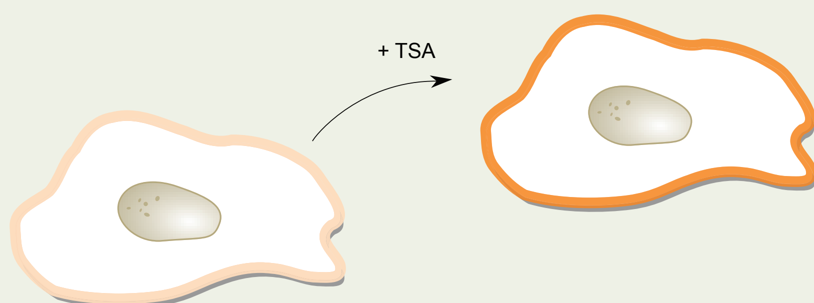

APPLICATION NOTE

Signal Amplification Strategies for Rapid and Sensitive High Content Analysis

Key Features

- Amplifies weak fluorescence signals
- Easily integrated into standard IF protocols
- Reduces exposure time by a factor of 10
- Reduces consumption of primary antibody

TSA reduces exposure time in an imaging assay for PKCα

Background

Protein kinase C alpha (PKCα) is associated with a wide variety of cellular processes including proliferation, adhesion and motility. Over expression of PKCα appears to be associated with certain forms of cancer, and it has been identified as a biomarker for poor prognosis in breast cancer [Lønne *et al*., 2010]. Upon activation with phorbol esters, PKCα translocates from the cytosol to the plasma membrane [Chun *et al*., 1996]. For this reason, it is best studied with a high content analysis method that provides quantitative data on subcellular localization.

Tyramide Signal Amplification™ (TSA) is a widely referenced technology for providing greatly enhanced sensitivity in an extensive range of assays. It can be used in any application that allows the addition of horseradish peroxidase (HRP) to the protocol, such as *in situ* hybridization [Thompson *et al*., 2008], ELISA [Luk *et al*., 2009], microarray-based differential gene and protein expression studies [Karsten *et al*., 2002], immunohistochemistry [Zaidi *et al*., 2000] and immunofluorescence (IF) [Brouns *et al*., 2002].

Using TSA with a standard High Content Screening (HCS) assay for PKCα results in a significant increase in sensitivity without loss of resolution or increase in background. For these reasons, TSA technology is a useful tool in HCS, especially when detecting low-abundance targets in confocal mode. TSA allows the reduction of exposure time by an order of magnitude, resulting in a reduction of the time required for reading a plate.

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Application

We have used the TSA Plus Cyanine 3 Kit (PerkinElmer) for signal enhancement in the PKCα activation assay (Cellomics® PKCα Activation HCS Reagent Kit, Thermo Scientific®) which is a standard IF protocol. HeLa cells were seeded into a 384 CellCarrier™ microtiter plate (PerkinElmer) at a density of 10,000 cells per well and cultured overnight. PKCα was activated by treatment with phorbol 12-myristate-13-acetate (PMA). After 10 minutes of compound incubation at various concentrations, cells were fixed using 3.7 % formaldehyde. The cells were permeabilized with 0.2 % Triton X-100 and labeled with anti-PKCα primary monoclonal antibody followed by an HRP labeled secondary antibody (PerkinElmer). Finally, cells were incubated in Cyanine 3 TSA working solution. Both antibodies were used at various concentrations to identify the optimal staining conditions.

To reference against a non-amplified signal, cells were labeled with DyLight 549 coupled secondary antibody. Nuclei were stained using 10 µM Hoechst 33342 dye solution. The plate was imaged on the Operetta® High Content Screening system in confocal fluorescence mode using the 20X high NA objective.

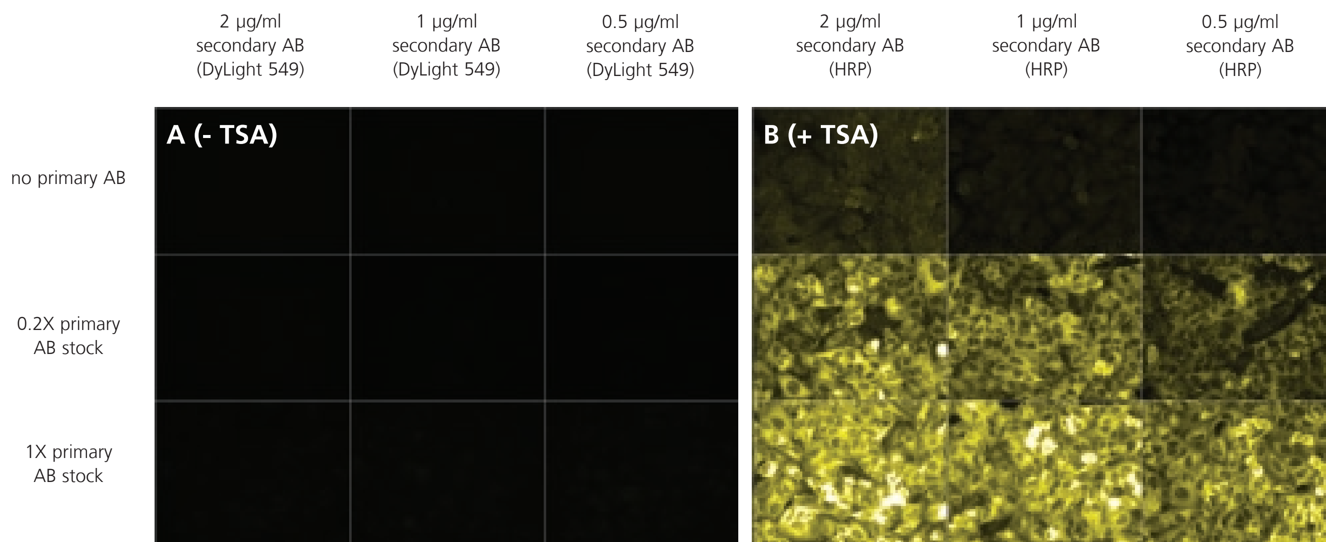

Figure 1. TSA signal amplification of PKCα imaging. Fluorescence images (PKCα channel) of non-stimulated cells labeled with various concentrations of primary / secondary antibodies either with or without the amplification reagent. A| Without TSA. B| 50X diluted TSA amplification reagent stock. The rows show various concentrations of primary PKCα antibody and the columns show different dilutions of secondary antibody, DyLight 549 labeled (A) or HRP labeled (B). The Operetta images were taken in confocal mode using the 20X high NA objective and an exposure time of 800 ms.

Five fields per well were imaged and analyzed (Figures 1-3).

PKCα is localized in the cytoplasm and then translocates to the plasma membrane upon compound stimulation. We showed that TSA amplification did not influence the localization of PKCα signal (Figure 2), but reduced the exposure time by a factor of ten. Furthermore, TSA amplification allowed for 5X less primary antibody to be used compared to the standard protocol. Translocation of PKCα upon PMA treatment was clearly detectable both with and without amplification.

For quantification of PKCα activation, we used the texture image analysis module "SER Ridge" in the Harmony® image analysis software to identify typical membrane shaped “ridges” of 3 pixel width (Figure 3). The calculated signal, based on the frequency and intensity of identified ridges, represented PKCα association with cell membranes. The assay showed the same dynamic range with and without TSA amplification, however, the TSA results had a slightly increased signal to background ratio.

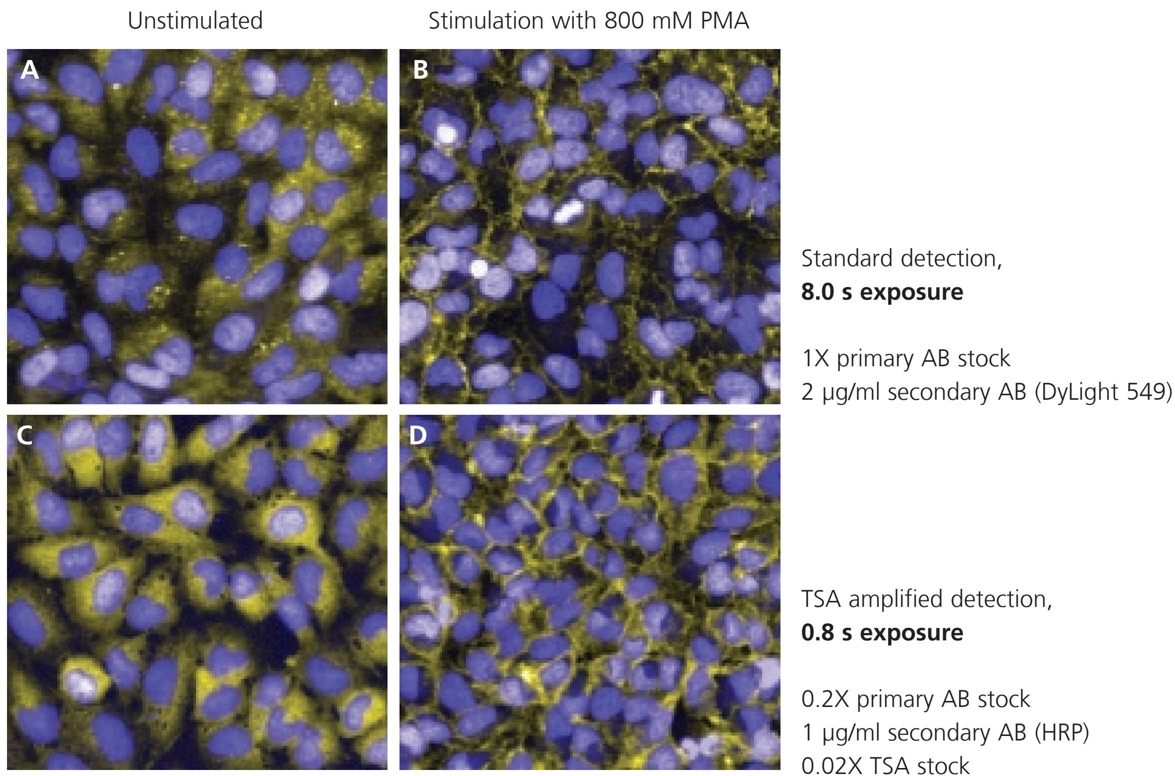

Figure 2. Comparison of PKCα signal without and with TSA amplification. Images show false color overlays of nuclei (Hoechst, blue) and PKCα (yellow). A, B | Non-amplified PKCα signal obtained using an exposure time of 8 s. C, D | TSA amplified PKCα signal obtained using an exposure time of 0.8 s using the 20X high NA objective in confocal mode. B, D | Cells were stimulated with 800 mM PMA. A, C | Cells were not stimulated.

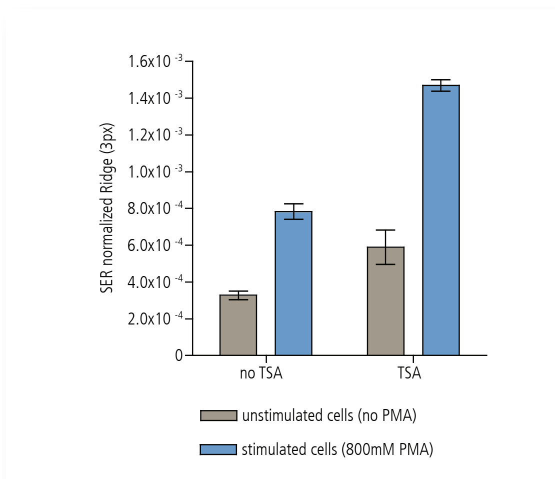

Figure 3. Quantification of signal amplified PKCα activation in HeLa cells stimulated with PMA. The texture analysis module in the Harmony software was used to identify typical membrane shaped "ridges" of 3 pixel width.

Conclusions

We present here the integration of the Tyramide Signal Amplification (TSA) kit into a typical antibody-based high content imaging assay, activation of PKCα. The TSA enhanced signal of PKCα resulted in a significant increase in sensitivity, with an increased signal to background ratio and without loss of resolution. No adaptation of the image analysis strategy was necessary and cytosolic and plasma membrane signal detection allowed for reliable quantification of PKCα activation. Fluorescence signal amplification using TSA is a valuable tool for high content assays that suffer from weak fluorescence signals and require long exposure times.

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