A Differential Cytolocalization Assay for Analysis of Macromolecular Assemblies in the Eukaryotic Cytoplasm*

Daniel Blanchard‡§¶, Harald Hutter¶, Jamie Fleenor¶, and Andrew Fire‡¶**

We have developed a differential cytolocalization assay (DCLA) that allows the observation of cytoplasmic protein/protein interactions in vivo. In the DCLA, interactions are visualized as a relocalization of a green fluorescent protein-tagged "prey" by a membrane-bound "bait." This assay was tested and utilized in Caenorhabditis elegans to probe interactions among proteins involved in RNA interference (RNAi) and nonsense-mediated decay (NMD) pathways. Several previously documented interactions were confirmed with DCLA, whereas uniformly negative results were obtained in several controls in which no interaction was expected. Novel interactions were also observed, including the association of SMG-5, a protein required for NMD, to several components of the RNAi pathway. The DCLA can be readily carried out under diverse conditions, allowing a dynamic assessment of protein interactions in vivo. We used this property to test a subset of the RNAi and NMD interactions in animals in which proteins central to each mechanism were mutated; several key associations in each machinery that can occur in vivo in the absence of a functional process were identified. Molecular & Cellular Proteomics 5: 2175–2184, 2006.

The substantial importance of protein interactions has driven the development of numerous technologies to monitor such interactions. These technologies range from the ability to measure binding of two proteins in complete isolation (1) to the capability, using tools such as yeast two-hybrid assay, to determine association within a living cell (for reviews, see Refs. 2 and 3). Each of these assays has provided important insights into the spectrum and details of protein/protein interactions. Despite the broad assortment of assays, work in this field is constrained by the fact that virtually all approaches require some perturbation or manipulation of the physiological situation to allow assessment of interaction. Thus measurement of protein/protein interactions in isolation requires that the two factors be studied in the absence of other cellular components and under solution conditions (buffer, salt, etc.) that must be chosen by the investigator. Likewise standard two-hybrid assays are feasible only in a limited set of intracellular circumstances, in many cases very distinct from those expected to exist at the physiological site of protein interactions. As a promising start to devise assays for protein interactions in the cytoplasm, a number of methods have been developed based on specific assays for protein-protein proximity (e.g. recruitment of RAS or STAT (signal transducers and activators of transcription) signaling factors by chimeric complexes (4, 5)). Assays for reuniting split proteins likewise appear promising (dihydrofolate reductase (6), green fluorescent protein (GFP)1 (7, 8), luciferase (9), β-lactamase (10), etc.). Although each of these assays entails constraints on the geometry of protein/protein interactions that can be observed and on the cell types in which the interaction can be observed, each will certainly have substantial applications.

As an alternative means to study protein/protein interactions in a physiological environment with minimal perturbation, numerous studies have made use of fluorescence resonance energy transfer (FRET) between protein molecules labeled with distinct fluorochromes (for a review, see Ref. 11). FRET assays (in common with two-hybrid approaches) generally require that the proteins of interest be engineered through coupling or fusion and expressed within cells. Other than the expression of foreign proteins, FRET assays do not entail any specific genetic constraint on the host cell types. This provides the distinct advantage of allowing folding and subsequent events to occur in a physiologically appropriate situation. FRET assays still entail challenges; in particular, these assays are most effective in cases where two proteins can be expressed at very high levels (to maximize signal) and associate very closely. Depending on the geometry of interactions and response of cells to overexpression, these constraints can substantially decrease the utility of FRET for

1 The abbreviations used are: GFP, green fluorescent protein; DCLA, differential cytolocalization assay; MTLS, membrane tether localization signal; RNAi, RNA interference; NMD, nonsense-mediated decay; FRET, fluorescence resonance energy transfer; ds, double-stranded.
certain applications (for a review, see Ref. 12).

In our analysis of diverse RNA-based silencing mechanisms that operate in the eukaryotic cytoplasm, we have found a particularly pressing need for sensitive and functionally relevant assays for in vivo interactions between protein assemblies. Many of the key events in RNA-triggered gene silencing can apparently occur outside the nucleus (13, 14). Although biochemical analysis of this pathway has indicated a number of relevant interactions, conflicting data (attributed by some to modest nonspecific interactions of pure proteins) have produced some confusion as to the precise nature of macromolecular assemblies (15–21). The assay system we describe should complement existing assays for protein/protein interactions while being applicable to the wide variety of organisms and cell populations that are manipulable by DNA-mediated transformation.

MATERIALS AND METHODS

Plasmid Constructs

Except as noted, all of the DNA constructs described in this study contain a coding region for a specified chimeric protein inserted downstream of the C. elegans myo-3 promoter in a vector that provides reproducible expression in C. elegans body muscles (22). To express C. elegans proteins we used intron-containing genomic coding regions (from cosmid DNA or amplified by PCR from C. elegans genomic DNA). The gfp coding region used was a standard intron-containing C. elegans vector component (23) that produces a rapidly folding and photostable isoform of the protein (S6SC (24)). In some cases, errors from PCR required correction to produce a functional clone; eventually all segments were confirmed either by complete sequencing or by demonstrating an ability to fully rescue the corresponding genetic mutation.

Derivation and Analysis of Transgenic Lines

Transgenic strains were selected in a pha-1(e2123ts) background in one of two ways. Injection mixtures including plasmid pC1 (pha-1(+)) rescue the temperature sensitivity of the pha-1(e2123ts) strain (25), allowing growth at 23 °C. Mixtures containing plasmid pRF4 (rol-6-su1006gf) (26) confer a rolling phenotype that can be selected at the permissive temperature (16 °C) and scored under either permissive or non-permissive conditions. Crosses between transgenic strains with distinct markers were carried out as follows: pha-1(e2123ts)III him-5(e1467)V males (27) were reared at 16 °C and mated at 23 °C with lines expressing the pha-1 rescue construct. F1 progeny males were then mated to animals from a transgenic strain expressing rol-6-su1006gf (reared at 16 °C and shifted to 23 °C on matting). Rolling F2 progeny that were viable at 23 °C were selected from this cross to ensure that both transgenes were present.

In several experiments, as noted, co-transformation was obtained in a single step by co-injecting two test constructs together with both marker genes (rol-6 and pha-1) into pha-1(e2123ts) animals. In such experiments, it is critical to avoid situations in which homologous recombination between injected plasmids (a common event in transgene array formation in C. elegans (26)) could create a novel chimeric construct in which the membrane anchor signal might become directly fused to the gfp reporter. The “bait” and “prey” constructs used in this work lack homologs that would allow confounding recombination. Experiments in which we co-injected non-interacting bait and prey constructs strongly indicate that confounding recombination events are absent under the conditions of the assay.

Advantages of the co-injection protocol include the use of fewer transgenic lines to look at a small number of interactions (because there is no need to make both bait and prey lines) and the avoidance of genetic crosses. Advantages of two-strain protocols (where distinct bait and prey transgenes are initially established and then combined using genetic crosses) include the ability to compare a single bait or prey transgene with several potential partners without the uncertainty of examining different transgenic lines and the ability (with large datasets) to obtain a matrix of interactions using a set of transgenic lines that increases as a linear (rather than \( n^2 \)) function of the number of interaction candidates. For reference (26), standard protocols for injection and isolation of transgenic C. elegans allow an experienced individual to readily isolate transgenic lines (usually several from each construct) for 20–40 constructs over a 2-week period of injection and screening.

Images

Animals were immobilized in 2.5 mM levamisole (23) and photographed using a Nikon S Flur 100×/1.3 numerical aperture objective. Unless otherwise stated, pictures were obtained using a DAGE-MTI CCD 300 ET-RC digital camera coupled to a Nikon TV lens 0.45× attachment. To complement standard fluorescence microscopy, a subset of interactions were also characterized using confocal microscopy (Nikon E600/PCM-2000).

Relocalization was in many cases very dramatic with the pattern switching between a relatively uniform distribution in cytoplasm and/or nucleus to a tightly membrane-associated distribution. In cases where a relocalization was less obvious, a careful analysis of localization of the GFP-fused prey in the absence of any membrane-tethered bait component was critical. Some factors can demonstrate membrane enrichment without any bait (and should be considered potential membrane-associated components, certainly confounding the assay). In other cases, a moderate nuclear or cytoplasmic preference may generate a discontinuity at the nuclear boundary that is just the result of a somewhat lower fluorescence level in the adjacent compartment. Careful analysis easily allows a distinction between such a discontinuity and true perinuclear enrichment (evidenced by greater levels at the nuclear/cytoplasmic boundary than in either individual compartment).

Rescue Assays

Rescue assays for MTLS and GFP constructs were performed as follows.

\( \text{mtf-1, rde-1, and rde-4:} \) The corresponding mutant strain (mtf-1(pk1417)), rde-1(ne300) or, rde-4(e2939)) was injected with a mixture of the following plasmids: (a) the coding region of interest driven from the myo-3 promoter, (b) a construct driving the sense strand of unc-22 (28) from the myo-3 promoter, and (c) a construct driving the antisense strand of unc-22 driven by the myo-3 promoter. RNAi-competent transgenic animals carrying the myo-3:unc-22 sense + antisense mixture twitch vigorously due to production of unc-22 dsRNA (29).

\( \text{dcr-1:} \) dcr-1 mutant animals are sterile, therefore an alternative measure of activity was needed. dcr-1 rescue was tested in two ways; dcr-1(ok247) mutants carrying an array with gfp::dcr-1 were first observed for viability and then assayed for RNAi with unc-22 by direct injection of dsRNA. The broadly expressed let-858 promoter (30) was used to achieve ubiquitous expression and hence viability.

\( \text{ego-1:} \) ego-1 function was tested by assaying complementation of mutations in the related mtf-1 gene; this was done by expressing the ego-1 coding region in body wall muscle (using the myo-3 promoter) in an mtf-1 mutant background (31, 32) and testing for rescued RNAi. smg-2, smg-5, and smg-7. These components, which are involved
We use the term bait to describe a second molecule of interest tethered to internal cytosolic membrane surfaces by some type of defined localization signal. A number of preliminary studies in which alternative intracellular tethers were tested for suitability in such an assay (data not shown) led us to choose the chimeric N-terminal membrane tether localization signal (MTLS) described in Fig. 1D. The signal sequence and transmembrane portion of the MTLS are derived from disparate regions of the C. elegans membrane-spanning protein PAT-3 (38). Based on the sequence of the MTLS, we expected a final protein product with a highly charged luminal domain of ~25 amino acids followed by a hydrophobic transmembrane tether (27 amino acids), an 11–18-amino acid cytosolic linker, and the protein sequence of interest.

To study the behavior of the prey and bait tethered factors in C. elegans, we started with a number of well characterized C. elegans promoters (39). The myo-3 promoter (which is an endogenous myosin promoter that is expressed strongly in body wall muscle cells of transgenic animals (39)) has been used for the majority of our analysis to date. This promoter is particularly useful in that body wall muscles are numerous, easily viewed in live animals, and capable of suffering considerable derangement without compromising organismal viability. Both nonsense-mediated decay and RNAi are highly active in body wall muscles, hence we can use these cells to assay function in a physiological environment.

For this work, we analyzed interactions within and between cellular machineries for two cytosolic processes. Nonsense-mediated decay is an RNA surveillance mechanism that leads to the preferential degradation of mRNAs with a premature termination codon or “aberrant” 3′-untranslated terminal regions (for a review, see Ref. 40). RNAi is a mechanism whereby mRNAs are selectively degraded in the presence of cognate dsRNA in a sequence-specific manner (41). Although RNAi and NMD seem to cause the degradation of mRNA through independent pathways, some studies have suggested a genetic connection between the two pathways (42). RNAi appears to be functional both in the nucleus and cytoplasm (13, 14). Although the situation with NMD is somewhat less clear, portions of this process are likewise thought to involve both nucleus and cytoplasm (for reviews, see Refs. 43 and 44).

As a first step toward detecting interactions, we obtained one or more lines of C. elegans expressing each of the GFP-tagged prey constructs (Figs. 1A, 2A, and 3). Observation of the GFP patterns of these transgenic animals gives a broad idea of the subcellular distribution of each of the proteins to be analyzed and is a prerequisite (and control) for the localization-dependent detection of in vivo interactions. As expected, we observed a mixture of nuclear and cytoplasmic localization for RNAi components (e.g. Fig. 2, A and C; data not shown). Mixed nuclear/cytosolic distributions were also obtained for three NMD-related components (SMG-2, SMG-5, and SMG-7; Fig. 3 and data not shown). The diffuse cytosolic patterns appeared to be independent of the quantity...
of expression for the GFP fusion; in particular the distributions maintained a uniform character in individual cells and individual transgenic lines with widely divergent expression levels (data not shown).

The second step in the procedure entails generating transgenic *C. elegans* lines each expressing one of the bait constructs (Fig. 1B). The bait plasmids are generally co-injected with a marker plasmid that produces a dominant “rolling” phenotype (26) that can be scored independently of the *pha-1* marker used to make the prey lines.

To carry out assays for interaction, we mated prey and bait lines to obtain transgenic animals expressing both constructs driven in a single tissue. Alternatively, prey and bait constructs may be co-injected to generate a double transgenic animal shortening the assay to one step (see “Materials and Methods”). If the fusion proteins interact, the ubiquitous distribution of the GFP fusion will change to reflect the membrane-bound localization of the MTLS-fused protein (Fig. 1C). We stress that the precise partitioning of each individual construct between different intracellular membrane surfaces is likely to reflect details of protein structure and interactions on the cytoplasmic face. Positive results (a dramatic change in localization of the GFP-tagged prey protein in the presence of the membrane-localized bait) are clear indicators of potentially significant interaction regardless of the precise details of the membrane partitioning. Negative results, as always, require more careful interpretation and will be discussed in greater detail below.

As a first test for the procedure, we assayed interaction between two proteins whose association has been well documented. *rde-4* and *dcr-1* are two components of the RNAi machinery that have been shown to associate in vivo (45). Transgenic animals expressing a *gfp::rde-4* construct showed the uniform nucleus + cytoplasm GFP distribution described above (Fig. 2A), whereas co-expression with a membrane-tethered version of *dcr-1* dramatically altered the GFP::RDE-4 distribution to a membrane-based pattern (Fig. 2B). This relocalization indicated a strongly positive interaction. In the resulting double transgenic strain, most of the GFP fluorescence was concentrated in a perinuclear halo. This type of localization was frequently observed in our analyses of positive protein/protein interactions (Figs. 2 and 3 and Supplemental Figs. S2 and S3); we assume that the perinuclear appearance represents a preponderance of early exocytic apparatus (endoplasmic reticulum or Golgi) in the vicinity of the muscle nucleus. When the membrane tether and *gfp* were reversed (*gfp::dcr-1*/MTLS::*rde-4*), we observed a comparable interaction as evidenced by dramatic relocation of the *gfp::dcr-1* fusion in the presence of the MTLS::*rde-4* fusion (Fig. 2, C and D).
little data overlap. As controls for false positive results, several factors with no expected ability to interact failed to show any interaction in the assay. In particular, neither MTLS-tagged RDE-4 nor MTLS-tagged SMG-5 showed an interaction with a pair of GFP-tagged ribosomal proteins (Fig. 4). Native GFP also failed to interact with each of the MTLS-tethered proteins used in the assay (Fig. 4 and Supplemental Fig. S1).

Among the novel interactions observed, it is noteworthy that SMG-5 readily interacts with several proteins central to the RNAi pathway (Figs. 3 and 4) suggesting a functional connection between the latter mechanism and NMD. As can be seen in Fig. 3, the degree of relocation of GFP::SMG-5 (as assessed by GFP enrichment at intracellular membranes and depletion of GFP from the remainder of the cytosol) varied depending on the MTLS fusion with which it was co-expressed. We used a qualitative scale to reflect such variations (Fig. 4).

For the majority of transgene pairs with a positive interaction, GFP relocation was comparable in most of the observed body wall muscles (e.g. Supplemental Figs. S2, A–C, and S2E). Some exceptions to this uniformity, where only a fraction of muscle cells shows relocation in a particular animal (e.g. Supplemental Fig. S2D and data not shown), were found with weakly expressing constructs (assessed by GFP fusion levels). These constructs could conceivably exhibit non-uniformity due to transgene mosaic expression (39).

Some indication of the specificity of the assay is provided by a reciprocity test: conceptually one would expect the majority of interactions to be retained when the identities of bait and prey are switched. We observed this to be the general case for the interactions we analyzed. We have not further investigated the rare exceptions (ego-1/rde-1, rrf-1/smgs-5, and ego-1/smg-5), although we note that these proteins are among the more weakly expressed from transgene constructs tested.

As with any assay involving chimeric proteins, a major concern is that the chimeric protein may lose interactions or functions that are characteristic of the original (non-tagged) factor. To address this issue we tested the biological activity of GFP and MTLS fusion constructs by determining their ability to rescue the null mutations for the corresponding endogenous genes. As noted in Table I, complete rescue of the loss-of-function phenotype was observed for each of the nine GFP constructs tested. The MTLS might be expected to alter activity more severely, but we note that the MTLS-containing constructs assayed were sufficient to rescue wild type function. The exceptions were the MTLS::rde-4 fusion (Table I), which appears to act as a dominant negative (data not shown), and MTLS::smg-2. It should be noted that the observed rescue could result from a small amount of protein that is produced lacking the membrane tether (or where the membrane tether

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![Table: Membrane Tether Localization Signal (MTLS)](image)

| GFP     | rde-1 | rde-4 | smg-5 | smg-7 | dcr-1 | rrf-1 | ego-1 |
|---------|-------|-------|-------|-------|-------|-------|-------|
| rde-1   | ++/−  | ++++  | −/−   | −/−   | ++/−  | +/−   | −/−   |
| rde-4   | ++++  | ++++  | ++/−  | −/−   | ++++  | ++++  | −/−   |
| smg-5   | ++    | ++++  | ++++  | ++++  | +/−   | +/−   | ++/−  |
| smg-7   | −/−   | −/−   | −/−   | −/−   | −/−   | −/−   | −/−   |
| dcr-1   | −/+++ | −/−   | −/−   | −/−   | −/−   | −/−   | −/−   |
| rrf-1   | −/−   | ++/−  | +/−   | +/−   | −/−   | +/−   | ++/−  |
| ego-1   | ++    | ++++  | ++++  | ++++  | +/−   | +/−   | ++/−  |
| smg-2   | N/D   | −/−   | −/−   | N/D   | N/D   | N/D   | N/D   |
| S9 Ribosomal | N/D | −/− | −/− | N/D | N/D | N/D | N/D |
| L1 Ribosomal | N/D | −/− | −/− | N/D | N/D | N/D | N/D |

Fig. 4. DCLA interaction matrix. Several factors involved in RNAi and NMD were expressed as fusions to GFP or to the MTLS and assayed for interactions as described in the text. For each combination of components, we analyzed at least eight doubly transgenic animals. In all cases where no interaction is reported in the figure, we observed no GFP relocation in any of these animals. Cases in which positive interactions were observed produced clear evidence of GFP relocation in a majority of cells of each observed animal. For several cases (denoted with a filled square in the lower right quadrant of the data entry (■)), we confirmed the interaction or lack thereof using an independent doubly transgenic strain. Although somewhat arbitrary, the qualitative assessments of interactions in the figure provide a rough measure of the level of interaction in the transgenic strains used for this analysis. ++++, complete or near complete interaction. All or almost all visible GFP signal relocalized to membrane sites. ++ and ++++, strong but incomplete interaction. Visible GFP predominantly relocalized to membrane sites with some GFP remaining cytosolic. +, weak interaction. All observable amounts of GFP relocate to membrane sites, the majority remains cytosolic. −, no interaction. No change in GFP pattern. +/-, variable interaction. Some cells present clear GFP relocalization, whereas others show no evidence of relocalization. N/D, not done.

To assess the specificity and generality of the cytolocalization assay, we extended our test to a larger family of proteins. Each coding region was incorporated into separate expression vectors tethered to GFP and to the MTLS. Production of two sets of transgenic lines (each protein as bait and prey) then allowed the assembly of a matrix of potential interactions by carrying out the appropriate genetic crosses (Fig. 4).

As this work was being completed, different groups used co-immunoprecipitation and mass spectrometry to identify interactions between a number of the proteins under study (RDE-1/RDE-4, DCR-1/RDE-4 (45, 46), RDE-1/DCR-1 weak interaction (46), and SMG-5/SMG-7 (47)). As can be seen in Fig. 4, the DCLA was effective in detecting three of these four interactions. The RDE-1/DCR-1 interaction that was not detected using DCLA is of some interest: this was a relatively weak interaction in the original immunoprecipitation study (1% peptide coverage as compared with 35% coverage for the RDE-4/DCR-1 interaction). In contrast, a comparison between our interaction results and those obtained in a high throughput genome wide yeast two-hybrid assay (48) showed
is severed). Significantly, however, the relocalization of many of the GFP constructs in our interaction assays was visually complete, indicating that untethered protein (although certainly possible) is likely to be present at low concentration.

One of the most useful aspects of the cytolocalization assay is the ability to monitor protein/protein interactions under different conditions. For the pathways we analyzed, the most robust manipulations involved the use of genetic ablations, which alter specific aspects of the response. We thus assayed a subset of interactions in animals with putative null mutations in genes encoding proteins required for RNAi or NMD. If a protein bridges the interaction between two peptides or if it acts as a scaffold for the structural integrity of a multiprotein complex, then its removal will cause the complexes or individual interactions to dissociate. As can be seen in Table II, none of the assayed mutations caused a change in the interactions tested. These experiments define examples in which complex formation in RNAi and NMD and between both pathways is independent of the ablated protein components and of the functionality of the pathway.

### Table I

| Construct | Rescue assay | Result |
|-----------|--------------|--------|
| myo-3::gfp::rde-4 | unc-22 RNAi | Full rescue |
| myo-3::gfp::rde-1 | unc-22 RNAi | Full rescue |
| myo-3::gfp::rfl | unc-22 RNAi | Full rescue |
| let-858::gfp::ego-1 | Viability | Full rescue |
| myo-3::gfp::smg-5 | unc-54 (c293) suppression | Full rescue |
| myo-3::gfp::smg-7 | unc-54 (c293) suppression | Full rescue |
| myo-3::MTLS::smg-2 | unc-54 (c293) suppression | Full rescue |
| myo-3::MTLS::rde-4 | unc-22 RNAi rescue | No rescue |
| myo-3::MTLS::rde-1 | unc-22 RNAi rescue | Full rescue |
| myo-3::MTLS::rfl | unc-22 RNAi rescue | Full rescue |
| myo-3::MTLS::ego-1 | unc-22 RNAi rescue | Full rescue |

### Table II

| Pairs of interactors | Interactions in WT (Table I) | Tested backgrounds | Interactions in mutant background |
|----------------------|-----------------------------|-------------------|----------------------------------|
| gfp::rde-1-MTLS::rde-4 | +++++ | rf-1 | +++++ |
| gfp::rfl-MTLS::rde-4 | ++ | rde-1 | ++ |
| gfp::smg-5-MTLS::smg-7 | ++ | smg-2 | ++ |
| gfp::rde-1-MTLS::smg-5 | ++++ | rde-4 | ++++ |
| gfp::rde-1-MTLS::smg-5 | ++++ | rf-1 | ++++ |
| gfp::rde-1-MTLS::smg-7 | ++ | rde-1 | ++ |
| gfp::rde-4-MTLS::smg-5 | ++++ | rf-1 | ++++ |
| gfp::rde-4-MTLS::smg-5 | ++++ | rde-1 | ++++ |
| gfp::rde-4-MTLS::smg-7 | ++ | rde-1 | ++ |
| gfp::smg-2-MTLS::rde-1 | ND | smg-7 | ND |
| gfp::smg-2-MTLS::rfl | ND | smg-7 | ND |

*To ensure that the co-injection protocol used in these assays was equivalent to the original double transgenic protocol (see 'Materials and Methods'), two pairs of MTLS- and GFP-fused constructs that failed to interact in our previous results (Fig. 3) were co-injected into wild type animals. These two combinations also failed to interact in the co-injection assay as shown in this table.
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we expect that the assay will yield a proportion of false positives if scaled up to examine larger numbers of interactions, these preliminary data indicate that such situations may be relatively rare. False negatives in the assay are also possible, and perhaps more likely, with further work required to determine a relevant “risk factor.” Based on these concerns, we suggest that DCLA be used only as a guide. Just as with two-hybrid assays, it will be essential to obtain corroborating data before committing resources based on assuming the validity of any given interaction (49).

In addition to the overall potential for false positives and negatives in DCLA tests, we note several other limitations of the assay. First, we cannot distinguish between direct protein/protein interactions or those bridged by other peptides. Any two proteins that are frequently associated in the cell as part of a larger complex will be scored as interacting.

Second, the assay as a physiological test is limited to proteins that normally exist as part of free-floating (i.e. mobile) complexes in the cytoplasm. This rules out both interactions that occur in other organelles and interactions involving proteins that normally reside at the membrane. DCLA is based on sequestering proteins to the cytoplasmic faces of cellular membranes. Such relocation might render some of the chimeric peptides non-functional (as observed for MTLS::rde-4) or cause deleterious effects to the cell. Furthermore, DCLA analysis of interactions that normally occur outside the cytoplasm may generate false negatives due to lack of cofactors and overall difference in surrounding environment. Because the assay relies on transgenesis or transfection, we expected some variability in quantity of the individual proteins. For the C. elegans expression system used in this work we indeed observed significant expression level differences as assessed by substantial variability in GFP intensity in individual cells for each transgenic line (data not shown; also see Fig. 2A). Although MTLS-tethered protein levels were not assessed, one might expect expression level variations similar to those observed with GFP-tagged proteins. Several types of experiments can serve to increase confidence in the functionality of the MTLS-fused bait, including the ability to rescue the corresponding null mutation for biological function (which we observed for all but two cases tested; Table I) and the ability to relocalize at least one interactor protein (which we were able to observe for all seven tested baits; Fig. 4). As an additional means to examine MTLS chimeras more directly, one test is to insert GFP into the corresponding fusion construct and examine expression in singly transgenic animals (e.g. Supplemental Fig. S4). Although the MTLS::GFP::bait expression assay (or immunofluorescence with bait-specific antibodies) is rather straightforward, it is generally of limited value in the absence of functional information (rescue or at least one interaction with a given bait); hence an inactive bait could be misfolded or otherwise inaccessible.

Concerns also arise at the higher end of the expression level spectrum. Certainly interactions may be sensitive to concentrations of the interacting partners and, by inference, to the expression levels of the bait and prey constructs in the assay. From this perspective, the use of a transgenesis/transfection system can provide both concerns and opportunities. For C. elegans, as with many expression systems, we observed considerable variation in expression levels from cell to cell (and to some extent from animal to animal) even with the use of a single well defined promoter. A robust interaction that occurs in all tested cells ranging in expression level from highly intense to barely visible is certainly most straightforward to interpret. In other cases, a cell-to-cell variability in the interaction may be observed, indicating that the efficiency of the interaction is controlled either by physiological properties of individual cells or by expression levels. Despite the uncertainties inherent in this (or any) transformation-based assays, we observed robust interactions over a wide natural expression range for the majority of interacting pairs of proteins that were examined (Figs. 2 and 4, Supplemental Fig. S2, and data not shown).

Many different parameters will influence the observed modifications in GFP distribution. As a first approximation, the fraction of membrane-associated prey (GFP) chimera will be determined by the steady state occupancy of the corresponding factor in complexes containing the bait (MTLS) protein. Entering into this calculation are the “on” and “off” rates in forming a relevant complex, the accumulation levels of the two proteins, the degree to which the transmembrane domain perturbs the assaysed interaction, the degree to which the prey protein has access in the cytosol to membrane-tethered bait, and the potential in vivo for alternative interactions of either chimera.

Flexibility in the DCLA—In principal, any combination of subcellular localization tag, visual reporter, and host cell type might be used to develop a similar cytolocalization assay. We note, however, several significant constraints on this flexibility.

To be useful, the localization tag must allow the bait protein to access the pool of visually tagged partner protein. For a cytosolic interaction, this entails keeping the bulk of the bait in contact with the cytosol. Because all but the strongest and most stable interactions presumably require that this contact be continuous, we were not surprised to find in our preliminary work that alternative localization signals where the bulk of the bait protein was taken out of the cytosolic pool (e.g. by transport to the nucleus, inner mitochondrion, or extracellular space) were not effective in this assay.

Although tethering of specific cellular components to the cytosolic faces of cellular membranes had the theoretical capability of preserving the ability to capture partners, we were still somewhat surprised by the efficiency with which the MTLS described in this work was capable of such capture. These data combined with the function of many of the MTLS-tethered constructs in rescue assays indicate that diverse cellular functions may be relatively indifferent to a defined
subcytoplasmic localization. There will certainly be limits on the flexibility of the system, and we note that at least two of the components that we describe lose their normal activity (and MTLS::rde-4 gains a counteracting activity) upon tethering.

The specific MTLS tether sequence used in this work caused no obvious deleterious effects and provided a strong and dramatic protein relocalization within the cell. We note that early attempts to use an alternative membrane anchor sequence (the C-terminal 15 amino acids of LET-60ras) with GFP as a "neutral" fusion partner led to axon misguidance (from expression in neuronal precursors) and movement defects (from expression in muscle precursors). Certainly it will be useful in the future to have additional functional tethers.

It should be noted that different cell types will have distinct patterns of cytoplasm/membrane interface and that different tethered proteins complexes will have distinct partitioning patterns even within a single cell type. Consistent with the latter expectation, we observed some variability in localization patterns of membrane-tethered complexes in C. elegans muscle. Thus certain complexes concentrate near the sites of presumed synthesis at the periphery of the cell nucleus, whereas others show distinct distributions among the membranous areas within cells (Figs. 2 and 4, Supplemental Figs. S2 and S3, and data not shown). This variation provides both opportunities and complications in the assay. The opportunities derive from the possibility that MTLS-tethered proteins may sample the cytoplasmic surfaces of various membrane compartments within cells until they locate (and are perhaps restricted in their mobility by) an appropriate protein complex. The challenges arising from this feature will be evident as automated routines are developed for high throughput screens and quantitation: although an experienced cell biologist can readily detect relocalization within a cell, developing quantitative means to routinely detect this may require some creativity and care.

Although C. elegans striated muscle provides a cytologically and genetically appropriate system in which to use DCLA, it would certainly be valuable to be able to use the assays in other cell types and with an arbitrary promoter. We note that the analysis is not specific to the myo-3 promoter but is most reliably carried out with promoters that (like myo-3) yield reproducible expression in a tissue of interest in transgenic lines. We were readily able to extend these assays to a neuronal cell type in C. elegans (using the endogenous mec-7 promoter, which functions in touch neurons (50)) with the only added challenge being a somewhat smaller distance of relocalization due to the small size of the cell (Supplemental Fig. S3). Not every tissue is appropriate for this procedure. In C. elegans, we failed to detect relocalization when the relevant protein chimeras were expressed in the gut (data not shown). The failure to relocalize could reflect various characteristics of gut tissue. One possible explanation is that the extensive distribution of large and small membranous organelles within the cytoplasm of that tissue may generate a uniform optical pattern within cells even for a membrane-bound reporter. Based on these data, we expect that DCLA and related assays might be most effective in relatively large cells in which membranous networks show sufficient spatial distinctiveness to allow perception of differential localization. Alternatively one might expect that different DCLA signals (perhaps limited to specific cellular compartments) may facilitate analysis in particularly challenging cell types. Finally we note that DCLA may, in theory, be useful to study context-dependant interactions in a real time manner.

Comparison with Other Protein Binding Assays—To better illustrate the properties of DCLA and therefore its potential usefulness, we offer the following comparison with some existing techniques.

FRET is certainly useful for measuring interactions where abundant fluorescent probes are within 70 Å of each other (12, 51). DCLA could complement FRET when looking at groups of interactions as some of the molecular constructs and control experiments (e.g. rescue of the relevant null mutations) are common between the two approaches. DCLA would add to FRET data in providing information on interactions at lower (and potentially physiological) protein levels and in detecting interactions that occur at a greater distance or those bridged by other peptides.

Biochemical peptide purification and interaction assays provide valuable biophysical data on specific interactions with the utilization of highly purified proteins providing one of the most convincing means of determining whether an interaction is direct. Moreover the combination of biochemistry with detection methods such as mass spectrometry provides a powerful tool to identify known and novel interactions. Almost by definition, such biochemical assays are performed under non-physiological conditions. Thus, there is ample opportunity to overlook interactions that are context-dependant or that require a specific cellular environment. Biochemical assays with pure proteins can also produce “false positive” results when two proteins that do not normally interact are mixed (potentially at higher than physiological concentration or in the absence of other proteins with competing binding specificity). DCLA provides a rapid in vivo corroboration for interactions that are identified biochemically: the possibility of testing such interactions in vivo (and under different conditions) should support the ability to focus on biologically significant interactions.

Yeast two-hybrid assays are similar to DCLA in that they utilize two sets of chimeric proteins: a bait fused to a DNA binding domain and a prey fused to a transcriptional activator domain (52). The yeast two-hybrid method is particularly well suited for screening large libraries of proteins and therefore has been utilized in numerous applications (48, 53–55). Along with the many advantages of the assay, there are a number of

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2 H. Hutter, unpublished data.
idiosyncrasies. The classic yeast two-hybrid assay is carried out in the nucleus of Saccharomyces cerevisiae. If the assayed proteins are native to organisms other than yeast, real interactions might be missed by the lack of cofactors, or alternatively spurious interactions with yeast proteins can lead to false positives (49). To address these caveats several two-hybrid assays have been successfully performed in mammalian systems (56–58). DCLAs should provide a valuable alternative and extension in many situations where classic two-hybrid assays are not feasible.

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