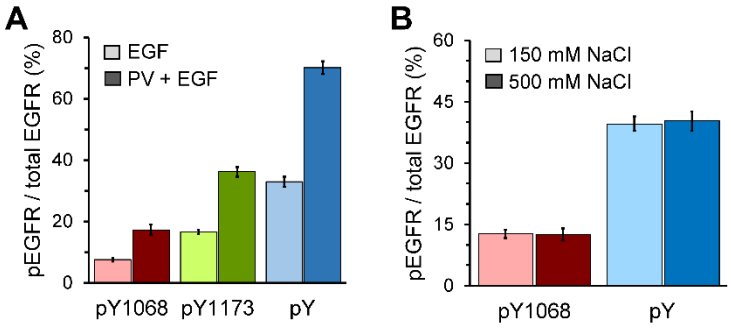
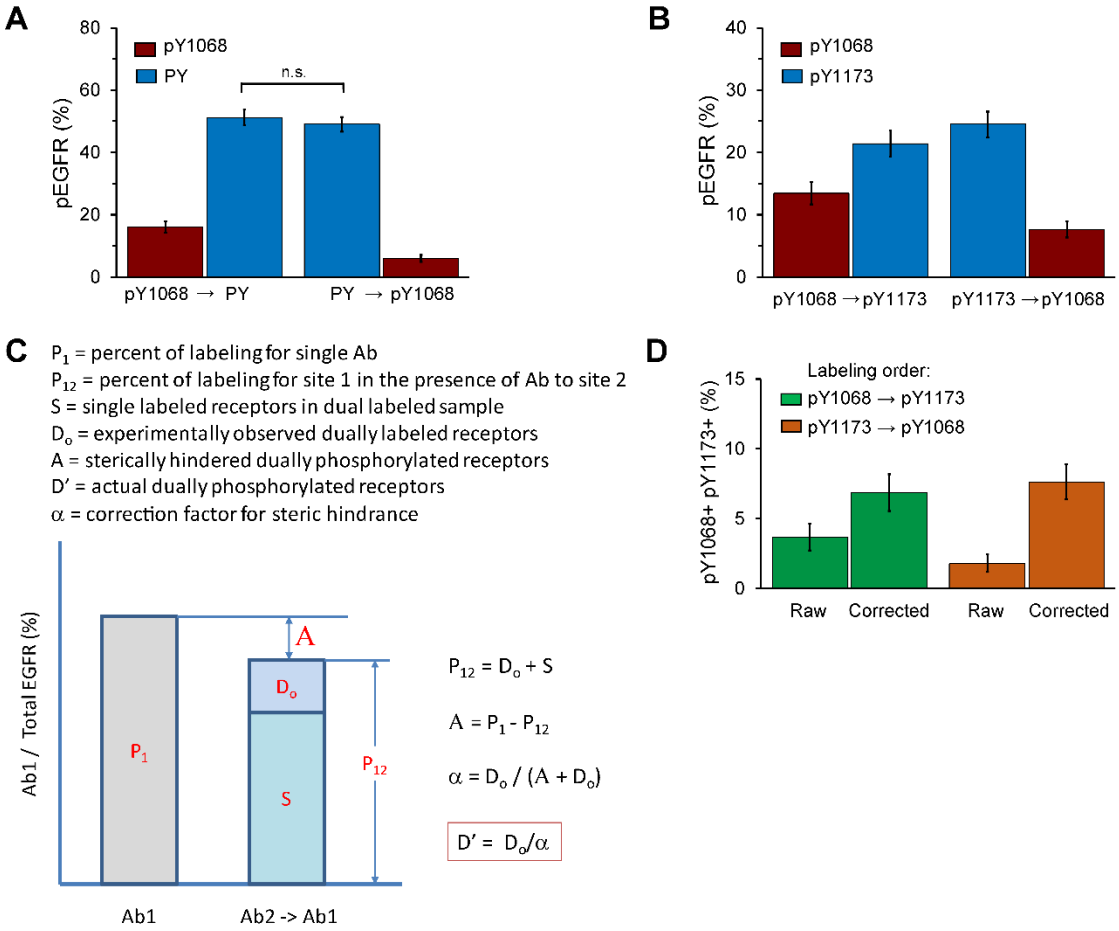
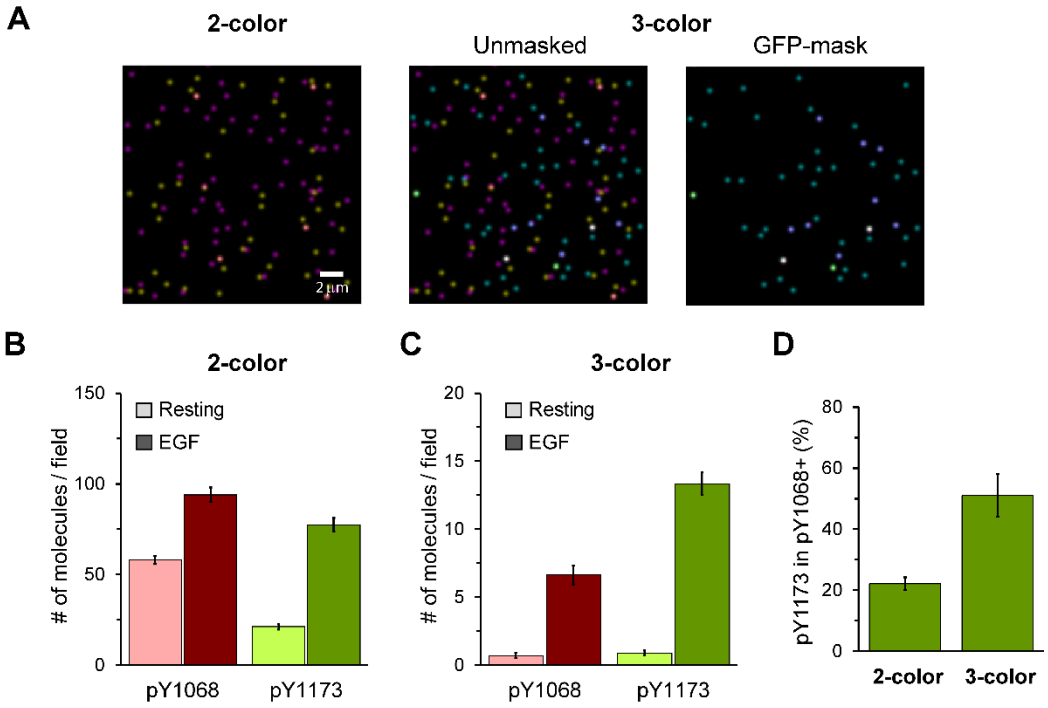
SUPPLEMENTARY FIGURES



Supplementary Figure 1. Effect of phosphatase inhibition or cell lysate salt concentration on detected phosphorylation levels. (A) CHO-EGFR-GFP cells were stimulated at 37°C with either 50 nM EGF for 5 min or pre-treated with 1 mM pervanadate (PV) for 15 min and then stimulated with 50 nM EGF and 1mM PV (PV + EGF) for 5 min. Considering that pervanadate treatment induces EGFR phosphorylation that may not be restricted to the plasma membrane, no surface correction was applied for this figure. Number of receptors per condition, 690 < N < 3400. **(B)** CHO-EGFR-GFP cells were stimulated at 37°C with 25 nM EGF for 1 min and protein extraction was performed with either regular lysis buffer containing 150 mM NaCl (see Methods) or 500 mM NaCl. High NaCl concentrations have been shown to promote disruption of interactions between SH2-containing proteins and their phosphorylated binding partner sites (Grucza, R. A., et al., *Biochemistry*, 39(33), 10072-10081). 670 < N < 1600. Error bars are standard error of measured phosphorylation percentages.



Supplementary Figure 2. Assessment and correction of steric hindrance in sequentially incubated antibodies for 3-color SiMPull. (A) Evaluation of steric hindrance between anti-pY1068-CF555 and anti-PY-AF647 (PY) antibodies. CHO-EGFR-GFP cells were stimulated with 25 nM EGF for 5 min at 37°C and EGFR phosphorylation quantified using 3-color SiMPull. Labeling with anti-pY1068 first did not reduce subsequent labeling by anti-PY. However, a reduction in pY1068+ receptors is seen when the labeling order is reversed. Number of receptors analyzed per measurement, N>800. n.s. not significant, P = 0.5187. **(B)** Evaluation of steric hindrance between anti-pY1068-CF555 and anti-pY1173-CF640R antibodies. Cells were stimulated as described in (A) and receptor phosphorylation assayed by 3-color SiMPull. A reduction in labeling was observed for the antibody that is applied second in the labeling sequence. N>780 per measurement. **(C)** Diagram describing estimation of correction factor (α) to calculate actual fraction of receptors with dual phosphorylation (D'). The observed reduction in labeling with Antibody 1 alone (left bar) as compared to Antibody 1 following Antibody 2 (right bar) indicates the level of steric hindrance. From this information, the correction factor can be calculated. **(D)** Validation of the correction factor by exchanged labeling order. After applying the correction factor ("Corrected" bars), the percentage of pY1068+pY1173+ receptors is similar. Error bars are standard error of measured phosphorylation percentages.



Supplementary Figure 3. Importance of multi-color imaging for accurate quantification of phosphorylation percentages. (A) Representative images displaying raw data and blob-reconstructed localized molecules from a 3-color SiMPull experiment. CHO-EGFR-GFP cells were stimulated with 25 nM EGF for 5 min at 37°C and assayed using anti-pY1068-CF555 (yellow) and anti-pY1173-CF640R (pink) antibodies. **(B)** Quantification of total number of pY1068 and pY1173 localizations per field of view when only those two channels are examined. EGFR-GFP channel was ignored for this quantification to emulate a 2-color SiMPull experiment. **(C)** Quantification of total number of pY1068 and pY1173 localizations per field of view using 3-color SiMPull. Here, the EGFR-GFP channel was used to identify pY1068 and pY1173 localizations overlapping with EGFR molecules, removing contributions from non-specific antibody binding. **(D)** In the absence of the EGFR-GFP channel to identify receptor locations, the 2-color SiMPull underestimates protein multi-phosphorylation. Number of receptors per condition, N>2400. Error bars are standard error of measured phosphorylation percentages.