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Virus-derived Platforms for Visualizing Protein Associations inside Cells*

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Protein-protein associations are vital to cellular functions. Here we describe a helpful new method to demonstrate protein-protein associations inside cells based on the capacity of orthoreovirus protein μNS to form large cytoplasmic inclusions, easily visualized by light microscopy, and to recruit other proteins to these structures in a specific manner. We introduce this technology by the identification of a sixth orthoreovirus protein, RNA-dependent RNA polymerase L3, that was recruited to the structures through an association with μNS. We then established the broader utility of this technology by using a truncated, fluorescently tagged form of μNS as a fusion platform to present the mammalian tumor suppressor p53, which strongly recruited its known interactor simian virus 40 large T antigen to the μNS-derived structures. In both examples, we further localized a region of the recruited protein that is key to its recruitment. Using either endogenous p53 or a second fluorescently tagged fusion of p53 with the rotavirus NSP5 protein, we demonstrated p53 oligomerization as well as p53 association with another of its cellular interaction partners, the CREB-binding proteins, within the inclusions. Furthermore, using the p53-fused fluorescent μNS platform in conjunction with three-color microscopy, we identified a ternary complex comprising p53, simian virus 40 large T antigen, and retinoblastoma protein. The new method is technically simple, uses commonly available resources, and is adaptable to high throughput formats. *Molecular & Cellular Proteomics* 6:1027–1038, 2007.

Protein-protein associations are vital to cellular functions. Numerous methods are available for addressing whether proteins associate either directly ("interaction") or indirectly (through bridging molecules), but many of these methods can be technically demanding, can be limited by the strength of the association, or cannot resolve whether the association indeed occurs inside cells. In addition, many current objectives and strategies demand methods that are simple and adaptable to high throughput formats. In this report, we describe a new such method that is based on an unusual property of the orthoreovirus-encoded μNS protein.

Double-stranded RNA viruses of the taxonomic family Reoviridae (e.g. orbiviruses, orthoreoviruses, and rotaviruses) form characteristically shaped, non-membrane-bound inclusions in the cytoplasm of infected cells. These structures are variously called viral inclusion bodies (orbiviruses) (1), viral factories (orthoreoviruses) (2, 3), or viroplasms (rotaviruses) (4) and are thought to be the sites of viral RNA replication and packaging into assembly intermediates that can later mature into infectious virions. The viral factories of some orthoreoviruses (such as certain isolates of strain Type 3 Dearing (T3D))

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1 The abbreviations used are: T3D, Type 3 Dearing; FLS, factory-like structure(s); VLS, viroplasm-like structure(s); EGFP, enhanced green fluorescent protein; RdRp, RNA-dependent RNA polymerase; SV40, simian virus 40; TAg, large T antigen; CBP/p300, CREB-binding proteins; pRb, retinoblastoma protein; T1L, Type 1 Lang; mAb, monoclonal antibody; HA, hemagglutinin; mRFP, monomeric red fluorescent protein; CREB, cAMP-response element-binding protein; HRP, horseradish peroxidase.
were our laboratory stocks, originally obtained from Dr. B. N. Fields. Rabbit polyclonal antiserum and puriﬁed polyclonal antibodies specific for μNS or λ3 have been described previously (18, 19). Monoclonal antibody (mAb) HA.11 speciﬁc for inﬂuenza virus hemagglutinin (HA) epitope was obtained from Covance Research Products, and rabbit polyclonal antibody speciﬁc for the HA epitope was obtained from Zymed Laboratories Inc. mAb DO-1 speciﬁc for p53 (Santa Cruz Biotechnology) was a generous gift from Dr. D. M. Knipe (Harvard Medical School). mAb Pab101 speciﬁc for SV40 TAg was obtained from Santa Cruz Biotechnology. pRb-speciﬁc Ab-4 (Clone XZ104) and CBP/p300-speciﬁc Ab-1 (Clone NM11) mAbs were obtained from Lab Vision. mAb G99-549 against underphosphorylated pRb was purchased from BD Biosciences. The following secondary antibodies were used as appropriate for different experiments: Alexa 488-, Alexa 594-, or Alexa 350-conjugated goat anti-mouse or anti-rabbit IgG (Molecular Probes) and horseradish peroxidase (HRP)-conjugated donkey anti-mouse or anti-rabbit IgG (Pierce). For microscopy, antibodies were titrated to optimize signal-to-noise ratios. All restriction enzymes were obtained from New England Biolabs.

2 C. L. Miller and M. L. Nibert, unpublished data.

### EXPERIMENTAL PROCEDURES

**Cells, Viruses, Antibodies, and Other Reagents—CV-1 and COS-7 cells were maintained in Dulbecco’s modiﬁed Eagle’s medium (In-vitro) containing 10% fetal bovine serum (HyClone) and 10 μg/ml gentamicin (Invitrogen). Reovirus strains Type 1 Lang (T1L) and T3D...**
infections or transfection in 6-well plates (9.6 cm²/well) containing complete p53 coding sequence after which the PCR product and sequences of all portions of plasmids amplified by PCR were determined. For transfections, a total of 4 × 10⁵ CV-1 cells were seeded the day before infection or transfection studies, 1.5 × 10⁵ CV-1 cells were seeded the day before infection or transfection in 6-well plates (9.6 cm²/well) containing 18-mm round glass coverslips. For immunoprecipitation studies, 3 × 10⁵ cells were seeded onto 60-mm dishes the day before infection or transfection.

Immunostaining and Microscopy—The following steps were performed at room temperature. Infected or transfected cells were fixed for 10 min with 2% paraformaldehyde in PBS. Fixed cells were washed three times with PBS and permeabilized for 5 min with 0.2% Triton X-100 in PBS. Cells were again washed three times with PBS and blocked for 5 min with 1% bovine serum albumin in PBS. Primary and secondary antibodies were diluted in 1% bovine serum albumin in PBS. After blocking, cells were incubated for 1 h with primary antibodies, washed three times with PBS, and then incubated for 1 h with secondary antibodies. Immunostained cells were washed a final three times with PBS, incubated for 5 min in 300 nM 4',6-diamidino-2-phenylindole (Molecular Probes), and mounted on slides with ProLong reagent (Molecular Probes). Immunostained samples were examined using an Optiphot-2 epifluorescence upright microscope (Nikon) or an Axiosvert 200 inverted fluorescence microscope (Zeiss). Images were collected digitally either using a Photometrics CoolSnap™ camera (Roper Scientific) and MetaVue imaging software (Molecular Devices) or an AxioCam MR Color camera and Axiovision software (Molecular Probes) and MetaVue imaging software (Molecular Probes) and MetaVue imaging software. Images were prepared for presentation using Photoshop and Illustrator (Adobe Systems).

Immunoprecipitation and Immunoblotting—Infected or transfected cells were lysed by incubation for 30 min on ice in non-denaturing lysis buffer (20 mM Tris (pH 8.0), 137 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40) containing protease inhibitors (Roche Applied Science). The protein concentration of each lysate was measured by Bradford assay (Bio-Rad) and normalized for relative protein concentration within each sample. Immunoprecipitating antibodies that had been incubated for 2 h with protein A-conjugated magnetic beads (Dynal Biotech) and washed six times with Raf buffer were added to the cell lysates, which were then rotated overnight at 4 °C. Immunoprecipitated proteins were washed four times with Raf buffer and resuspended in sample buffer (125 mM Tris (pH 6.8), 10% (v/v) sucrose, 1% (w/v) sodium dodecyl sulfate, 0.02% (v/v) β-mercaptoethanol, 0.01% bromphenol blue). Samples were then boiled for 3 min and separated on denaturing 10% (w/v) polyacrylamide gels containing 0.01% (w/v) sodium dodecyl sulfate. Proteins were transferred from the gels to pieces of nitrocellulose by electroblotting in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3). Nitrocellulose was then blocked by incubation with 5% milk in Tris-buffered saline (20 mM Tris (pH 7.6), 137 mM NaCl) containing 0.5% Tween 20 (Sigma) (TBS-T) for 30 min at room temperature. Primary antibodies were allowed to bind during an overnight incubation at room temperature in TBS-T containing 1% milk. HRP-conjugated secondary antibodies were allowed to bind during a 2-h incubation at room temperature in TBS-T containing 1% milk. Before and after incubation with secondary antibodies, the nitrocellulose was washed three times with TBS-T. Binding of HRP conjugates was detected by incubation with chemiluminescence substrate (Bio-Rad) according to the manufacturer’s recommendation followed by exposure to film (Fuji).

RESULTS

Orthoreovirus Protein λ3 Associates with FLS—The orthoreovirus RdRp λ3 has been reported to localize to viral factories in infected cells (21). In this study, although we did not have appropriate λ3-specific antibodies for directly immunostaining this protein, we found that plasmid-encoded λ3 tagged C-terminally with influenza virus HA epitope (λ3/HA) colocalized with viral factories when expressed in infected cells (data not shown). Based on our published findings with other orthoreovirus proteins (7, 14, 16), we hypothesized that λ3 is recruited to viral factories through an association with μNS. To test this idea, we infected CV-1 cells with orthoreovirus strain T1L or T3D, and after a 24-h growth period, we immunoprecipitated μNS and associated proteins from the cell lysates. In cells infected with either viral strain, λ3 was coimmunoprecipitated by the μNS-specific antibodies (Fig. 1b), lending support to our hypothesis that λ3 localizes to viral factories through an association with μNS.

Because μNS associates with at least five other orthoreovirus proteins (7, 14, 16), the preceding result does not rule out the possibility that another viral or cellular protein is bridging μNS and λ3 to provide the observed association. To rule out the involvement of other viral proteins, we exploited the capacity of μNS to form FLS when expressed in the absence of other viral proteins in transfected cells to determine whether coexpression of λ3 would result in its colocalization within these structures as visualized by immunostaining and fluorescence microscopy. When expressed alone, λ3/HA was diffusely distributed throughout transfected CV-1 cells (Fig.
When coexpressed with μNS, however, λ3/HA colocalized with μNS in FLS (Fig. 1c, middle and right panels). To validate our use of FLS for identifying μNS-λ3 association in the absence of other viral proteins as well as to confirm our findings, we additionally performed immunoprecipitation studies. In these experiments, λ3/HA was coimmunoprecipitated with μNS (Fig. 1b, middle panel).
tated with μNS-specific antibodies only when both proteins were coexpressed in the transfected CV-1 cells (Fig. 1d).

The x-ray crystal structure of λ3 has been determined (22), revealing three domains: an N-terminal 380-residue domain of unknown function, a central 510-residue domain (381–890) that encompasses the polymerase subdomains (fingers, palm, and thumb), and a C-terminal 377-residue domain (891–1267) that forms a bracelet around the RNA product exit channel. To identify the domain(s) of λ3 involved in μNS association, we first constructed and tested mutants lacking the N-terminal 380 or 890 residues (λ3(381–1267)/HA and λ3(891–1267)/HA, respectively). Both of these mutants lost the capacity to colocalize with μNS in FLS and instead were distributed diffusely throughout transfected CV-1 cells when coexpressed with μNS or expressed individually (Fig. 1e, top row, and data not shown). These findings suggested that the N-terminal domain of λ3 is necessary for association with μNS. To determine whether this domain of λ3 may be sufficient for the association, we constructed and tested a mutant that contained only the N-terminal domain of λ3 (λ3(1–380)/HA). In this case, the mutant colocalized with μNS in FLS when coexpressed with μNS but was distributed throughout the transfected CV-1 cells when expressed individually (Fig. 1e, bottom row, and data not shown).

In summary, the results of this section provide strong evidence that the orthoreovirus RdRp λ3 associates with μNS in viral factories within infected cells as well as in FLS within transfected cells in which μNS and λ3 are coexpressed. The results also show that the N-terminal domain of λ3 is both necessary and sufficient for the association with μNS in FLS. Importantly for this report, the results here demonstrate the utility of this system not only for easily visualizing the association between two proteins inside cells but also for mapping the regions of one or both proteins that are involved in their association.

**FLS as a Cytoplasmic “Bait”-presenting Platform**—We reasoned that it should be possible to exploit the capacity of μNS to form FLS to detect associations of proteins from many different sources, not just those between μNS and other orthoreovirus proteins. Our idea was to fuse a bait protein to μNS such that it would be presented within cytoplasmic FLS where “fish” proteins with which the bait associates would then be recruited. We have reported that the C-terminal 251 amino acids of μNS (μNS(471–721)) are both necessary and sufficient to form FLS in transfected cells (12). Moreover when fused at its N terminus to EGFP (EGFP/μNS(471–721)), this protein remained capable of forming large-sized FLS (Fig. 2a). We therefore chose EGFP/μNS(471–721) as the platform for testing the broader effectiveness of this technology.

One of the major benefits of using the described technology to characterize protein-protein associations is the simplicity of the assay as outlined in Fig. 2, b and c. A plasmid expression vector with the gene encoding the bait protein cloned in-frame upstream of the EGFP/μNS(471–721) sequences (Fig. 2b, Bait/EGFP/μNS(471–721)) is either singly transfected into cells that endogenously express the fish protein or cotransfected into cells with a plasmid expressing the fish protein (Fig. 2, Fish). The bait is localized to FLS by the fused C terminus of μNS and visualized by the inherent fluorescence of the fused EGFP (Fig. 2c, left panels, middle and bottom rows). The localization of each respective fish protein can be visualized in various ways depending on the situation: by immunostaining with fish-specific antibodies, by immunostaining with tag-specific antibodies if the fish is tagged with a non-fluorescent marker, or by the inherent fluorescence of an alternative fusion protein such as mRFP (23) if the fish is tagged in that manner. If the bait and fish proteins associate, they will colocalize in FLS (Fig. 2c, middle row). If they do not associate, the fish protein will retain its normal distribution in the transfected cells (Fig. 2c, bottom row). As a control, each fish protein is also coexpressed with unfused EGFP/μNS(471–721) to confirm that its association with FLS is dependent on FLS fusion with the bait and also to confirm that there is no cross-reaction of the bait/EGFP/μNS(471–721) fusion with any antibodies used in the assay (Fig. 2c, top row). Other variations of this general approach, including different tagging systems and adaptations for high throughput applications, are described under “Discussion.”

**SV40 Tag Associates with p53-fused FLS—**To test the general utility of this technology, we first took advantage of the well defined interaction between the mammalian tumor suppressor protein p53 and the SV40 tumor protein TAg (17). We first constructed a plasmid in which the full coding sequence of p53 was cloned in-frame upstream of EGFP/μNS(471–721) to allow expression of the fusion protein p53/EGFP/μNS(471–721). We next tested the localization of p53 in transfected CV-1 cells expressing p53/EGFP/μNS(471–721) compared with those expressing EGFP/μNS(471–721). In cells expressing EGFP/μNS(471–721), endogenous p53 localized primarily to the nucleus with no visible colocalization with FLS (Fig. 3a, top row). This result was important for showing that p53 did not associate with FLS on its own and that the p53-specific antibody used for immunostaining did not cross-react with EGFP/μNS(471–721). In contrast, in cells expressing p53/EGFP/μNS(471–721), p53 localized almost exclusively to FLS (Fig. 3a, bottom row). This result was important for showing that fusion of p53 to EGFP/μNS(471–721) did not disrupt FLS formation and that p53 was not cleaved from the fusion or otherwise lost to detection. Interestingly in most cells expressing p53/EGFP/μNS(471–721), the nuclear localization of endogenous p53 was greatly reduced, suggesting that the p53 fused to EGFP/μNS(471–721) was binding and sequestering endogenous p53 within FLS (also see Fig. 3b and associated text below). This finding was not unexpected because p53 is known to form dimers and tetramers in mammalian cells (24).

COS-7 cells contain an integrated copy of the SV40 genome that lacks a replication origin and therefore endog-
enously express SV40 TAg (25), which localizes largely to the nucleus (26). In transfected COS-7 cells expressing EGFP/H9262 NS(471–721), we observed that TAg remained localized to the nucleus and did not colocalize with FLS (Fig. 4a, top row). This result was important for showing both that TAg did not independently associate with FLS and that the TAg-specific antibody used for immunostaining did not cross-react with EGFP/H9262 NS(471–721). In contrast, in transfected COS-7 cells expressing p53/EGFP/H9262 NS(471–721), we observed that much more TAg was found in the cytoplasm where it exactly colocalized with FLS (Fig. 4a, bottom row). Moreover in many cells expressing p53/EGFP/H9262 NS(471–721), the amount of TAg localized to the nucleus was either greatly reduced or absent.

The smallest contiguous region of TAg shown to retain strong interaction with p53 comprises 377 centrally located amino acids (TAG(250–627)) (27). Furthermore deletion of 11 amino acids within this region of full-length TAg (TAG(Δ434–444)) has been shown to ablate the interaction with p53 (28). We tested our ability to reproduce these findings using the FLS platform technology. In CV-1 cells expressing p53/EGFP/H9262 NS(471–721), consistent with the published results obtained by other methods (27), TAG(250–627) was sufficient to associate (i.e. colocalize) with p53 in FLS (Fig. 4b, bottom row), whereas TAG(Δ434–444) did not associate with p53 in FLS and instead remained localized to the nucleus (Fig. 4c, bottom row).

In summary, the results in this section demonstrate the utility of the μNS-based, FLS platform technology for more general studies of protein-protein associations inside cells extending beyond the studies of orthoreovirus proteins. In addition, they demonstrate the utility of this system not only for easily visualizing the association between two proteins but
also for mapping the regions of one or both proteins that are involved in their association.

p53/EGFP/NSP5 Associates with p53-fused FLS—In our initial examination of the localization of p53 in CV-1 cells expressing p53/EGFP/µNS(471–721) (see Fig. 3a), we noted that antibodies against p53 often did not stain the nucleus, where p53 is typically localized, but instead stained only the cytoplasmic structures formed by p53-fused FLS. These results suggested that in the presence of these platforms, endogenous p53 was oligomerizing with the cytoplasmically anchored p53 and as a result was retained in the FLS. To examine further the possibility that p53-p53 oligomers could form within p53-fused FLS, we constructed two additional expression vectors for p53 fusion proteins, one in which p53 was fused to EGFP/µNS(471–721) and EGFP/NSP5 (top row) or p53/mRFP/µNS(471–721) and p53/EGFP/NSP5 (bottom row), FLS, p53-fused or not, were detected by the inherent fluorescence of the fused EGFP (left column, as indicated at bottom), and p53 was detected by immunostaining with p53-specific mAb followed by Alexa 594-conjugated anti-mouse IgG (α-p53; middle column). b, CV-1 cells were transfected with plasmid expressing p53/mRFP/µNS(471–721) and EGFP/NSP5 (top row) or p53/mRFP/µNS(471–721) and p53/EGFP/NSP5 (bottom row), VLS, p53-fused or not, were detected by the inherent fluorescence of the fused EGFP (left column). FLS were detected by the inherent fluorescence of the fused mRFP (middle column). Merge, overlay of green and red fluorescence signals (right column). Scale bars, 10 µm.

Fig. 3. p53 forms oligomers with p53-fused FLS or VLS. In each experiment, cells were processed at 18 h post-transfection. a, CV-1 cells were transfected with plasmid expressing EGFP/µNS(471–721) (top row) or p53/EGFP/µNS(471–721) (bottom row). FLS, p53-fused or not, were detected by the inherent fluorescence of the fused EGFP (left column, as indicated at bottom), and p53 was detected by immunostaining with p53-specific mAb followed by Alexa 594-conjugated anti-mouse IgG (α-p53; middle column). b, CV-1 cells were transfected with plasmid expressing p53/mRFP/µNS(471–721) and EGFP/NSP5 (top row) or p53/mRFP/µNS(471–721) and p53/EGFP/NSP5 (bottom row), VLS, p53-fused or not, were detected by the inherent fluorescence of the fused EGFP (left column). FLS were detected by the inherent fluorescence of the fused mRFP (middle column). Merge, overlay of green and red fluorescence signals (right column). Scale bars, 10 µm.

CBP/p300 Associate with p53-fused FLS—As one of the key regulators of cellular stress, p53 interacts with a large number of cellular proteins, including CBP and p300, a pair of homologous proteins that act as transcriptional coactivators with p53 upon cellular DNA damage (29). In an attempt to demonstrate the utility of the FLS technology for identifying cellular protein associations beyond those of p53 and TAg, or p53 oligomers, we examined the localization of CBP/p300 in the presence of p53-fused FLS. Either EGFP/µNS(471–721) or p53/EGFP/µNS(471–721) expression vectors were transfected into CV-1 cells, and endogenous CBP/p300 localiza-
tion was examined using a mAb that reacts with both proteins. In cells expressing EGFP/ p33/NS(471–721), CBP/p300 were pri-
marily localized in the nucleus (Fig. 5, top row). In many cells expressing p53/EGFP/ p33/NS(471–721), however, CBP/p300 colocalized within the cytoplasmic p53-fused FLS (Fig. 5, bottom row). It is important to note that the localization of CBP/p300 in FLS did not occur in every cell expressing the p53 fusion protein. The reason for this cell-to-cell variation is not currently understood but may in fact correctly represent a cell-to-cell difference in p53 association with partner proteins (see “Discussion”). Taken together, these data demonstrate the widespread utility of the FLS technology in examining a diverse range of binary protein associations, including ones with proteins that are viral-viral, viral-cellular, and cellular-cellular in origin.

A Ternary Complex Comprising p53, TAg, and pRb in p53-fused FLS—Previous reports have shown that in addition to p53, TAg associates with a second cellular tumor suppressor protein, pRb (30). We reasoned that the FLS technology may be useful for demonstrating a ternary complex comprising p53, TAg, and pRb. Taking advantage of the expression of TAg in COS-7 but not CV-1 cells, we first examined the 627)/HA (bottom row). FLS were detected as in a (left column), and TAg was detected by immunostaining with HA-specific mAb followed by Alexa 594-conjugated anti-mouse IgG (a-HA; middle column). c, CV-1 cells were transfected with plasmids expressing EGFP/ p33/NS(471–721) and HA/TAg (top row), p33/EGFP/µNS(471–721) and HA/TAg (middle row), or p33/EGFP/µNS(471–721) and TAg(250–627)/HA (bottom row). FLS were detected as in a (left column), and TAg was detected by immunostaining with HA-specific mAb followed by Alexa 594-conjugated anti-mouse IgG (a-HA; middle column). c, CV-1 cells were transfected with plasmids expressing EGFP/ µNS(471–721) and HA/TAg (top row), p33/EGFP/µNS(471–721) and HA/TAg (middle row), or p33/EGFP/µNS(471–721) and TAg(434–444) (bottom row). FLS (left column) and TAg (middle column) were detected as in a. Merge, overlay of green and red fluorescence signals (right column). Scale bars, 10 μm.
localization of endogenous pRb in the absence and presence of TAg in cells expressing either EGFP/μNS(471–721) or p53/EGFP/μNS(471–721) (middle row), or CV-1 cells were transfected with plasmid expressing p53/EGFP/μNS(471–721) (bottom row). FLS, p53-fused or not, were detected by the inherent fluorescence of the fused EGFP (left column). pRb was detected by immunostaining with pRb-specific mAb followed by Alexa 594-conjugated anti-mouse IgG (merge; middle column). Merge, overlay of green and red fluorescence signals (right column). Scale bars, 10 μm.

To demonstrate the TAg-p53-pRb complex more directly,
we tested our ability to combine the FLS technology with three-color microscopy. In these experiments, we transfected CV-1 cells with either HA/TAg and EGFP/μNS(471–721) or HA/TAg and p53/EGFP/μNS(471–721) expression vectors and examined the localization of both HA/TAg and pRb in the transfected cells. We found that in cells expressing FLS not fused to p53 both TAg and pRb were primarily localized in the nucleus and did not localize to the FLS (Fig. 6b, top row). In cells expressing HA/TAg and p53-fused FLS, the HA/TAg was completely localized to the p53-fused FLS in most cells confirming our previous results (see Fig. 3, b and c). Additionally endogenous pRb was again recruited to the p53/TAg-containing FLS (Fig. 6b, bottom row) in many cells. These data show that TAg can associate with both p53 and pRb simultaneously in cells and further expand the utility of the FLS technology to include identifying associations among proteins in ternary or higher order complexes.

**DISCUSSION**

There are numerous methods for detecting protein-protein associations, each with advantages and disadvantages. Advantages of the FLS platform technology presented here are its versatility and simplicity with the capacity to evaluate proteins from many different sources yet without the need for purified proteins, unusual technical expertise, or highly specialized equipment. The most demanding requirement is for a fluorescence microscope with standard imaging capabilities. Regarding ease of use, it is also significant that this technology is designed to work upon transient expression of proteins from transfected plasmids and does not require the generation of stably expressing cell lines. Another advantage is that associations are visualized inside cells in a setting that approximates the native environment of the proteins. In addition to CV-1 and COS-7 cells, we have shown the technology to work as well in other cell types (BHK-21 and NIH 3T3) (data not shown) so that cell type restrictions seem not to be a general limitation. Because of the multivalent nature of the FLS platform, we believe this technology might be distinctively useful for identifying weakly associating partners by tending to concentrate two associating proteins within discrete areas of the cytoplasm.

For some applications, the FLS platform technology can be applied after constructing a single plasmid to express the bait/EGFP/μNS region or peptide that is sufficient to recruit a particular protein. Additionally wild-type or mutant fish proteins can be coexpressed from plasmids with the FLS-forming bait fusion, allowing investigators to detect an association between two specified proteins and then to map the regions of association within one or both proteins. The latter approach makes it possible to test for associations with fish proteins for which protein-specific antibodies are not available by adding a fusion tag (e.g. HA) to the fish and using tag-specific antibodies to detect it. The fish could alternatively be tagged with a fluorescent protein (e.g. mRFP) distinct from that in the bait fusion (primarily EGFP in this study), removing the need for immunostaining. In fact, the inherent fluorescence of both bait and fish in this case would provide the potential for live cell imaging and dynamic studies of protein associations. Another variation is to include a different tag, or even no tag, in the FLS-forming bait fusion, adapting to the needs of the particular investigators in light of reagents at hand (e.g. plasmids for expressing EGFP-tagged fish). Indeed we have also demonstrated SV40 TAg association with FLS by using p53/mRFP/μNS(471–721) as the bait-presenting platform (data not shown). Demonstrations of ternary or even higher order associations in the FLS are also apparently possible by use of appropriate tags and/or antibodies and controls as shown in this study for p53-TAg-pRb.

**Published work has provided strong evidence that localizations of orthoreovirus proteins λ1, λ2, μ2, α2, and αNS to FLS result from their specific associations with μNS and not from FLS acting as “sinks” for nonspecific associations (7, 14–16). Further supporting evidence is found in this report. First, we showed that a specific domain of λ3 (residues 1–380) is necessary (and also sufficient) for the association with μNS in FLS. Second, we showed that a small deletion in TAg (removal of residues 434–444), which has been shown previously to ablate its interaction with p53, also ablates its association with p53-presenting FLS and allows TAg to retain its normal nuclear localization.**

The technology we describe in this report must certainly have some limitations as does any single method. Similar to yeast two-hybrid and coimmunoprecipitation assays, this method does not distinguish whether an association between proteins is direct (an interaction) or requires one or more bridging molecules present in the cell. It is also possible that some bait proteins when fused to EGFP/μNS(471–721) may not fold properly, may not remain otherwise competent for associating with particular fish (such as by losing the capacity to be modified post-translationally), or may disrupt the capacity of μNS(471–721) to form FLS. For example, in the current study using p53/EGFP/μNS(471–721), we were unable to recapitulate the previously described association of p53 with its known interaction partner MDM2 (data not shown) for reasons that remain unclear. We did not directly test the phosphorylation status of p53 fused to FLS; however, the binding of CBP/p300 to p53 is known to be strongly enhanced by phosphorylation of serine 15 in p53 (29). Thus, our data demonstrating CBP/p300 association with p53-fused FLS suggest that at least some post-translational modification of proteins fused to FLS (i.e. p53 phosphorylation in this case) may occur.
Improper folding or failure of the bait to function fully could result in some cases from fusion of tag/µNS(471–721) to the C terminus of the bait, and we are therefore now testing whether µNS(471–721)/tag/bait constructs can retain the basic features of the system described here, allowing fusion to the N terminus of a bait when desired or necessary. Fusing EGFP to the C terminus of µNS has little effect on FLS formation, and so this variation seems likely to succeed. Another possible limitation is that certain fish proteins may associate with FLS independently of the bait; however, the routine control of coexpressing the fish with the non-bait fused FLS-forming protein (EGFP/µNS(471–721)) in this study is designed in part to test for this possibility with each respective fish. Some fish proteins that are normally localized to specialized structures in cells may not be free for recruitment to FLS. Membrane-anchored fish may be one such class, for example. This general concern highlights an interesting point from the presented results, however, in that we have shown that several fish that are normally localized to the nucleus, SV40 TAg, endogenous p53, and CBP/p300, can be sequestered in the cytoplasmic FLS when an interacting protein, p53, is presented on the FLS platform. Whether this reflects sequestration of these target proteins after their return to the cytoplasm by shuttling through the nuclear pore or during cell division and/or recruitment of newly synthesized target proteins accompanied by normal turnover of their nuclear forms remains to be determined.

Although in this report we show how the FLS platform technology can be used to characterize the association between two or three specified proteins in each example, we envision that it can be broadened for other purposes and formats as well. For example, we are currently screening a library of EGFP-tagged cellular proteins in an effort to demonstrate that a differentially tagged version of the FLS technology can be used to identify new protein interactors of a specified bait protein. For such screening applications, adaptations to high throughput formats for data acquisition and analysis should be feasible and could make this a useful new approach for extending or validating “interactome” maps. Particularly when applied in high throughput formats, the FLS technology may also prove useful for screening libraries of small molecules for those that inhibit the association of any two specified proteins. Lastly because of the “sequestering” nature of the FLS, it may be possible to use this technology to deplete bait-associating proteins from their normal sites of intracellular localization and thereby to exploit or to study the functional effects of this sequestration.

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