Mapping of adherens junction components using microscopic resonance energy transfer imaging

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SUMMARY
Quantitative microscopic imaging of resonance energy transfer (RET) was applied for immunological high resolution proximity mapping of several cytoskeletal components of cell adhesions. To conduct this analysis, a microscopic system was developed, consisting of a highly stable field illuminator, computer-controlled filter wheels for rapid multiple-color imaging and a sensitive, high resolution CCD camera, enabling quantitative data recording and processing. Using this system, we have investigated the spatial inter-relationships and organization of four adhesion-associated proteins, namely vinculin, talin, α-actinin and actin. Cultured chick lens cells were double labeled for each of the junctional molecules, using fluorescein- and rhodamine-conjugated antibodies or phalloidin. RET images were acquired with fluorescein excitation and rhodamine emission filter setting, corrected for fluorescein and rhodamine fluorescence, and normalized to the fluorescein image. The results pointed to high local densities of vinculin, talin and F-actin in focal adhesions, manifested by mean RET values of 15%, 12% and 10%, respectively. On the other hand, relatively low values (less than 1%) were observed following double immunofluorescence labeling of the same cells for α-actinin. Double indirect labeling for pairs of these four proteins (using fluorophore-conjugated antibodies or phalloidin) resulted in RET values of 5% or lower, except for the pair α-actinin and actin, which yielded significantly higher values (13-15%). These results suggest that despite their overlapping staining patterns, at the level of resolution of the light microscope, the plaque proteins vinculin and talin are not homogeneously interspersed at the molecular level but form segregated clusters. α-Acctin, on the other hand, does not appear to form such clusters but, rather, closely interacts with actin. We discuss here the conceptual and applicative aspects of RET measurements and the implications of the results on the subcellular molecular organization of adherens-type junctions.

Key words: resonance energy transfer, vinculin, talin, α-actinin, actin, focal contact, cell adhesion

INTRODUCTION
Adherens-type junctions (AJ) are a family of cell-cell and cell-matrix adhesions which are characteristically associated with the actin-based cytoskeleton through a multimolecular submembrane plaque (Geiger and Ginsberg, 1991). The molecular composition and diversity of AJ have been extensively studied during the last several years. It was shown that essentially all AJ share some common components such as actin, α-actinin and vinculin, while other junctional molecules, notably the plaque proteins talin, paxillin, plakoglobin and catenins, are selectively associated with either cell-matrix or cell-cell adhesions (Geiger et al., 1985, 1990). The transmembrane receptors of AJ, namely members of the integrin and cadherin families (mediating contacts with the matrix or with neighboring cells, respectively), show further tissue-specific heterogeneity which reflects their individual binding specificities (Geiger and Ayalon, 1992; Burridge et al., 1988; Hynes, 1992).

In vitro binding studies with isolated junctional proteins have provided some insight into the possible molecular interactions in adherens junctions. For example, integrin was shown to bind to both talin (Horwitz et al., 1986) and α-actinin (Otey et al., 1990), and cadherin was shown to bind to catenins (Kemler and Ozawa, 1989). Among the plaque proteins, vinculin was shown to interact with several proteins including talin (Burridge and Mangeat, 1984), paxillin (Turner et al., 1990) and α-actinin (Belkin and Koteliansky, 1987), and possibly with itself (Bendori et al., 1989). α-Acctin, also, binds to another junctional protein, zyxin (Crawford and Beckerle, 1991), as well as to actin filaments (Baron et al., 1987). AJ contain, in addition, actin capping proteins such as tensin (Butler and Lin, 1989) and a multitude of enzymes (mostly kinases) which are known to participate in the transmembrane transduction of growth and differentiation signals (Geiger et al., 1992).

Mainly on the basis of these biochemical data, a general structural model was offered for adherens junctions (e.g. Burridge et al., 1988; Geiger et al., 1990), though the actual molecular topology and substructure of these sites was not directly demonstrated experimentally. This is mainly due to
the fact that the currently available methods fail to resolve the molecular architecture of these sites. Fluorescence microscopy, which is the most commonly used technique for visualizing AJ components, has a rather poor resolution of 0.2 µm at best. Immunoelectron microscopy, on the other hand, has an excellent spatial resolution, yet the stoichiometric representation of the cellular antigens, using the state-of-the-art methodologies (namely, the number of immuno-gold particles per antigen molecule), is insufficient to provide reliable molecular information (see Discussion). Since cellular ultrastructure is still not amenable for high resolution analytical methods (like crystallography), novel approaches for high resolution molecular mapping are essential for studying the molecular structure and assembly of adhesion sites in situ.

The approach applied here for the study of AJ organization is fluorescence resonance energy transfer (RET). This approach has long been utilized as a spectroscopic ruler for measurements of nanometer-scale proximities using visible light (Förster, 1946, 1948, 1959, 1964; Stryer and Haugland, 1967; Stryer, 1978; Stryer et al., 1982; Kuhn, 1982). The phenomenon occurs due to electron orbital dipole-dipole coupling between an excited donor and adjacent acceptor fluorophores whose respective emission and absorption spectra overlap. A characteristic distance between the dyes (r0), typically in the range of 4-6 nm (Stryer and Haugland 1967; Stryer 1978), defines the scale factor in the 6th power, which determines the inverse relationships between RET probability and the distance (for review see Lakowicz, 1983). RET provides, therefore, a sensitive measure for inter- or intra-molecular proximity (Stryer 1978), rendering it a useful tool for examination of the structure of molecular complexes including membranes (Fernandez and Berlin, 1976; Yguerabide and Förster, 1981; Szollősi et al., 1984; Johnson et al., 1984; Jovin and Arndt-Jovin, 1990; Uster and Pagano, 1986; Kubitscheck et al., 1991; Wang et al., 1993), cytoskeletal filaments (Taylor et al., 1981), chromatin (Jovin, 1979; Arndt-Jovin and Jovin, 1989; Cardullo et al., 1988; Ludwig et al., 1992), and many other interactions. The double conjugated molecules were separated from free dye by gel filtration on Sephadex G-50 (Pharmacia, Sweden). The molecules selected for immunolabeling contained an average of 2 moles of each fluorophore per mole of antibodies as determined spectrophotometrically from the 280, 495 and 575 nm absorptions. FITC- and TRITC-conjugated phaloidin were purchased from Fluorescent Imaging Systems (Petaluma, CA). The filters used here were bandpass interference filters (50% transmission at 485 and 590 nm, 20% at 430 and 650 nm), dichroic mirror (DAPI, FITC, Texas Red and Cy5; purchased from Omega Co., Brattleboro, VT).

**MATERIALS AND METHODS**

**Immunohistochemical reagents**

Primary antibodies used in this study were as follows: monoclonal anti-huvinulin (bVin1) was obtained from Sigma Immunochemicals (St Louis, USA); polyclonal rabbit antibodies (R694) were prepared against pure chicken vinculin. Monoclonal antibodies to α-actinin were from Sigma Immunochemicals (clone BM-75.2) and polyclonal antibodies were prepared in rabbits; both raised against the chick gizzard protein. Anti-talin antibodies were either monoclonal (clone 27.2) or rabbit antibodies kindly supplied by Dr K. Burridge (U. North Carolina, Chapel Hill) and by Dr M. Beckerle (U. Utah, Salt Lake City). Fluorescent secondary antibodies (goat antibodies to mouse or rabbit IgG), conjugated to either fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) were purchased from Jackson Laboratories (West Grove, USA). Double conjugated goat anti-mouse IgG, containing comparable levels of fluorescein and rhodamine, were prepared by mixing the commercial rhodamine-labeled antibodies (3 mg/ml) in 0.2 M carbonate buffer, pH 9.0, and at room temperature, with a 10-fold molar excess of dichlorotriazinyl amino fluorescein (DTAF, Research Organics, Cleveland, USA). The double conjugated molecules were separated from free dye by gel filtration on Sephadex G-50 (Pharmacia, Sweden). The molecules selected for immunolabeling contained an average of 2 moles of each fluorophore per mole of antibodies as determined spectrophotometrically from the 280, 495 and 575 nm absorptions. FITC- and TRITC-conjugated phalloidin were purchased from Molecular Probes (Eugene, Or).

**Immunolabeling of cultured cells**

Cultured chicken lens cells were prepared as previously described (Volk and Geiger, 1984) from 7- to 8-day old embryos and immunofluorescently labeled at 30-50% confluence. Cells were simultaneously permeabilized and fixed (for 5 minutes) in 3% paraformaldehyde solution containing 0.5% Triton X-100 in 50 mM MES buffer, pH 6.0, and then post-fixed for an additional 30 minutes in 3% paraformaldehyde. This procedure was selected to optimize preservation of antigenicity and the structure of focal adhesions.

For immunolabeling, fixed cells on cover slides were inverted on antibody droplets (cells facing down) and incubated at room temperature for 30 minutes. Following extensive wash with phosphate buffered saline (PBS) the cells were incubated for 20 minutes with the secondary, fluorescently conjugated, anti-IgG, at an appropriate dilution, rinsed again and mounted in 90% glycerol in 0.1 M Tris buffer, pH 8.0. Antifade agents were not added to the mounting medium, since they could interfere with RET efficiency. Primary antibody concentrations used in this study ranged between 20 and 100 µg/ml, depending on the antibody. The secondary antibodies were usually used at a concentration of 5 µg/ml. Fluorescent phalloidin was used for labeling at saturating concentrations, as determined directly by titration, followed by measurement in the digital microscopic system.

**Digital microscopic system**

Images were recorded with a Zeiss Axiomat microscope coupled to a cooled CCD camera (CC/CE 200, Photometrics, Tucson, AZ). The computerized microscope system (previously described by Hiraoka et al., 1991; Kam et al., 1993) included fast computer-controlled step motors driving the excitation and emission filter wheels (Compumotor, Petaluma, CA). The filters used here were bandpass interference filters DF type (to minimize cross talk) excitation: 488 (bandwidth=30) nm and 580(25) nm; emission: 535(50) nm and 623(40) nm for fluorescein and rhodamine imaging, respectively, and a matching quadruple-wavelength dichroic mirror (DAPI, TRITC, Texas Red and Cy5; purchased from Omega Co., Brattleboro, VT).
To reduce the bleed-through between fluorescein and rhodamine signals, the filters used for rhodamine imaging were the ones originally recommended for Texas Red recording.

For RET analysis three images were consequently recorded. The first two employed the conventional fluorescein (F) and rhodamine (R) channels and the third used the fluorescein excitation and the rhodamine emission filters (denoted here as X channel). As the images detected through the three filter settings are often different in their brightness, we adjusted the length of exposure times to keep the number of measured photons (and thus the noise) roughly constant. To avoid changes in the profile of light illumination intensity we have used a fiber optics illumination scrambling system (Kam et al., 1993).

Furthermore, for each channel a homogeneous layer of fluorescent solution was imaged as a reference for variations in the illuminated field profile, and all acquired images were pixel-by-pixel normalized, thus eliminating effects of residual inhomogeneities in illumination as well as in the CCD response (Hiraoka et al., 1987).

Despite the high optical quality of the filters we found small lateral shifts between images recorded through the different filters. They were determined using 0.1 μm Nile Red fluorescent beads (L-5198 Fluorospheres, Molecular Probes, Eugene, OR, USA). To correct these shifts, images were aligned using the Simplex algorithm (Amoeba routine taken from Numerical Recipes, Press et al., 1986). Correlations (or r-factor) between two images were maximized following high-pass filtering and Hanning. Displacements were found reproducible within ±0.1 pixel and depended only on the emission filter. For ×100/1.3 NA Axiomat objective (0.047 μm/pixel) the displacements between images recorded through the fluorescein and rhodamine emission filters are:

\[ \Delta x = 2.5 \pm 0.1, \quad \Delta y = -1.7 \pm 0.1 \text{ pixels.} \]

Similar correlation analysis for a focal series at 0.2 μm steps did not point to a measurable shift in focus between fluorescein and rhodamine images.

**Image processing**

Due to partial overlap between the excitation and emission spectra of the donor (fluorescein) and acceptor (rhodamine) fluorophores, the image recorded through the X channel contains, in addition to RET, contributions from the donor and acceptor direct fluorescence. These contributions can be corrected (see Trón et al., 1984) if the fluorescein and rhodamine fluorescence images are measured, and the bleed-through coefficients for the particular filter sets are known. To experimentally determine these coefficients, chicken lens cells were labeled with antibodies with one fluorophore only (fluorescein or rhodamine) and recorded through the F, R and X channels. Following alignment, ratio images were calculated for all pixels above a threshold level setting at about 30% of the maximum image intensity. Average background was evaluated at the excluded area below the threshold, and subtracted. For focal contacts, signal to background ratios were determined using 0.1 mm diameter masks.

**Direct measurement of \( \alpha \)**

A 2 μl sample of labeled antibodies (1.4 μg/ml, 3 fluorophores/antibody molecule) was dried on a slide, the area measured at low magnification (1× objective and ×0.5 magnification factor for the microscope-CCD coupling optics) and the average fluorescence intensities measured in 25 fields within each drop using 20× objective, giving the measured fluorescence intensities per fluorescein and rhodamine dye molecule (I\(_F\), I\(_R\) in the F and R channels, respectively). The ratio of fluorescence intensity between the X and F channels, for fluorescein, and the X and R, for rhodamine-labeled antibodies (R\(_0\) and R\(_3\)) was measured, yielding:

\[ \alpha = \frac{(R_0R_3)}{(R_1R_3)} = 0.24 \pm 0.04. \]

The error is dominated by the variations in fluorescence intensity within the dried drop area.

**RESULTS**

**Microscopic resonance energy transfer (RET) in immunofluorescently double-labeled cells**

To determine the dependence of microscopic RET on the molecular proximity between antibody-bound fluorophores we
have compared RET values as a function of antibody concentrations using 3 distinct immunolabeling procedures.

(1) Labeling of cells with a monoclonal anti-vinculin antibody followed by a secondary antibody which is double-conjugated to both fluorescein and rhodamine. Since both fluorophores are bound to the same molecule, it is expected that most of the RET in the labeled cells will be contributed by intramolecular (rather than inter-molecular) energy transfer, and thus will be largely insensitive to antibody dilution (Fig. 1A).

(2) Labeling of cells with a monoclonal anti-vinculin antibody, followed by a mixture of fluorescein- and rhodamine-labeled anti-mouse IgG antibodies. In such samples only intermolecular interactions contribute to RET, though the labeled secondary antibodies could be associated either with the same primary antibody, or with antibodies bound to different vinculin molecules (Fig. 1B).

(3) Labeling of cells with a mixture of two distinct primary anti-vinculin antibodies (monoclonal, mouse antibody; and a polyclonal, rabbit antibody) followed by fluorescein-labeled anti-mouse IgG and rhodamine-labeled anti-rabbit IgG antibodies (or vice versa). In this experiment RET may occur only between secondary antibody molecules that are bound to different primary antibodies. When the primary antibodies are used at sub-saturation levels it is conceivable that each fluorophore represents a different vinculin molecule (Fig. 1C). Antibody saturation concentrations were established experimentally by serial dilutions. For example, for vinculin the intensity of fluorescence labeling reached a plateau at an antibody concentration of about 30 µg/ml, yielding 4×10^6 counts/µm² per second, corresponding to approximately 4×10^4 fluorophores per cell. This number of fluorophores at saturation per focal contact unit area is comparable to the estimated number of molecules according to Kreis et al. (1982, 1985). To reduce the chances of two antibodies bound to the same antigen molecule (in self-RET experiments) primary antibody concentration used were selected to yield fluorescence levels of 20-30% below saturation (defined here as subsaturation). The saturation is less critical for hetero-RET measurements.

It should be emphasized that RET values, described in this work, are defined as relative energy transfer intensities, not absolute efficiencies, E. RET dependence on secondary antibody dilution is presented in Fig. 1. It is shown that dilution of the double-conjugated antibodies causes an initial decrease in the RET calculated according to Eq. (1) from 28% to 16% (Fig. 1A), probably due to the loss of the intermolecular transfer, and then reaches a plateau, due to RET between donor and acceptor fluorophores present on the same antibody. In contrast, RET values in cells double labeled with antibodies, each conjugated to a different fluorophore (Fig. 1B,C), were highly sensitive to secondary antibody dilution (especially to the dilution of the antibody conjugated to the acceptor-fluorophore). Although this argument is not rigorous, due to the non-additive nature of RET, it is noteworthy that the maximal RET values of about 13% obtained when donor and accep ter fluorophores were conjugated to different secondary antibody molecules (linked either to the same or to different primary antibodies Fig. 1B,C) were comparable to what we interpret as the intermolecular RET (about 16%) obtained with the double-conjugated antibody (see plateau in Fig. 1A). It was thus concluded that RET values indeed represent the local neighborhood of the fluorophore-conjugated antibodies and hence may provide information on the average distances between (or density of) adjacent molecules of the respective antigens.

Molecular organization of vinculin, talin, α-actinin and actin in AJs and along stress fibers: self-RET images

To obtain information concerning the local densities of each of the different junctional molecules (see below) we have labeled cells for each of these proteins using both mouse and rabbit primary antibodies, followed by a mixture of the respective fluorophore-conjugate secondary antibodies. As a rule we have examined the RET values at subsaturating levels of the two primary antibodies, to avoid binding of two different primary antibodies to the same antigen molecule, and saturating levels of the secondary antibodies. Double labeling for actin was
Resonance energy transfer imaging carried out using a mixture of fluorescein- and rhodamine-conjugated phalloidin.

The patterns of double fluorescent labeling and the calculated RET images in pseudo-color for actin, vinculin, talin and α-actinin are shown in Fig. 2 and summarized in Table 1. F-actin, labeling with fluorescein- and rhodamine-labeled phalloidin, was carried out using a mixture of fluorescein- and rhodamine-conjugated phalloidin.

**Fig. 2.** Double labeling of chick lens cells for one of the four proteins: actin, vinculin, talin and α-actinin (self-RET). Actin double labeling was carried out by mixture of fluorescein- and rhodamine-conjugated phalloidin. Immunolabeling for the last three proteins (rows designated, respectively) was carried out with mixture of the mouse and rabbit primary antibodies (see Materials and Methods) followed by fluorescein-labeled goat anti-mouse Ig (column designated F) and rhodamine-labeled goat anti-rabbit Ig (R). The RET images calculated from Eq. (1) in Materials and Methods are shown in the column designated RET. The pseudo color scale is linear in RET values (24% full scale). Bar, 10 µm.
loin (Fig. 2 actin/F and R, respectively), yielded essentially identical and largely uniform patterns both along stress fibers and at their focal contact-associated termini. RET values, on the other hand, were quite variable, ranging from 5 to 15% (Fig. 3). Characteristically, the highest RET values were observed at or close to the termini of actin cables, where association with the junctional plaque occurs. These regions did not, necessarily, coincide with those showing highest phalloin staining. RET between anti-actin antibodies and phalloin was comparable to RET between fluorescein- and rhodamine-labeled phalloin.

Vinculin immunolabeling (Fig 2, vinculin) was most prominent in focal contacts and intercellular AJ, with high RET values of up to 25% (average value was 14%, as shown in Fig. 3). Careful examination indicated that nearly all focal contacts as well as intercellular AJ (not shown), regardless of their size or subcellular location, displayed similar RET values. We did, however, detect non-uniform RET levels within individual focal contacts, manifested, for example, by the fine red streaks (see Fig. 2) which indicate RET values of 20% or higher. These streaks were aligned parallel to the direction of the bundle. It should be mentioned that switching the fluorophores conjugated to the secondary antibodies had no apparent effect on the results of self-RET.

Talin double labeling gave consistently somewhat lower RET values compared to vinculin (average of about 12% and nowhere reaching local values as high as those of vinculin), despite the fact that the overall labeling intensity for talin was comparable or higher. It was also noted that central areas of focal contacts exhibited lower RET (green) compared to the periphery (yellow).

In contrast to actin, vinculin and talin, self-RET values for α-actinin were very low, typically below 1%. Again, the low values could not be attributed to a low labeling intensity, since the immunostaining was similar to that of the other proteins.

Spatial inter-relationships between different junctional proteins

Further experiments were conducted to determine the apparent proximity of pairs of the different AJ-associated molecules (Figs 4, 5). The relationships of vinculin, talin and α-actinin to actin were examined using immunolabeling in conjunction with fluorescent phalloidin. As shown in Fig. 4, and quantitatively by the histograms in Fig. 6, both vinculin and talin displayed low RET values with actin (below 3%). The actin-vinculin values were somewhat higher than those obtained for actin-talin, yet the significance of these differences is not clear. It should be emphasized that these low values were obtained also when the donor and acceptor labeling was switched, despite the extensive overlap between the areas labeled with the two fluorophores, represented by the yellow areas in the superimposed images (Fig. 4, F/R). In contrast, α-actinin-actin RET values were very high when the former molecule was labeled with the donor fluorophore, reaching an average value of about 13%. RET was significantly lower (about 5%) when α-actinin was labeled with the acceptor fluorophore, most likely due to the vast excess of actin in these sites (see Discussion). The highest RET values for these two proteins were locally detected in association with striations along the stress fibers.

The analysis carried out to determine the cross inter-relationships between vinculin, talin and α-actinin is shown in Fig. 5 and, quantitatively, in Fig. 6 (see also Table 1). RET values between these proteins were relatively low, of the order of 5%.

DISCUSSION

There is now growing awareness of the fact that cellular processes occur in a highly specific structural context (for review see Ingber, 1993). Thus the assembly of supramolecular structures such as enzyme-substrate or receptor-ligand complexes, as well as cytoskeletal or karyoskeletal networks, depends on highly specific and accurate subcellular targeting and compartmentalization. Elucidation of cellular events at the molecular level requires, therefore, detailed information not only on the expression and post-translational processing of the
relevant components but also on their subcellular distribution at the highest possible resolution and sensitivity.

The most common approach used for subcellular localization of molecules in, or on, cells is immunofluorescence microscopy, which has a spatial resolution of approximately 0.2 μm, namely, more than one order of magnitude larger than the size of most globular proteins. Thus, information about packing patterns and fine molecular organization is not readily attainable by light microscopy (LM). The alternative experimental approach, namely electron microscopy (EM), has an intrinsic resolution comparable to the size of globular proteins and may be combined with immunochemical labeling for the identification of specific molecules in cells at an approximate resolution of about 200 Å (limited by the size of the antibodies and the gold particles). However, a major limitation of immuno-EM is the low ratio between the electron-dense particles resolved and the number of antigen molecules present. This is usually attributed to EM sample preparation procedures, including strong fixation and ultrathinning sectioning. For example, in our previous studies (Volberg et al., 1986), indirect immunolabeling of smooth muscle for vinculin and talin yielded approximately 500 gold particles per μm² of the dense plaques. Similarly, immuno-EM labeling of wet-cleaved focal contacts (Brands et al., 1990; Feltkamp et al., 1991) yielded maximal values of 400 particle per μm². This value is almost two orders of magnitude lower than the estimated density of vinculin molecules in focal contacts (Kries et al., 1985) and therefore the immunolabeling of EM samples cannot faithfully represent the distribution of vinculin.

Immunofluorescence labeling, on the other hand, appears to be much more efficient, reaching at saturation fluorophore densities comparable to those of vinculin. We therefore propose that the application of RET may yield valuable information on intermolecular proximity and submicroscopic organization of AJ components. As shown, the regions with high RET values do not, necessarily, coincide with those displaying highest staining levels, in line with the claim that RET does not merely report on the average local concentration but playing highest staining levels, in line with the claim that RET values close to the termini of bundles were higher than along the filaments, suggesting that F-actin in these regions may be more tightly packed than along the bundle, resulting in inter-filament RET.

(b) The lengths of the antibody arms, connecting the fluorophore to the antigen, are considerably longer than r₀ and thus may have an over-riding effect on the dependence of RET on the distances between the antigen molecules. For example, indirect immunolabeling may place the fluorophores at a maximal distances of ≈200 Å from the antigen, namely 5-6 times r₀. It should, however, be emphasized that most of the fluorophores are expected to be located closer to the antigen, that there are 2-3 fluorophores bound per secondary antibody and that there might be several labeled antibodies attached to each of the primary antibodies. The configurational possibilities of secondary antibody-conjugated fluorophores can be simulated by donors and acceptors each attached at the ends of two-segment arms with flexible hinges, and anchored at the other ends, again via flexible rotating hinges. RET dependence on the separation between the two anchor points can be estimated by averaging on arm angles. For 100Å arm length, at a separation of d_{0}=90 Å, RET decays to half of its maximal value. Compared to a donor-acceptor pair at distance r₀, RET probability is expected to be enhanced by having multiple fluorophores per primary antibody molecule, but smaller due to possible 3-dimensional distribution, according to: n²(r₀/d_{0})³ ≈ 0.5. The rather small compromise on resolution and RET probability is apparently compensated by a significant increase in flexibility of labeling configuration and labeling intensity. It is conceivable that the use of Fab fragments and of direct labeling (rather than indirect labeling with intact antibodies) might somewhat improve the resolution, though it is still not clear whether the availability of only about one fluorophore per antigen at saturation (rather than several, as described in this study) will yield RET values that will be too low for accurate density evaluations. Ultimately, one may conjugate fluorophores to the molecules of interest and microinject them into cells for RET analysis. This approach has the attractive potential of reporting molecular interactions and probing dynamic molecular processes in vivo. However, in several preliminary attempts, the RET signals obtained, using such approach, were very low. The reason for that is not clear yet, though it could be due either to the dilution of the microinjected labeled molecules in the endogenous unlabeled pool, or to the difficulty, encountered by rigidly bound fluorophores, of approaching each other at the right distance and orientation.

(c) Another major factor affecting RET is the saturation level of antigenic sites, namely the molar ratio between antigen molecules and antibodies bound to them. Oversaturation of primary polyclonal antibodies may result in binding of more then one antibody to the same molecule, which could artificially raise the self-RET values. Under-saturation of labeling may similarly be misleading, since it may erroneously suggest large distances between the respective antigenic molecules. In principle, undersaturation may stem from limited accessibility, partial loss of antigenicity, or the use of insufficient amounts of primary and/or secondary antibodies. While the effect of the
first two variables cannot be readily evaluated, the latter factor can be experimentally controlled. In this study, we have measured the intensity of labeling versus primary antibody concentration, determined the saturation levels and used antibody concentrations below this level. The dilution experiment relates to this issue. It was found

![Fig. 4. Double labeling of chick lens cells for pairs of actin with vinculin, talin or α-actinin. Actin was labeled using fluorescein-conjugated phalloidin in conjunction with monoclonal antibodies to vinculin and talin (first and second columns), or rhodamine-phalloidin in conjunction with monoclonal anti-α-actinin (third column). The former two samples were then labeled with rhodamine-conjugated anti-mouse IgG and the latter with the fluorescein-conjugated secondary antibody (for details, see Materials and Methods). The upper two rows show the fluorescein (F) and rhodamine (R) channels and the third depicts the superimposed images of the two. The bottom row shows the RET image. The pseudo color scale is linear in RET values (24% full scale). Bar, 10 µm.](image-url)
that RET values declined following dilutions of both donor- and acceptor-conjugated antibodies. This is in contrast with homogeneous solutions of small dyes, where RET efficiency reflects the distances between acceptor molecules neighboring each donor fluorophore and is therefore independent of donor concentration (e.g. Herman, 1989). A possible explanation for

**Fig. 5.** Double labeling and RET analysis of chick lens cells for the other three pairs of the four focal adhesion molecules. The antibodies used were as follows (from left to right): talin (left column), and α-actinin (middle column)-specific monoclonal antibodies in conjunction with rabbit anti-vinculin, followed by fluorescein anti-mouse IgG and rhodamine anti-rabbit IgG. The cells shown in the right column were labeled with rabbit anti-α-actinin and monoclonal anti-talin followed by rhodamine anti-mouse IgG and fluorescein anti-rabbit IgG. The upper two rows show the fluorescein (F) and rhodamine (R) channels and the third depicts the superimposed images of the two. The bottom row shows the calculated RET image. The pseudo color scale is linear in RET values (24% full scale). Bar, 10 µm.
low for α-actinin, despite their comparable labeling intensity, is interpreted as indicating the presence of clustered domains of the first three, and dispersion of the latter. Low RET values between all pairs of vinculin, talin and actin imply that focal contacts are composed of segregated clusters of these proteins. The size of such clusters is not known, yet it could be roughly estimated based on the ratio between self-RET and hetero-RET. This ratio reflects the ratio between the size of the domains occupied by each protein, and the extent of the boundary between domains where antibodies against one protein may closely approach antibodies against the second protein. Taking the antibody arm length as 20 nm, and the self to hetero ratio of vinculin and talin as 4, the estimated diameter of vinculin and talin domains is about 80 nm.

The high RET values between α-actinin and actin support the notion that the primary interaction of α-actinin is indeed with actin filaments. An intriguing question is related to the low self-RET values obtained for α-actinin, which is known to be a dimer, and thus may bind pairs of antibodies at close range. One possible explanation is that the binding of one antibody molecule interferes with the binding of the second. However, even if the efficiency of labeling was high enough to allow most or even all dimers to be doubly labeled, and half of them bind a donor and acceptor pair, the expected RET value might still be low compared to RET from many neighboring molecules. Apparently, the dimer-to-dimer spacing in situ is too high to allow for such inter-molecular RET to occur. How do these findings relate to the various structural models proposed for the molecular organization of focal contacts? Based mainly on the binding between isolated junctional molecules, these models predict that the intrinsic transmembrane adhesion molecules, integrins, bind to the microfilament system through a complex chain of molecular interactions. It has been shown that two cytoplasmic components of focal contacts, talin and α-actinin, can bind to the cytoplasmic tail of integrin. Talin might further bind to vinculin, which interacts with α-actinin and through it with actin (Burridge et al., 1988; Geiger and Ginsberg, 1991). Alternatively, α-actinin was shown to be capable of directly interacting with integrin, thus linking it to actin (Turner et al., 1990; Burridge et al., 1988). These linear models are challenged by the present findings, which suggest that the submembrane plaque is dominated by submicroscopic patches, each containing multiple copies of either vinculin or talin. The presence of vinculin clusters is in line with previous studies, which showed that vinculin has a tendency to oligomerize through its C-terminal tail (Milam, 1985; Molony and Burridge, 1985), as well as with the demonstration that binding of vinculin to the plaque is a highly cooperative process (Bendori et al., 1989). It has been argued (Geiger et al., 1990) that cell-substratum adhesion sites are composed of three laminated domains: a transmembrane region with specific adhesion receptors, mostly of the integrin family, a vinculin-containing junctional plaque and an actin-containing cytoskeletal domain. It had been further shown that actin, together with α-actinin, can be dissociated from the plaque by severing proteins such as fragmin, without affecting the organization of vinculin (Avnur et al., 1983), suggesting that the two belong to distinct subcellular domains each with close intermolecular packing (see Bendori et al., 1989). This notion is supported by the present results, which show low RET values between vinculin antibodies and actin-bound phalloidin.

![Fig. 6. Histograms of RET values presenting the distributions in the RET images shown in Figs 4-5 for the six possible pair-labeled samples of the four proteins, α-actinin, talin, vinculin and actin.](image-url)
In summary RET, based on indirect antibody labeling, is not only simple to apply, but also yields measurable fluorescence intensities which reflect submicroscopic patterns of assembly of cellular structural proteins. Future analysis along the lines set in this work will be used to probe differences in the assembly of the various junctional components, as well as their organizational rearrangements during cell spreading, locomotion and growth stimulation.

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REFERENCES


Kubitscheck, U., Kirchheis, M., Schweitzer-Stenner, R., Dreybrodt, W.,


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