Reagent-based Signal Amplification for High Content Analysis of PKCα Localization in HeLa Cells.

Michael Campisano, Stefan Letzsch

1 Abstract

High content analysis (HCA) is a powerful technique for automated cell biology that enables extraction of quantitative data from cellular images. In some cases, sensitivity limitations have prevented broader application of the technique. Examples include measurement of rare proteins and transcripts, as well as those that are expressed over a very broad dynamic range.

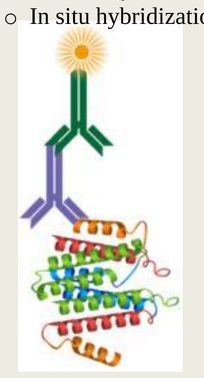
Tyramide signal amplification (TSA™) is an enzymatic technique that is widely used in fixed cell assays like immunocytochemistry and *in situ* hybridization for improved sensitivity and specificity. TSA generally provides 2-3 orders of magnitude in sensitivity enhancement over conventional methods like fluorophore labeled secondary antibodies with similar resolution. TSA allows use of very dilute primary antibody solutions for detection which often improves the specificity of the assay.

Here we demonstrate use of TSA for quantification of PKCα in unstimulated and stimulated HeLa cells using the Operetta® High Content Imaging System.

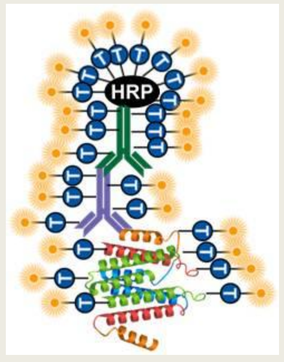
Operetta High Content Imaging System features large image field with high resolution, eight color excitation and emission, and network compatibility in a small footprint. Harmony® software was used for data analysis.

2 What is TSA?

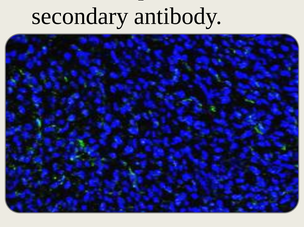
- Enzyme-catalyzed reporter deposition
- 100-1000 fold higher sensitivity than most standard detection methods
- Use more dilute primary antibody for detection
- Signal is covalently bound and well localized to the target
- Widely referenced for
  - Immunocytochemistry (ICC)
  - In situ hybridization (ISH)

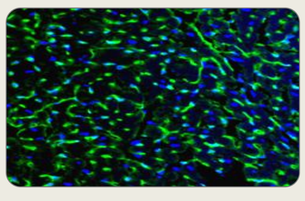


Standard detection with fluorophore labeled secondary antibody.



TSA detection with TSA Plus Fluorescein.

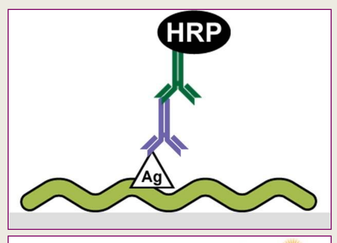




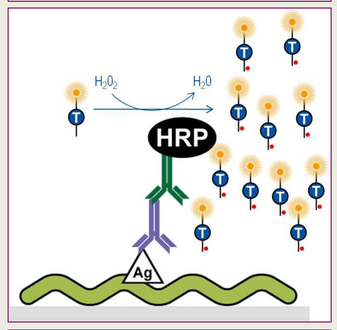
Comparison of standard and TSA detection of CD-31 (PECAM1) in mouse embryo heart tissue. Courtesy of Bin Zhao, Harvard Stem Cell Institute.

3 How does TSA work?

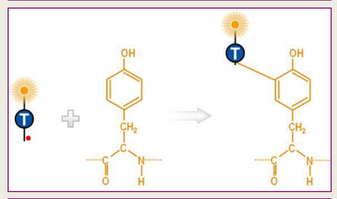
Introduction of HRP
Horseradish peroxidase (HRP) catalyzes the TSA labeling reaction. In this case, HRP is introduced as a secondary antibody conjugate directed against the species of the primary antibody.



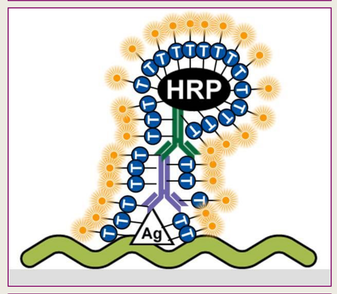
3-10 minute incubation with cyanine 3 TSA reagent
HRP catalyzes reaction of TSA reagent with H_2O_2 yielding highly reactive TSA free radicals.



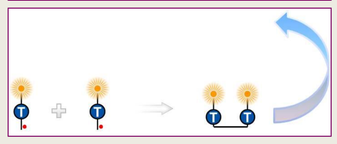
TSA free radicals form covalent bonds to TYR residues proximal to HRP…



…resulting in specific, amplified labeling of the target antigen.



Unused TSA free radicals form dimers that are washed away, ensuring localization of the signal to the target.



4 Protein kinase C alpha (PKCα)

- Associated with a wide variety of cellular processes including proliferation, adhesion and motility
- Over expression 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]

5 Method Development

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 Plus 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 Imaging system in confocal fluorescence mode using the 20X high NA objective.

References

van Gijlswijk, R, Zijlmans, H, Wiegant, J, Bobrow, M, Erickson, T, Adler, K, Tanke, H, and Raap, A. (1997) **Fluorochrome-labeled Tyramides: Use in Immunocytochemistry and Fluorescence In Situ Hybridization** J Histochem Cytochem 45: 375-382

Lønne GK, Cornmark L, Zahirovic IO, Landberg G, Jirström K, Larsson C (2010): **PKCa expression is a marker for breast cancer aggressiveness**. Molecular Cancer, 9(76)

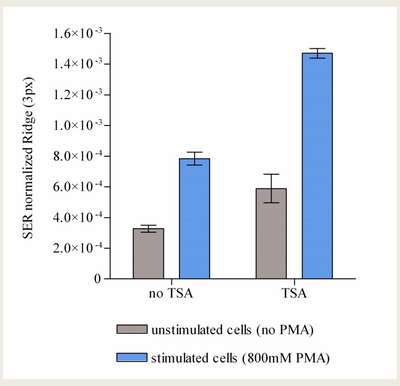
Chun J, Ha M, Jacobson BS (1996): **Differential Translocation of Protein Kinase C during HeLa Cell Adhesion to a Gelatin Substratum**. Journal of Biological Chemistry, 271, 13008-13012

	2µg/ml sec AB (DyLight549)	1µg/ml sec AB (DyLight549)	0.5µg/ml sec AB (DyLight549)	2µg/ml sec AB (HRP)	1µg/ml sec AB (HRP)	0.5µg/ml sec AB (HRP)
no primary AB	A (- TSA)			B (+ TSA)		
0.2x primary AB stock						
1x primary AB stock						

Optimization experiment: Fluorescence images (PKCα channel) of non-stimulated cells labeled with various concentrations of primary / secondary antibodies either with or without the amplification reagent. 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.

Unstimulated	Stimulated with 800mM PMA	
A	B	Standard detection, **8.0s exposure** 1x prim AB stock 2µg/ml sec AB (DyLight549)
C	D	TSA amplified detection, **0.8s exposure** 0.2x prim AB stock 1µg/ml sec AB (HRP) 0.02x TSA stock

Comparison of PKCα signal without and with TSA amplification under optimized conditions. 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 | Unstimulated cells.



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.

6 Conclusions

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.

While enhancing the sensitivity of the assay, use of TSA in place of a fluorophore labeled secondary antibody also

- reduced exposure time by 10-fold.
- reduced consumption of primary antibody by 5-fold.

Signal amplification with TSA is a valuable tool for high content assays limited by weak fluorescence signals.

PerkinElmer, Inc., 940 Winter Street, Waltham, MA USA (800) 762-4000 or (+1) 203 925-4602 www.perkinelmer.com