interaction blues: protein interactions monitored in live mammalian cells by \(\beta\)-galactosidase complementation

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In mammalian cells, protein–protein interactions constitute essential regulatory steps that modulate the activity of signalling pathways and many other intracellular processes. Although efficient genetic methods for identifying interacting partners exist and have been successfully applied by a number of laboratories, there has been a need for a technique that allows protein interactions to be monitored in real time in the cellular compartment in which they normally take place. The \(\beta\)-galactosidase-based intracistronic complementation methodology described here is the first technology that might fulfil these requirements, and can be applied to live mammalian cells. In addition, it holds promise for applications in high-throughput screens for agonists and antagonists of specific interactions and for the development of a ‘mammalian two-hybrid’ screen for novel protein partners. Cell physiology and development is controlled by well-regulated cascades of protein–protein interactions. For example, the activation and subsequent auto-phosphorylation of growth-factor receptors is often dependent on their ligand-induced homo- or heterodimerization[1]. Such protein interactions result, in turn, in the creation of docking sites for downstream components of the relevant signalling pathways and thus in additional protein–protein interactions[2]. Several systems have been developed for identifying and studying protein–protein interactions, including the yeast two-hybrid system[3], the split-ubiquitin system[4], the Sos-recruitment system, and dihydrolipoate reductase (DHFR) complementation[5,6]. As shown in Table 1, each of these systems has inherent advantages, as well as disadvantages, including lack of utility in mammalian cells or with membrane proteins, or lack of rapid quantitative analysis of the interaction owing to the absence of signal amplification, indirect readouts or other assay limitations. We have developed a novel assay for monitoring protein–protein interactions based on intracistronic \(\beta\)-galactosidase complementation. There are several advantageous properties of the intracistronic \(\beta\)-galactosidase complementation method:

- it works in live mammalian cells;
- it monitors interactions in the compartment in which they normally take place (e.g. membrane or cytoplasm);
- rapid sensitive assays are available that are amenable to high-throughput screening methods;
- it provides a quantitative readout, allowing the monitoring of interaction kinetics;
- it signals amplification, allowing physiological interactions to be monitored in the absence of overexpression.

Properties of \(\beta\)-galactosidase intracistronic complementation for monitoring protein–protein interactions

Intracistronic \(\beta\)-galactosidase complementation is a phenomenon whereby two mutants of the bacterial enzyme \(\beta\)-galactosidase that harbour inactivating mutations in different crucial domains are capable of recreating an active enzyme by sharing their intact domains[11,12]. It has long been known that, in Escherichia coli, specific mutants can complement one another more or less efficiently, depending on the nature of the mutations[11]. We have shown that the same holds true in mammalian cells[13]. Our protein-interaction detection method capitalizes on the expression of low levels of chimeric proteins incorporating weakly complementing \(\beta\)-galactosidase mutants. \(\beta\)-galactosidase activity is recreated only when physical interaction of the mutants is forced by the non-galactosidase components of the hybrids. Under these conditions, the complementation of \(\beta\)-galactosidase mutants does not drive, but rather monitors, the interaction of other proteins. To monitor the interaction between two proteins, each of the proteins is

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fused to one of a pair of β-galactosidase mutants (Fig. 1), and the two fusion proteins are expressed at low levels in mammalian cells. For use with this system, we have developed a specific pair of β-galactosidase deletion mutants (Da and Dv; Fig. 2) that display a low level of spontaneous complementation when coexpressed in mammalian cells.14 Low levels of the chimeric proteins are expressed in order to avoid perturbing the physiological balance of the cell and further reduce non-specific interactions, thereby minimizing basal β-galactosidase activity. This is achieved by introducing single copies of constructs into cells by using limiting dilution of the two retroviruses and by expressing the chimeric protein and a selectable marker from the same bicistronic messenger RNA.15,16

A particularly important characteristic of the intracistronic complementation method is that the enzyme that provides the readout of the assay is linked covalently to the interacting proteins. Thus, the signal is generated directly in the subcellular compartment in which the interaction takes place. Using this system, we have been able to monitor ligand-induced interactions between two cytoplasmic proteins (FKBP12 and the FRB domain of FRAP)16,17, between a cytoplasmic protein and a transmembrane receptor (FADD and FAS) (Ref. 18; A. Estellés et al., unpublished), and the formation of homodimers and higher-order complexes between transmembrane proteins (EGF receptor and FAS, respectively).19,20 Crucial to the future broad application of this assay was the finding that the kinetics of appearance of β-galactosidase activity in response to a particular inducer varied and reflected the properties of the protein pairs tested. When the dimerization of chimeric epidermal growth factor receptor constructs was monitored, an increase in β-galactosidase activity was detectable within 15 seconds after treatment with EGF, which peaked within one to two hours.20 By contrast, in the case of the FKBP12–FRAP interaction, β-galactosidase activity was first detected 0.5 hours after the inducer, rapamycin, was added to the culture medium, and this activity increased steadily over the subsequent 12 hours, in good accordance with previously published results.16,17,21 We conclude therefore that β-galactosidase activity reflects the characteristics of the interactions being monitored rather than the rate of the β-galactosidase

### Table 1 - Advantages and Disadvantages of Some Systems for Analysing Protein–Protein Interactions

<table>
<thead>
<tr>
<th>System</th>
<th>Works in mammalian cells?</th>
<th>Works with membrane proteins?</th>
<th>Pros</th>
<th>Cons</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-galactosidase complementation</td>
<td>Yes</td>
<td>Yes</td>
<td>Allows real-time measurements of interactions. Owing to enzymatic amplification of the signal, works as a tracer in the absence of overexpression.</td>
<td>Because it relies on re-assembly of a complex enzyme, it is likely to be subject to steric constraints.</td>
<td>16</td>
</tr>
<tr>
<td>Yeast two-hybrid</td>
<td>No (yeast)</td>
<td>No</td>
<td>Widely used and very successful in detecting novel interaction partners of a given test protein.</td>
<td>Has not been successfully used in mammalian cells. Requires nuclear localization of the hybrid proteins, thus it cannot be used with membrane proteins. Indirect readout limits the kinetic analysis of interactions.</td>
<td>3</td>
</tr>
<tr>
<td>Sos-recruitment system</td>
<td>No (yeast)</td>
<td>No</td>
<td>Good sensitivity and direct selection for positive colonies based on growth. Likely to be independent of steric constraints.</td>
<td>Cannot be used in mammalian cells or with transmembrane proteins. Indirect readout limits the kinetic analysis and subcellular localization of interactions.</td>
<td>7, 8</td>
</tr>
<tr>
<td>DHFR complementation system</td>
<td>Yes</td>
<td>Yes</td>
<td>In DHFR mutant cells, allows direct selection of positive clones based on growth.</td>
<td>No enzymatic amplification in cells containing wild-type DHFR, thus requires overexpression of the test proteins. Not yet shown to allow quantitative measurements of interactions in wild-type DHFR cells. Likely to be subject to steric constraints.</td>
<td>9, 10</td>
</tr>
<tr>
<td>Split-ubiquitin system</td>
<td>Yes</td>
<td>Yes</td>
<td>Good sensitivity owing to transcription-mediated signal amplification.</td>
<td>Indirect readout limits the kinetic analysis and subcellular localization of interactions. Likely to be subject to steric constraints.</td>
<td>5, 6</td>
</tr>
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Techniques
complementation reaction. This was not a foregone conclusion since the reconstitution of β-galactosidase activity by intracistronic complementation is thought to require partial refolding of the mutants and subsequent assembly of the tetrameric active enzyme. In theory, a considerable amount of time might be required for the development of a detectable β-galactosidase signal. Our experiments suggest that such a lag is on the order of seconds and should therefore be negligible for most applications.

Since β-galactosidase has been extensively used as a marker gene for the past 30 years, a number of diverse assays are available to detect its activity. The 'complemented' β-galactosidase can be detected by histochemistry with both the traditional chromogenic substrate X-gal and a more sensitive fluorogenic substrate (fluor-X-gal)24. These microscopic detection methods are useful for the visualization of the enzyme within cells. Sensitive quantitation of complemented β-galactosidase can be achieved by biochemical assays of cell lysates with commercially available chemiluminescence substrates (e.g. the GAL-Screen β-galactosidase detection kit from Tropix). Such assays can be readily adapted to a multiwell format, allowing a rapid test of the effect of a variety of pharmacological and biological agents on the protein interactions of interest. For monitoring enzyme activity in live cells, another sensitive and quantitative method uses the fluorogenic substrate fluorescein di-β-d-galactopyranoside (FDG), which is detectable by flow cytometry25. FDG can be introduced into living cells by hypotonic loading, which does not affect cell viability, and the fluorescent cleavage product of the reaction catalysed by β-galactosidase (free fluorescein) remains within the cells, as it is unable to cross the plasma membrane. A great advantage of the β-galactosidase complementation methodology is that it only requires a low level of expression of the chimeric proteins to generate a detectable signal upon interaction. In the case of the EGF receptor, the best results were obtained with cells that expressed levels of the chimeric proteins comparable with wild-type endogenous proteins26. Thus, β-galactosidase complementation can be used under conditions that should not alter the balance of cellular proteins or lead to nonphysiological results.

Potential applications and future directions

Intracistronic complementation assays are easily adapted to work in a multiwell format. Furthermore, some of the assays for detecting β-galactosidase activity can be fully automated. Thus, the most straightforward and immediate application of this method is likely to be in high-throughput screening of combinatorial chemical libraries for compounds that can either block or induce a specific protein–protein interaction. This method is particularly well suited for monitoring interactions between membrane proteins such as receptors, between receptors and cytoplasmic components of the downstream signalling pathway or between cytoplasmic proteins. For example, a chemical library could be screened for compounds that inhibit dimerization (and consequent activation) of the EGF receptor or related family members such as ErbB2, which

![FIGURE 1](image)

Schematic of experimental hypothesis. (a) When the Δw and Δα β-galactosidase mutants are fused to proteins that heterodimerize (represented by the yellow and orange shapes), their association in the active enzyme conformation is favoured. (b) When the two proteins fused to Δw and Δα cannot interact to form a complex, the formation of active β-galactosidase is not favoured. The reconstitution of β-galactosidase activity is dependent on the interaction of the non-β-galactosidase components of the chimeric proteins and allows it to monitor the kinetics of the interaction.

![FIGURE 2](image)

Three-dimensional structure of β-galactosidase in its active tetrameric form. Each monomer is represented in a different colour (green, light blue, cyan and dark blue). Two α domains are in red and two in yellow. The two w domains on the right-hand side of the molecule are represented in red and orange, respectively. Note the extensive intramolecular contacts of the α and w domains with the central portion of each monomer. In the absence of a forced juxtapositioning of the Δw and Δα mutants, these interactions are likely to sequester the w and α domains and render them unavailable for complementation.
might thus have anti-neoplastic activ-
ity in a subset of human carcinomas,
as such tumours of the breast.

The ability of intracistronic comple-
mentation to monitor interactions in
the absence of overexpression might make
it applicable to mapping pro-
tein interactions directly in transgenic
animals. The fusion point between
test proteins and β-galactosidase
mutants that function best in vitro
could be recreated in vivo by knock-in
of DNA segments in-frame with
the endogenous gene. Animals carrying
two transgenes should develop β-
galactosidase activity, given that in
those cells in which the endogenous pro-
teins are expressed and actually inter-
act. To date, the detection of mRNA
and protein localization has been possible in
developing organisms.

Histochemical detection of β-galac-
tosidase complementation would extend
these findings in animal mod-
els by allowing the mapping of spe-
cific protein interactions in single cells
both during embryogenesis and in
specific disease states.

Another exciting potential appli-
cation of intracistronic complementa-
tion is the development of a ‘mam-
malian two-hybrid system’. This
possibility stems from the ability to
use flow-cytometry-based techniques
to isolate β-galactosidase-positive cells
without compromising their vi-
ability. In theory, a chimeric library
obtained by fusing random cDNAs to
one of the β-galactosidase mutants
could be constructed. This library
could then be screened for gene
products that interact with a given
‘bait’ protein fused to the comple-
mentary β-galactosidase mutant.
Such a screen would be unique in
that it could be performed in cultured
mammalian cells, allowing the detec-
tion of interactions that need to be
facilitated by an endogenous protein
or that only take place in a specific
cellular compartment. Furthermore,
unlike the yeast two-hybrid system,
the readout of the assay would not be
dependent on nuclear localization of
the interacting partners, allowing
this method to be used to isolate partners
of membrane-bound proteins.

In theory, the detection of protein–protein interactions by
intracistronic complementation could be hindered by steric
constraints that prevent formation of an active enzyme. Given two
known polypeptides, a range of chimeric proteins could be generated in vitro
followed by selection of those that
display the best characteristics. This
limitation might preclude detection of
certain interactions in screens of
CDNA libraries, in which the CDNA–β-
galactosidase fusion products are
necessarily generated at random and
cannot be predicted or individually
optimized. Studies to overcome these
problems are under way: for example,
testing the insertion of flexible linkers of different length
between the test protein and β-galactosidase.

Concluding remarks

Clearly, the intracistronic β-galac-
tosidase complementation method
allows monitoring of protein–protein
interactions in real time, in live cells, as
shown by our analysis of protein-inter-
actions involved in cell signalling.
Although it is still in the early stages of its
development, high-throughput screens for antagonists of specific
interactions can be readily envisioned
at this time. Improvements will cer-
toniously be forthcoming, as well as
a deeper understanding of the underly-
ing molecular mechanisms. In vivo
applications in developing transgenic
mice are ongoing, and the development of a ‘mammalian two-
hybrid system’ might soon become a reality.

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