Single-Well Monitoring of Protein – Protein Interaction and Phosphorylation - Dephosphorylation Events

Experimental Methods
Recombinant proteins and reagents. Wild-type full length (unless indicated otherwise) recombinant protein kinases, phosphatases, and substrates with appropriate tags and respective activation state were purchased from various suppliers, as described in Supporting Information Table 1. All AlphaScreen beads (AlphaScreen Acceptor, AlphaLISA Acceptor and Donor) were from PerkinElmer, while all mouse monoclonal (pThr202/pTyr204 ERK1/2, pThr180/pTyr182 P38 and pSer217/pSer 221 MEK1/2) phosphorylation-specific antibodies were purchased from Cell Signaling Technology. Inhibitors staurosporine and U0126 were from Sigma. All assays were performed in solid white 384 well plates (OptiPlates, PerkinElmer) in 15 µL of kinase buffer containing 20 mM Tris pH 7.4, 100 mM NaCl, 1 mM MgCl₂, 1 mM Dithiothreitol (DTT) and 0.01% Tween 20. Typically, 5 µL of pre-incubated antibody and Acceptor beads were added to the enzymatic reaction, before the addition of 5 µL of Donor beads for a final detection volume of 25 µL.

Fluorescence spectroscopy. Unconjugated AlphaScreen or AlphaLISA beads were dissolved in water to a final concentration of 100 µg/mL. Fluorescence emission spectra were obtained on the LS 55 spectrofluorometer (PerkinElmer) with readings spaced by 0.5 nm, and by exciting bead samples at 350 nm to avoid Raman interference in the range of 450 to 680 nm. The narrow bandwidth filters at 572 nm (Dysprosium 572/7.5 filter) and 615 nm (Europium 615/8.5 filter) were respectively chosen to detect AlphaScreen and AlphaLISA signals, because of their commercial availability as off-the-shelf products. Although these two filters were designed for other applications, they can be seamlessly integrated in an Alpha detection protocol through the EnVision’s software.

Interaction assays. Increasing concentrations of enzyme and substrate were incubated 2 hours at 23°C. Detection reagents in kinase buffer were then sequentially added to the reaction: nickel chelate AlphaLISA Acceptor beads and glutathione-coated (GSH) Donor beads (Supporting Information Figure 1b). Beads were used at a final concentration of 20 µg/mL in a final volume of 25 µL. Each bead addition was followed by 1 hour incubation at 23°C, and the plates were read on an EnVision 2103 (PerkinElmer) using the preset AlphaScreen protocol.

Kinase assays. Phosphorylation reactions were performed under conditions similar to interaction assays, but were initiated upon addition of 10 µM ATP assay concentration. Substrate phosphorylation was detected by a corresponding phospho-specific antibody with a 1:1000 final dilution. Mouse monoclonal antibodies were captured with anti-mouse IgG AlphaScreen Acceptor beads (Supporting Information Figure 1d).

Phosphorylation-interaction assays. 1:1 ratios of 100 nM enzyme and substrate were prepared in kinase buffer and incubated with various ATP concentrations for 2 hours at 23°C. For the His-MEK1 and GST-ERK2 pair, nickel chelate AlphaLISA Acceptor beads, anti-pTpY ERK1/2 antibody and anti-mouse IgG Acceptor beads were pre-incubated together in kinase buffer prior to addition in the reaction. After 1 hour incubation with the detection cocktail, GSH Donor beads were added to a final concentration of 40 µg/mL, with each Acceptor bead at 20 µg/mL (Figure 1a). After a final 1 hour incubation, plates were read twice on an EnVision reader equipped with Dysprosium 572 nm and Europium 615 nm narrow bandwidth filters (PerkinElmer), thus enabling separation of signals generated respectively by AlphaScreen and AlphaLISA Acceptor beads. Hence, the AlphaScreen signal was read first using a modified AlphaScreen protocol with 572 nm filtering, and a similar protocol but with filtering at 615 nm was used in the second reading to detect the signal from the higher count-yielding AlphaLISA Acceptor beads.

The emission spectra of AlphaScreen and AlphaLISA signals partly overlap (Supporting Information Figure 1a). Interference might thus be observed in a dual detection experiment despite the use of narrow bandwidth emission filters. To ensure that the signals measured in each emission channel in the dual configuration assay were not due to cross-channel contamination, we performed experiments using two configurations of AlphaScreen and AlphaLISA Acceptor beads (Supporting Information Figure 2). These control experiments confirmed signal specificity in both detection channels, although some minor cross-talk from AlphaScreen signal was detected into the AlphaLISA channel under both assay configurations (Supporting Information Figure 2a, b, 3rd panels). This cross-talk from AlphaScreen beads could be significant in assays with low AlphaLISA signal. However, since there was no detectable cross-channel contamination from AlphaLISA signal into the AlphaScreen channel (Supporting Information Figure 2a, b, 3rd panels), we can easily eliminate any channel contamination by ensuring that all assays in a dual
configuration exhibit higher AlphaLISA than AlphaScreen signal by at least 2-fold.

**Phosphatase assays.** ERK2 and P38α dephosphorylation-interaction assays were performed analogously to the above described assays (Supporting Information Figure 3b). His-tagged phosphatase with concentrations ranging from 0.1 nM to 100 nM was added to 100 nM of pre-phosphorylated MAP kinase (final concentrations in 15 µL assay volume) to initiate dephosphorylation. Reaction proceeded 15 minutes at 23°C before addition of detection reagents.

**Software and data analysis.** All assay points were performed in triplicates, and each experiment was repeated on 3 to 5 independent occasions. All graphics shown were generated with GraphPad Prism® 4 software and display a single representative experiment with error bars indicating standard deviation. Data was fitted using nonlinear regression analysis. When incomplete inhibition curves were displayed, a bottom constraint equal to 0 was included in the curve fitting and calculations.

**Table 1.** Recombinant proteins used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Enzymatic class</th>
<th>Family</th>
<th>Phosphorylation state</th>
<th>Activity</th>
<th>Tag (N-terminal)</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERK2</td>
<td>Kinase</td>
<td>CGMC</td>
<td>Unphosphorylated</td>
<td>Unactive</td>
<td>GST</td>
<td>Carna Bio</td>
</tr>
<tr>
<td>MEK1</td>
<td>Kinase</td>
<td>STE</td>
<td>Phosphorylated</td>
<td>Active</td>
<td>His</td>
<td>Carna Bio</td>
</tr>
<tr>
<td>MEK1</td>
<td>Kinase</td>
<td>STE</td>
<td>Unphosphorylated</td>
<td>Unactive</td>
<td>His</td>
<td>SignalChem</td>
</tr>
<tr>
<td>MKP-2/DUSP4</td>
<td>Phosphatase</td>
<td>Dual specificity</td>
<td>Unphosphorylated</td>
<td>Active</td>
<td>His</td>
<td>BioMol</td>
</tr>
<tr>
<td>MKP-6/DUSP14</td>
<td>Phosphatase</td>
<td>Dual specificity</td>
<td>Unphosphorylated</td>
<td>Active</td>
<td>His</td>
<td>BioMol</td>
</tr>
<tr>
<td>MKP-7/DUSP16</td>
<td>Phosphatase</td>
<td>Dual specificity</td>
<td>Unphosphorylated</td>
<td>Active</td>
<td>His</td>
<td>BioMol</td>
</tr>
<tr>
<td>Raf-1 (kinase domain)</td>
<td>Kinase</td>
<td>TKL</td>
<td>Mutant</td>
<td>Active</td>
<td>GST</td>
<td>Carna Bio</td>
</tr>
<tr>
<td>P38α (kinase domain)</td>
<td>Kinase</td>
<td>CGMC</td>
<td>Unphosphorylated</td>
<td>Unactive</td>
<td>GST</td>
<td>Carna Bio</td>
</tr>
</tbody>
</table>

Supporting Information Figure 1. Optimization of interaction and phosphorylation assays. (a) Fluorescence emission spectra of AlphaScreen (gold) and AlphaLISA (red) Acceptor beads. Standard reading window is shown below in orange whereas narrow bandwidth detection channels used for dual readings AlphaScreen (572 nm filter) and AlphaLISA (615 nm filter) are respectively indicated in blue and green. (b) AlphaLISA assay configuration for the detection of His-tagged MEK1 and GST-tagged ERK2 interaction. Binding is measured using nickel chelate-coated AlphaLISA Acceptor beads and GSH-coated Donor beads. When the beads are within 200 nm of each other, excitation of Donor beads with a 680 nm laser triggers the diffusion of singlet oxygen to Acceptor beads resulting in light emission between 520 nm and 620 nm. (c) In the absence of ATP, increasing amounts of phosphorylated His-MEK1 were incubated with increasing concentrations of unphosphorylated GST-ERK2. (d) AlphaScreen assay configuration to monitor GST-ERK2 phosphorylation by MEK1. The dual phosphorylation-specific antibody is captured by an anti-mouse antibody-coupled AlphaScreen Acceptor bead, thereby allowing signal generation upon excitation of GSH-coated Donor beads. (e) Increasing amounts of phosphorylated and active His-MEK1 were incubated with increasing concentrations of unphosphorylated GST-ERK2 in presence of 10 µM ATP.
Supporting Information Figure 2. His-MEK1 – GST-ERK2 standard assay used as control experiments to determine cross-channel interference. (a) Signals generated with a complete assay (left panel), by substitution of nickel-coated with unconjugated AlphaLISA beads (2nd panel), by substitution of anti-mouse with unconjugated AlphaScreen beads (3rd panel), or by removal of the phospho-specific antibody (right panel). (b) Signals generated with a His-MEK1 – GST-ERK2 reverse assay configuration (red circles: AlphaLISA 615 nm signal/ERK2 phosphorylation; gold squares: AlphaScreen 572 nm signal/MEK1-ERK2 interaction) with a complete assay (left panel), by substitution of anti-mouse with unconjugated AlphaLISA beads (2nd panel), by substitution of nickel-coated with unconjugated AlphaScreen beads (3rd panel), or by removal of the phospho-specific antibody (right panel).

Supporting Information Figure 3. MKP selectivity and assay set-up. (a) Properties of the MKPs used for dephosphorylation-interaction assays. (b) Dual assay configuration to simultaneously monitor MKP dephosphorylation activity and MAP kinase binding. Pre-phosphorylated GST-tagged MAP kinase (ERK2 or P38α) binds a His-tagged MKP which can dephosphorylate it. Interaction is detected by GSH Donor beads and nickel AlphaLISA Acceptor beads, whereas MAP kinase phosphorylation is detected by phosphorylation-specific antibodies that are in turn captured by anti-mouse-coupled AlphaScreen beads. Dephosphorylation causes a decrease in AlphaScreen signal.
Supporting Information Discussion

ERK2 phosphorylation and MEK1 interaction curves do not intersect at midpoint, suggesting that a decrease in MEK1-ERK2 binding slightly precedes ERK2 dual phosphorylation. Previous studies have shown that phosphorylation of the tyrosine residue in the ERK2 activation loop occurs at a lower concentration than that of the threonine, and thus accumulation of a tyrosine mono-phosphorylated form can be observed (1, 2). This tyrosine is the most mobile residue in ERK2 and undergoes significant changes upon phosphorylation (3) or ERK2 docking interactions (4). We thus hypothesize that mono-phosphorylation of the tyrosine residue occurring at lower ATP could be sufficient to induce conformational changes within ERK2 that would affect its binding to MEK1. In contrast, MEK1 phosphorylation by Raf-1 is processive with both activating residues phosphorylated in a single reaction (5), and in a GST-Raf-1 and His-MEK1 phosphorylation-interaction experiment (Supporting Information Figure 4a), we observed the interaction and phosphorylation curves crossing at midpoint (Supporting Information Figure 4b). Nevertheless, we are currently investigating phosphorylation order in the ERK MAP kinase pathway and its impact on PPIs.

SUPPORTING INFORMATION REFERENCES