured. Then, double left-click on the line that was drawn. Make sure that the line is highlighted: the line should appear as a streaming dotted line. If you want to start again, then double left-click and press **Backspace** on the keyboard; the selection disappears. Repeat the above process until you are satisfied that you have correctly represented the entire length of the junction complex (Fig. 1B).

To measure the length of the junction in pixels, go to the **Measure** tool bar menu and select the **Region Measurements**... command. In the **Region Measurements** dialog box, the **Include** pulldown menu has **Active Region** selected. By selecting the **Configure** tab, you can select **Image Name** check box (uncheck the **Region Label** check box) if you want your data to contain your file name (but this is not necessary). Under **Color Channel**, the **Intensity** button should be selected, and under **Display and Log**, the **Region Measurements Only** button should be selected. Then, select the **F9: Log Data** button at the top of the dialog box. The **Distance** or length of the junction is given in pixels and should be entered into the spreadsheet program. The number may be a noninteger because the line may be curved. If the data were not entered into the spreadsheet, then click the **F9: Log Data** button again.

Many fields are quantified using the above method. Usually we do as many fields as needed to quantify 20 to 30 transfected (microinjected) junctions and 20 to 30 untransfected (uninjected) junctions. Similarly, many fields are quantified for cells that have been subjected to an experimental treatment (e.g., ATP depletion). Sufficient numbers of fields are analyzed to quantify 20 to 30 transfected (microinjected) junctions and 20 to 30 untransfected (uninjected) junctions that were subjected to an experimental treatment. To ensure good normalization to untransfected (uninjected) junctions, more than one untransfected (uninjected) junction is measured for each field analyzed. Using statistics for each individual junction, a ratio of fluorescence intensity per unit length for *an individual* transfected (microinjected) junction to fluorescence intensity per unit length for *an individual* untransfected (uninjected) junction is calculated. The two junctions used to calculate the ratio are always taken from the same microscopic field. We *do not* use averages for all transfected (microinjected) junctions divided by averages for all untransfected (uninjected) junctions.

After the 20 to 30 ratios of fluorescence intensity per unit length for individual transfected (microinjected) junctions to that for individual untransfected (uninjected) junctions are calculated, then *an average of these ratios is calculated*. This is termed the ***fluorescence intensity ratio***. A similar average for ratios (fluorescence intensity ratio) is calculated for junctions following an experimental treatment (e.g., ATP depletion). Comparison of these fluorescence intensity ratios for cells prior to an experimental treatment with those after an experimental treatment allows the investigator to determine whether the transfected or microinjected signaling molecule acts in concert with the pathway stimulated or inhibited by the experimental treatment (see below) (Fig. 2). Student's *t* test is used to statistically compare these ratios.

The fluorescence intensity ratio is the fold change in assembly from control (untransfected or uninjected) junctional complexes. There are many possible variations for this general application. For example, this
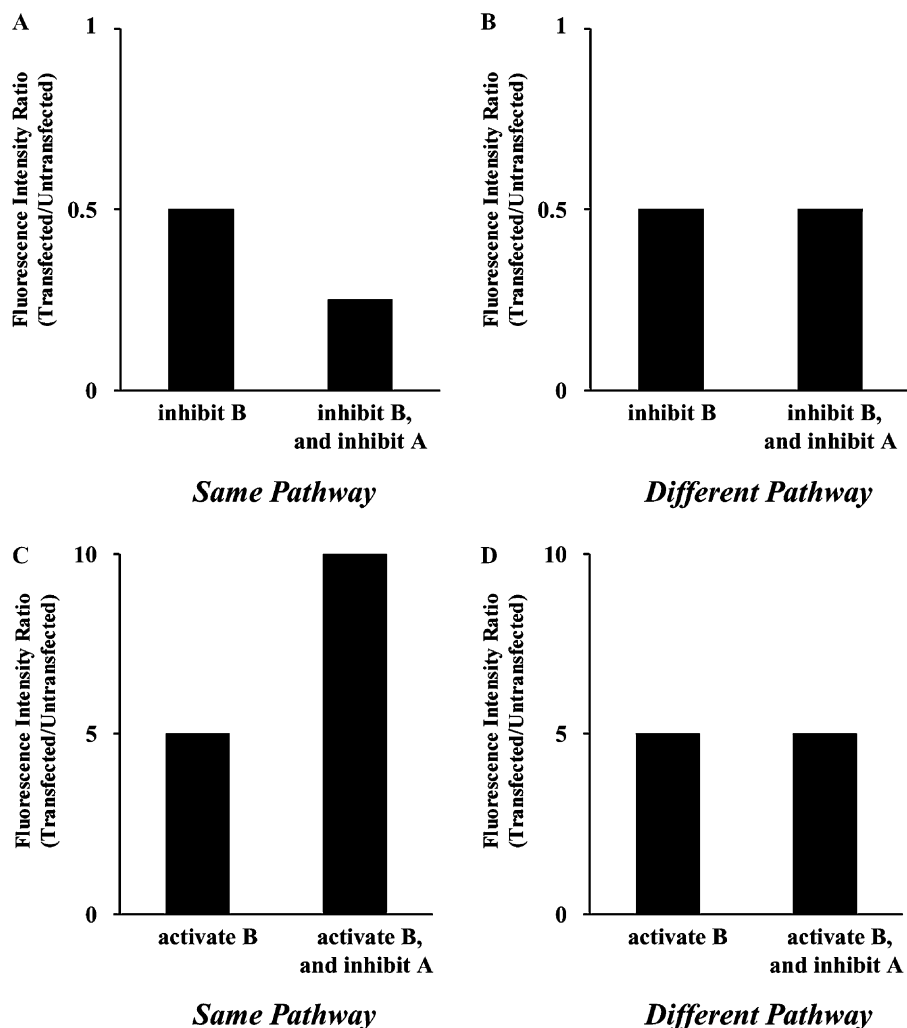
Fig. 2. Examples of data that could be generated using the quantitative image analysis method described and examples of signaling pathways and effects of treatments as outlined in the text.

fluorescence intensity ratios calculated for variants of a molecule (e.g., mutant signaling molecules generated by recombinant DNA methods) that are transfected or microinjected could be compared statistically using Student's t test.

3. Implications and applications of this quantitative image analysis methodology

This method provides a powerful method that can be fit into many experimental paradigms. We have used this method and have shown that it is robust [17,18]. Statistical tests for experiments that showed positive results had extremely high levels of significance. Also, the experimental system did not produce false positive results due to a defect in the statistical method; negative results were detected that did not approach statistical significance.

Specific requirements for this method include a confocal microscope and the MetaMorph software package (Universal Imaging Corporation). Confocal microscopy is becoming very common in cell biological research laboratories, and availability should not always be a limiting factor. MetaMorph software is often available within the microscopy facility that houses the confocal microscope, but other image analysis software packages may be used and adapted to the protocol outlined in this article. The description of the analysis that we perform is based on our use of MetaMorph Version 4.6. We have used previous versions of MetaMorph software for this analysis, and some commands may change in future versions of the software. However, we feel that this will be a generally adaptable protocol for many cell biology laboratories.

The potential combinations of treatments that could be used in this experimental system are extensive. Any molecule that affects junctional complex assembly and

can be transfected or microinjected into cells can be used. Molecular dissection of such a regulatory molecule could include the production of various mutant versions using recombinant DNA methods. These various mutants could be tested using the image analysis methods outlined above.

Treatments that affect junctional complexes in all cells in a culture, like a drug treatment, can be used in combination with transfected molecules. Drug treatments that affect signal transduction pathways, such as those that affect protein kinases or phosphatases, could be applied. In addition, any number of treatments could be adapted to this protocol, for example, environmental factors, pathological agents, and toxins.

This image analysis protocol could also be adapted to analyze other cellular structures. As an example, Rho signaling and ATP depletion effects on actin stress fibers and cortical actin cytoskeleton were studied using a similar approach [22]. When analyzing other cellular structures, changes in the specific protocol are necessary. For actin stress fiber analysis, only the basalmost image planes were used to reduce signal from other actin structures [22]. Also, the parameters for averaging and background subtraction should be adapted for the size of the structures.

Idealized examples of data that could be generated using this methodology are shown in Fig. 2. These examples are based on a model where two factors are affected by transfection and treatment, and the experiment tests whether these factors act in the same pathway or in a different, parallel pathway. Two factors (factor A and factor B) affect junctional complex assembly, and in the first set of examples, factor A is upstream of factor B (A → B → junctional complex assembly).

In this example, factor B can be affected by transfection; then the measurements of junction staining between transfected cells are made before and after treatment with a drug that affects factor A. If transfection inhibits factor B activity, then the effect is to reduce the fluorescence intensity ratio (transfected/untransfected; *fluorescence intensity ratio* defined above) to less than 1 (where the value of 1 represents the fluorescence intensity ratio for untransfected/untransfected junctions) (Fig. 2A). If the drug inhibits factor A activity and factor A acts within the same pathway as factor B, then the effect on the junctions between transfected cells is synergistic, further reducing the assembly of the junctions relative to the untranfected junctions (Fig. 2A). In contrast, if factor A is not in the same pathway as factor B, then the inhibitor of factor A activity has the same effect on both junctions between transfected cells and untransfected cells (Fig. 2B).

Alternatively, transfection could activate factor B activity; then the fluorescence intensity ratio (transfected/untransfected) increases to a value greater than 1 (where the value of 1 represents the fluorescence intensity ratio for untransfected/untransfected junctions) (Fig. 2C). If the same drug as used in the example above is used to inhibit factor A activity and factor A acts within the same pathway as factor B, then the effect on the junctions between transfected cells overrides the effect on factor A. However, if the effect of the drug on factor A on junctions between untransfected cells is not overridden, junction assembly is reduced. Consequently, the fluorescence intensity ratio is higher than the control ratio before drug treatment (Fig. 2C). If factor A is not in the same pathway as factor B, then the inhibitor of factor A activity has the same effect on both junctions between transfected cells and untransfected cells, and the fluorescence intensity ratio does not change as a result of drug treatment (Fig. 2D).

These examples are intentionally similar to the findings from our experiments studying Rho-GTPase signaling effects on cadherin and tight junctions in a model of renal ischemia, ATP depletion of MDCK cells [17,18]. However, the possible outcomes of experiments and potential combinations of treatments are nearly endless. Thus, there cannot be a simple formula for outcomes from experiments that combine treatments to examine a particular pathway that regulates junctional complex assembly mechanisms. Empirical findings are required, but this quantitative microscopy method is a useful way to study pathways that affect junction assembly.

Acknowledgments

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