

# Detection and Visual Localization of Individual Cellular Proteins Using a Proximity Ligation Assay



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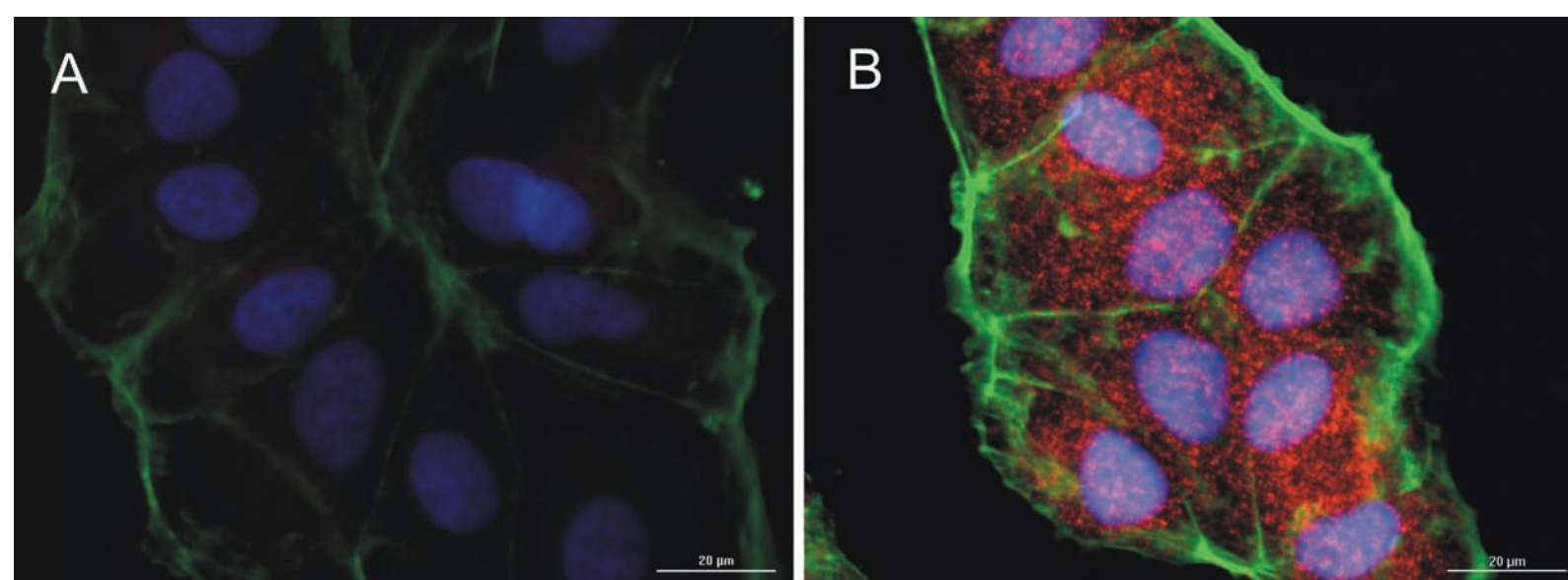
## Abstract

With the mapping of the human proteome the understanding of protein interaction and localization in mammalian cells grows ever more complex. The complexity of the proteome and the understanding of its inner workings create a need for more precise and accurate ways of detecting targets. Besides general detection and localization of a protein, it is necessary to investigate its interaction partners, posttranslational modifications or its role in protein complexes in order to understand the complete role a particular protein plays.

Proximity ligation assays (*in situ* PLA) combine the specificity of antibody based detection of antigenic epitopes with a signal amplification that allows the visualization of components through fluorescence microscopy [1-2]. Proximity ligation assay is a technology that extends the capabilities of traditional immunoassays to include direct detection of proteins, protein interactions and modifications with high specificity and sensitivity. Protein targets can be detected and localized with single molecule resolution and quantified in unmodified cells and tissues.

BioTek's Cytation™ 5 has a number of features that enable Duolink® imaging. Four separate LED positions allow for multiplex fluorescence imaging using a number of different magnification microscope objectives. Besides identification of the protein(s) of interest, counterstaining for cytoplasmic markers or nuclei provide cellular location information. In addition the imager holds 6 objectives with magnification up to 60X. Gen5™ software provides autofocusing of cells in microplates, capturing of images with both automatic or user defined parameters (LED intensity, CCD gain, integration time, etc.) and cellular analysis algorithms that allow for cell segmentation and cell counting. The Gen5 software used to control reader function is also capable of significant automated image analysis. Image analysis can be used to count numbers of cells that meet threshold and size criteria.

## Specificity of Duolink Staining



**Figure 1. Specificity of Duolink® Staining.** MDA-MB-175 cells plated in 96-well microplates and grown overnight at 37 °C, 5% CO<sub>2</sub> in Advanced DMEM, supplemented with in 10% FBS serum, 2 mM glutamine were fixed in 4% paraformaldehyde, permeabilized and blocked prior to antibody binding and Duolink® processing. **(A)** Control wells where 1° antibody was omitted and **(B)** primary mouse anti-TK1 monoclonal antibody was added prior to subsequent Duolink processing. Cells for both figures were treated with Duolink® anti-mouse PLUS and MINUS secondary antibodies and the signal amplified with the Red Detection System. Cells were counterstained with the DAPI and AlexaFluor 488- phalloidin. Images were taken with a 60X objective.

## Materials and Methods

### Cell Culture

MDA-MD-175 and U-2 OS cells were cultured in Advanced DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37 °C in 5% CO<sub>2</sub>. Cultures were routinely trypsinized (0.05% Trypsin-EDTA) at 80% confluency. For experiments, cells were plated into Corning 3904 black sided clear bottom 96-ell microplates at 2,500 to 10,000 cells per well depending on the experiment.

### Detection

Primary antibodies in conjunction with the Duolink® secondary PLA conjugated antibodies and the Red Duolink detection system were used to detect specific cellular targets. Mouse anti-TK-1 monoclonal antibody (cat. # WH0007083M2) was purchased from Sigma-Aldrich (St. Louis, MO), while mouse anti-elf4E monoclonal antibody (cat. #ab171091) and rabbit anti-elf4E(phospho S209) monoclonal antibody (cat. # ab76256) were obtained from Abcam® (Cambridge, MA). Duolink anti-mouse PLUS (cat.# DUO92001), anti-mouse MINUS (cat. DUO92004), anti-Rabbit MINUS (cat.# DUO92005) secondary antibodies, and the Red Detection Reagents (cat.# DUO92008), were obtained from Sigma-Aldrich (St. Louis, MO).

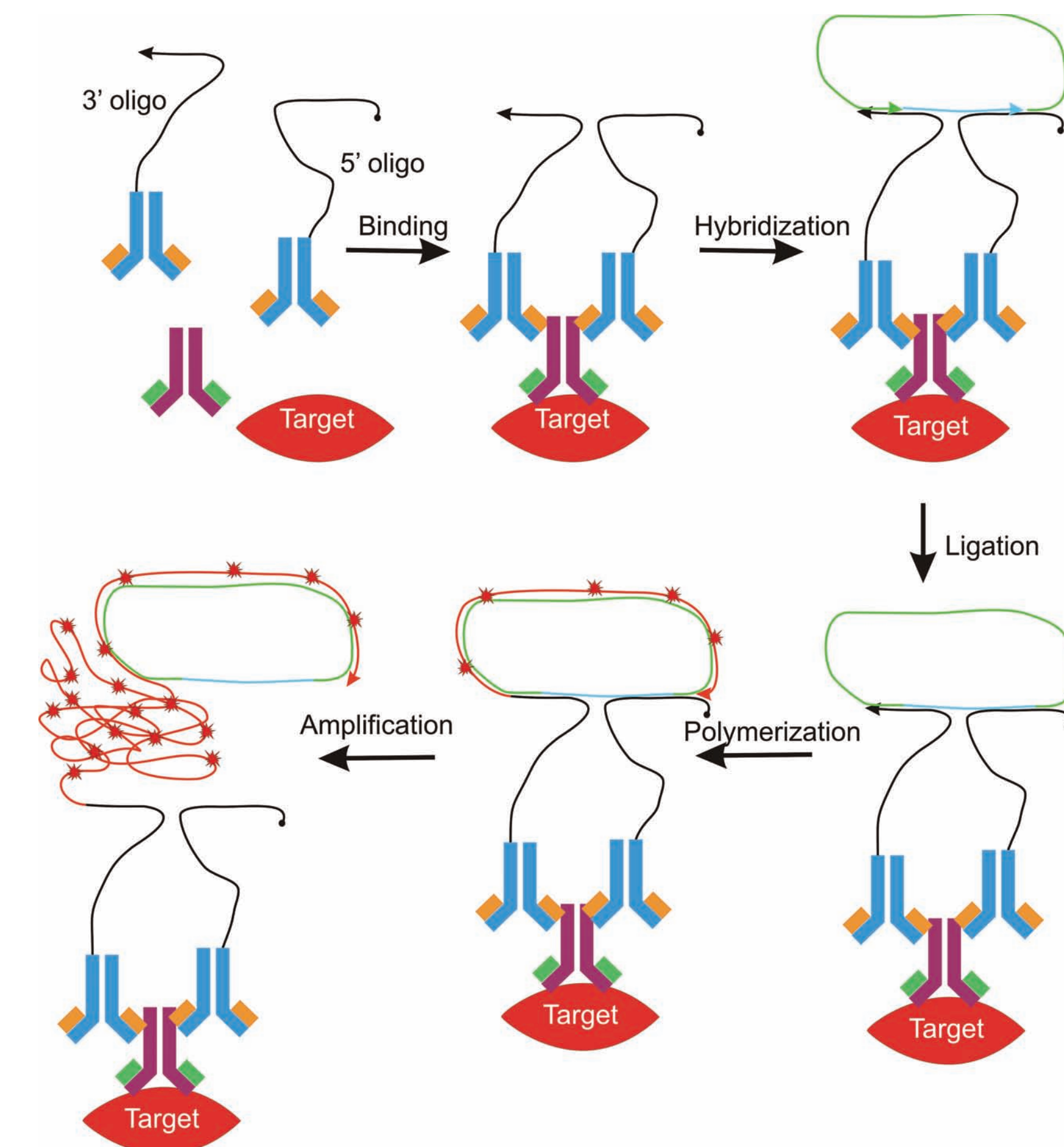
### Imaging

Cultures were imaged using a Cytation 5 Cell Imaging Multi-Mode Reader (BioTek Instruments, Winooski, VT) Configured with DAPI, GFP and TR light cubes. The imager uses a combination of LED light sources in conjunction with band pass filters and dichroic mirrors to provide appropriate wavelength light. The DAPI light cube uses a 337/50 excitation filter and a 447/60 emission filter. GFP light cube uses a 469/35 excitation filter and a 525/39 emission filter, while the TR light cube uses a 585/29 excitation and 624/40 emission filters.

### Image Analysis

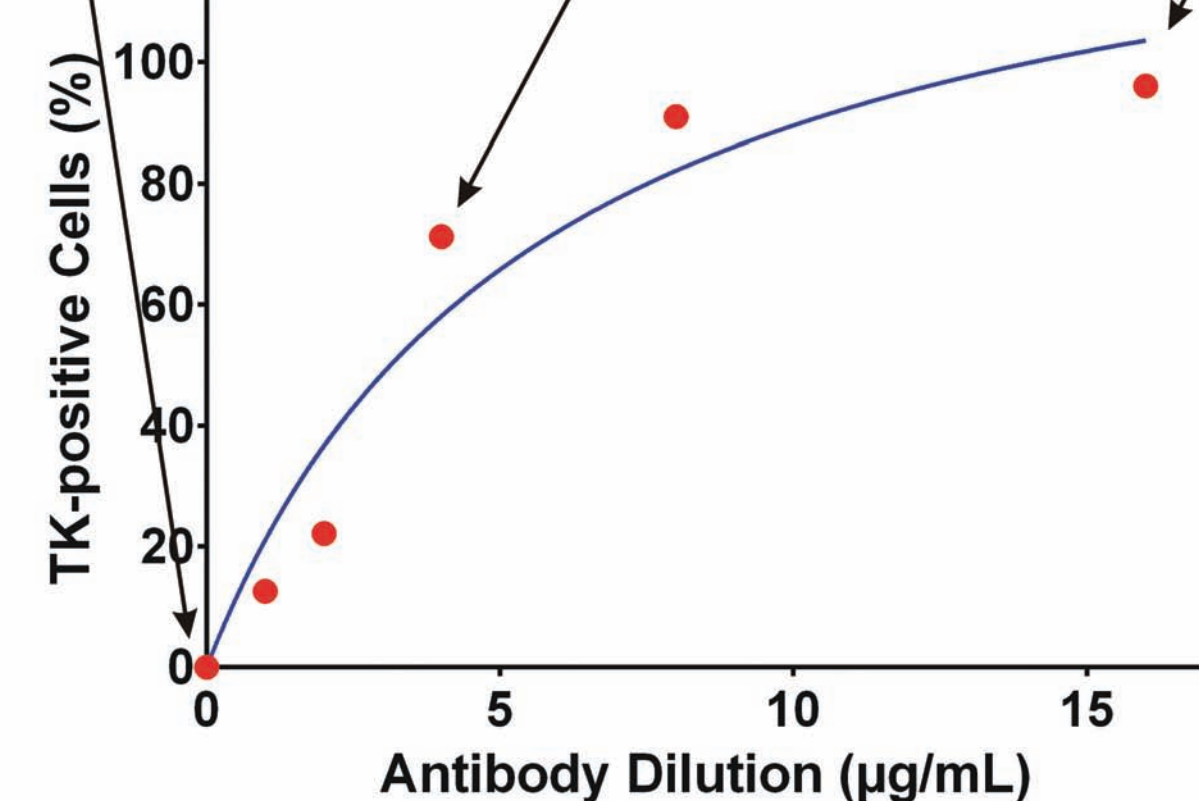
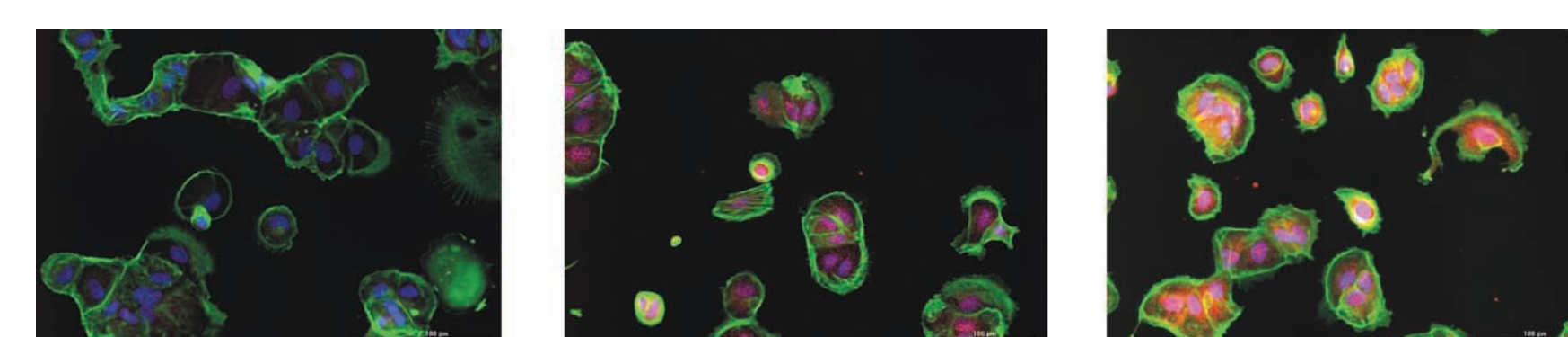
Multiple three-color overlaid digital images were electronically stitched using Gen5 software. Typically samples were imaged by making stitching several images into a single montage. Object cell counting of the DAPI channel was used to identify cell nuclei. Subpopulation analysis was used to determine the mean fluorescence intensity of the TR channel as a means to assess TK-1 positive cells.

## DuoLink® Technology



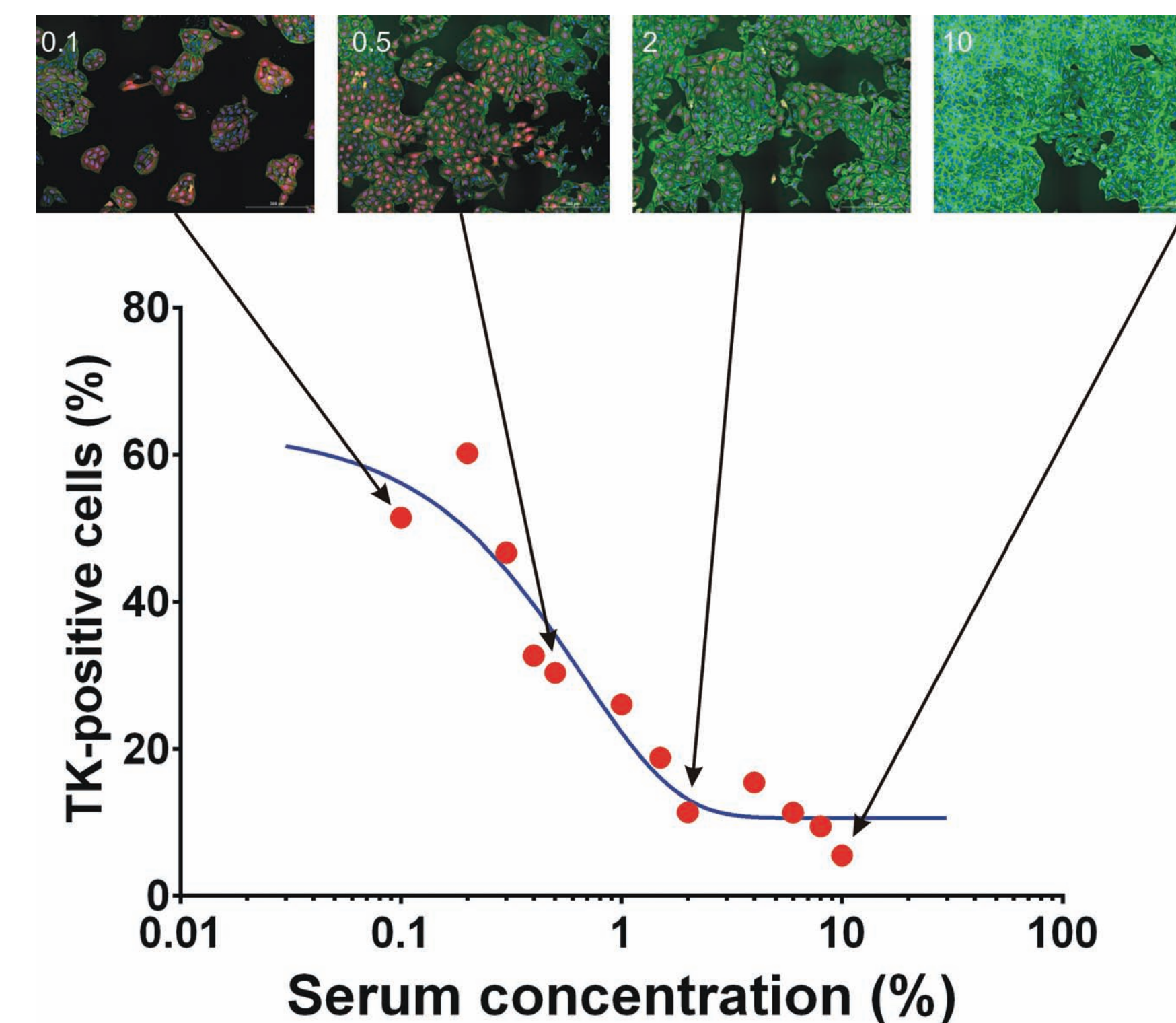
**Figure 2. Duolink technology.** The Duolink® In Situ reagents are based on *in situ* PLA®. One or two primary antibodies raised in different species recognize the target antigen or antigens of interest. Species-specific secondary antibodies, called PLA probes, each with a unique short DNA strand (PLUS and MINUS) attached to it, bind to the primary antibodies. When the PLA probes are in close proximity (<40 nm), the DNA strands can interact through a subsequent addition of two other circle-forming DNA oligonucleotides. After joining of the two added oligonucleotides by enzymatic ligation, they are amplified via rolling circle amplification using a polymerase. After the amplification reaction, several-hundredfold replication of the DNA circle has occurred, and labeled complementary oligonucleotide probes highlight the product. The resulting high concentration of fluorescence in each single-molecule amplification product is easily visible as a distinct bright dot when viewed with a fluorescence microscope.

## Primary Antibody Titration



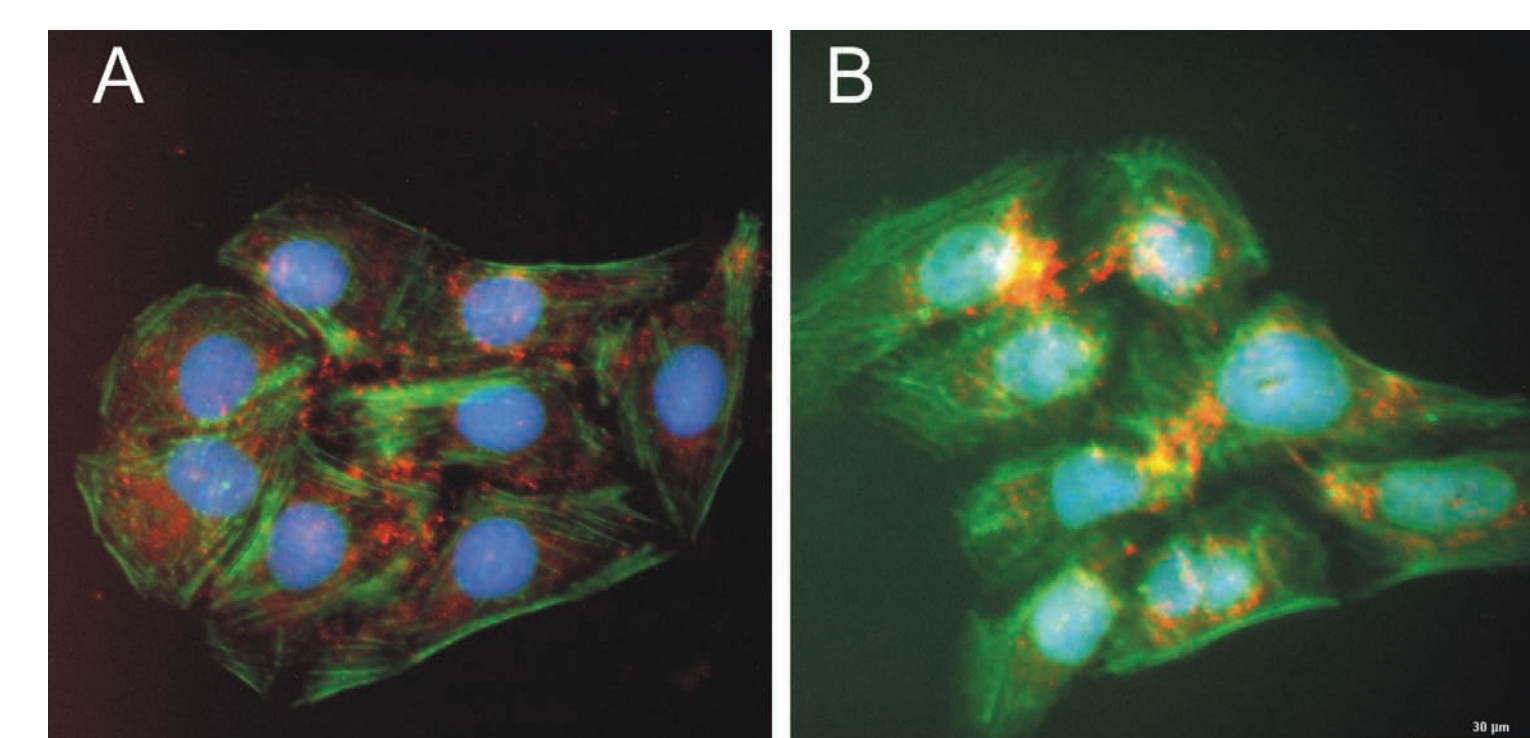
**Figure 3. Effect of Primary Antibody Concentration on Duolink® signal.** MDA-MB-175 cells were seeded into 96-well Microplates and grown overnight at 37 °C, in a humidified 5% CO<sub>2</sub> environment. Cells were then fixed with 4% paraformaldehyde and assayed using a mouse anti-Thymidine kinase monoclonal antibody with Duolink® red detection technology. Three color montages (40X) were obtained by stitching several overlapping images. Image analysis identified cell number using object counting of DAPI stained cell nuclei. Subpopulation analysis of nuclei exceeding a threshold (11,000) for mean RFP fluorescence identified TK positive cells. Data was expressed as a percent of the total. Data points represent the mean of 4 determinations at each serum concentration.

## Affect of Serum on Thymidine Kinase



**Figure 4. Effect of Serum stimulation on Thymidine Kinase Enzyme Levels.** U-2OS cells were serum starved for 24 hours after which various concentrations of serum was added. Cells were then fixed with 4% paraformaldehyde and assayed using Duolink technology using an anti-Thymidine kinase antibody. Image analysis identified cell number using object counting of DAPI stained cell nuclei. Subpopulation analysis of nuclei exceeding a threshold (20,000) for mean RFP fluorescence identified TK positive cells. Data was expressed as a percent of the total. Data points represent the mean of 4 determinations at each serum concentration.

## Localization of Total vs. phospho-ELF4



**Figure 5. Localization of Total and phospho-elf4 protein.** U-2OS cells plated in 96-well microplates and grown overnight at 37 °C, 5% CO<sub>2</sub> in Advanced DMEM, supplemented with in 10% FBS serum, 2 mM glutamine. Cells were then switched to 0.1% serum and serum starved for 24 hours. Cells were then treated with 100 ng/mL EGF for 15 minutes then fixed in 4% paraformaldehyde, permeabilized and blocked prior to antibody binding and Duolink processing. **(A)** Duolink staining using a mouse anti-IF4 monoclonal 1° antibody in conjunction with anti-mouse PLUS and MINUS secondary antibodies. **(B)** Duolink staining using a mouse anti-elf4 monoclonal and a Rabbit antiphospho-elf4 1° antibodies in conjunction with anti-mouse PLUS and an anti-rabbit MINUS secondary antibodies. Both reactions were treated with the Red detection system.

## References

- Söderberg, Ola; Gullberg, Mats; Jarvius, Malin; Ridderstråle, Karin; Leuchowius, Karl-Johan; Jarvius, Jonas; Wester, Kenneth; Hydring, Per; et al. (2006). Direct observation of individual endogenous protein complexes *in situ* by proximity ligation. *Nature Methods* **3** (12): 995–1000. doi:10.1038/nmeth947. PMID 17072308.
- Jarvius, M.; Paulsson, J.; Weibrecht, I.; Leuchowius, K.-J.; Andersson, A.-C.; Wahlby, C.; Gullberg, M.; Botling, J.; et al. (2007). *In Situ* Detection of Phosphorylated Platelet-derived Growth Factor Receptor Using a Generalized Proximity Ligation Method. *Molecular & Cellular Proteomics* **6** (9): 1500–9. doi:10.1074/mcp.M700166-MCP200. PMID 17565975.

## Instrumentation



**Figure 6. EL406™ Microplate Dispenser.** The EL406 is a modular upgradable full plate washer with reagent dispensing capabilities. Besides the 96/384 Dual Action washer manifold, the EL406 has a peri-pump (8 tube dispensers) dispenser and two additional syringe pump dispensers can be added. The syringe and washer manifolds can be configured for plate densities from 6- to 384-well.



**Figure 7. Cyation 5 Cell Imaging Multi-Mode Reader.** Cytation™ 5 is a modular, upgradable multi-mode reader that combines automated digital microscopy and conventional microplate detection. Cytation 5 includes both filter- and monochromator-based detection; the microscopy module provides up to 60X magnification in fluorescence, brightfield, color brightfield and phase contrast. Incubation to 65 ° and plate shaking are standard features. The imaging module uses a turret to hold up to 6 objectives. Excitatory light and emission wavelengths for fluorescence microscopy are provided using LED light cubes in combination with specific band pass filters are dichroic mirrors. The imaging module holds up to 4 LED cubes. In conjunction with the multimode reader, Gen5 software, which controls reader function, also provides image analysis and data reduction.

## Conclusions

- Duolink® Assay Technology is amenable to the 96-well microplate format
- Duolink® can be utilized to detect low copy number proteins in cells
  - High signal amplification
  - Discrete localization of signal
- Quantitative and phenotypic changes can be observed
  - Antibody Titration demonstrates requirement for saturation of signal
  - Serum stimulation demonstrates change in thymidine kinase enzyme
  - EGF stimulation demonstrates perinuclear localization of p-ELF4
- EL406 Washer Dispenser
  - Automates the washing steps required of the Duolink Assay Process
- Cytation 5 Cell Imaging Multi-Mode Reader has a number of features that enable live cell imaging
  - Auto-focus and auto-exposure
  - Multiple color imaging capabilities
- Quantitative image analysis using Gen5 Software
  - Montage stitching
  - Mean signal determination
  - Population analysis