## LOOKING AT THE FUNCTIONAL STATE OF PROTEINS INSIDE CELLS

Stephen W. Carmichael,[1] Mayo Clinic

Many intracellular proteins are catalysts that regulate cellular functions. These catalysts can be assayed to determine their functional state, but until now it was not possible to simultaneously obtain a functional analysis and spatial data. Tony Ng, Anthony Squire, and others, working in the laboratories of Phillippe Bastiaens and Peter Parker,[2] have combined Fluorescence Lifetime Imaging Microscopy (FLIM) with Fluorescence Resonance Energy Transfer (FRET) to spatially resolve the activation of a protein catalyst within living cells. Their technique was also applied to fixed cells.

Protein kinase CK (PKCK) is a protein catalyst that is specifically activated when the cell is treated with a phorbol ester. Phosphorylation of threonine at position 250 (and also phosphorylation of serine at 260, but this was not exploited) in the PKCK molecule results from its activation. Ng, Squire, *et al.* raised an antibody that was specific for the phosphorylated $Thr^{250}$ (this antibody is called T(P)250). In cultured cells they measured binding of the antibody to $Thr^{250}$ after activation with the phorbol ester, and also blocked the reaction with a specific inhibitor of protein kinase C. In additional experiments they monitored the dephosphorylation of PKCK by removing the relatively hydrophilic agonist, phorbol dibutyrate. Thus, the phosphorylation, blocking the phosphorylation, and the dephosphorylation of the $Thr^{250}$ site on PKCK could be followed through the immunoreaction with T(P)250.

Next, they tagged an antibody to the PKCK molecule with the fluorophore Cy3, which fluoresces green, and tagged T(P) 250 immunoglobulin G (IgG) with Cy5, which fluoresces red. In resting cultured cells that were fixed before they were observed, the PKCK molecule could be seen as a green fluorescence, but there was little or no red fluorescence. When the cells were stimulated with the phorbol ester prior to fixation, both green and red fluorescence were co-localized, indicating that the PKCK molecule was being phosphorylated at $Thr^{250}$. This was interpreted to be only an indication of association, but not necessarily on the same molecule. However, if the Cy3- and Cy5-labeled antibodies were attached to the same molecule, then Cy3 acted as a donor to Cy5 and there would be a measurable decay (on the order of a nanosecond) in the lifetime of Cy3, as measured by FLIM. There was no change in the lifetime when Cy3-anti-PKCK was used alone, but the lifetime of Cy3 was reduced when Cy3-anti-PKCK and Cy5-T(P)250-IgG were both present in stimulated cells. This could be monitored by FLIM and it indicated that the Cy3 and the Cy5 were on the same molecule, specifically PKCK. This provides a molecular proximity assay with resolution on a nanometer scale for the quantitative determination of the activation and intracellular localization of PKCK.

Labeled antibodies to PKCK or activated PKCK, or Cy3-tagged antibodies specific for the endoplasmic reticulum (anti-p62) or the Golgi (anti-galactosyl transferase) were employed to determine the specificity of the fluorophore lifetime changes. There was some overlap in localization of PKCK with both compartments, but the FRET as measured by FLIM showed no diminution in the lifetime of the Cy3 fluorescence, indicating that the PKCK is not in proximity (on the molecular scale) to the markers for the two compartments, although there was some overlap in localization. Additional experiments showed that when PKCK was down-regulated, immunoreactivity to the anti-PKCK was abolished, demonstrating that this antibody was specific for PKCK.

Green Fluorescent Protein (GFP) was used to tag PKCK to monitor activation in living cells. In resting cultured cells, the tag was localized in the cytoplasm and in a perinuclear location; after stimulation the tag accumulated in vesicular structures in the cytoplasm. When Cy3.5-tagged T(P)250-IgG was microinjected into cells with GFP-tagged PKCK, GFP acted as a donor to the Cy3.5 fluorophore. A decreased lifetime for GFP could be measured, showing by another means that the phosphorylation of $Thr^{250}$ was occurring on the PKCK molecule. More importantly, it was determined that the phosphorylation occurred at a low rate in resting cells, and increased when the cells were stimulated. Control experiments were done on fixed cells that gave results consistent with those on living cells. Experiments on fixed human breast tumor specimens demonstrated that PKCK is activated in some, but not all, specimens. Furthermore, PKCK was up-regulated in some tumors, but the protein kinase C content itself did not correlate with activation.

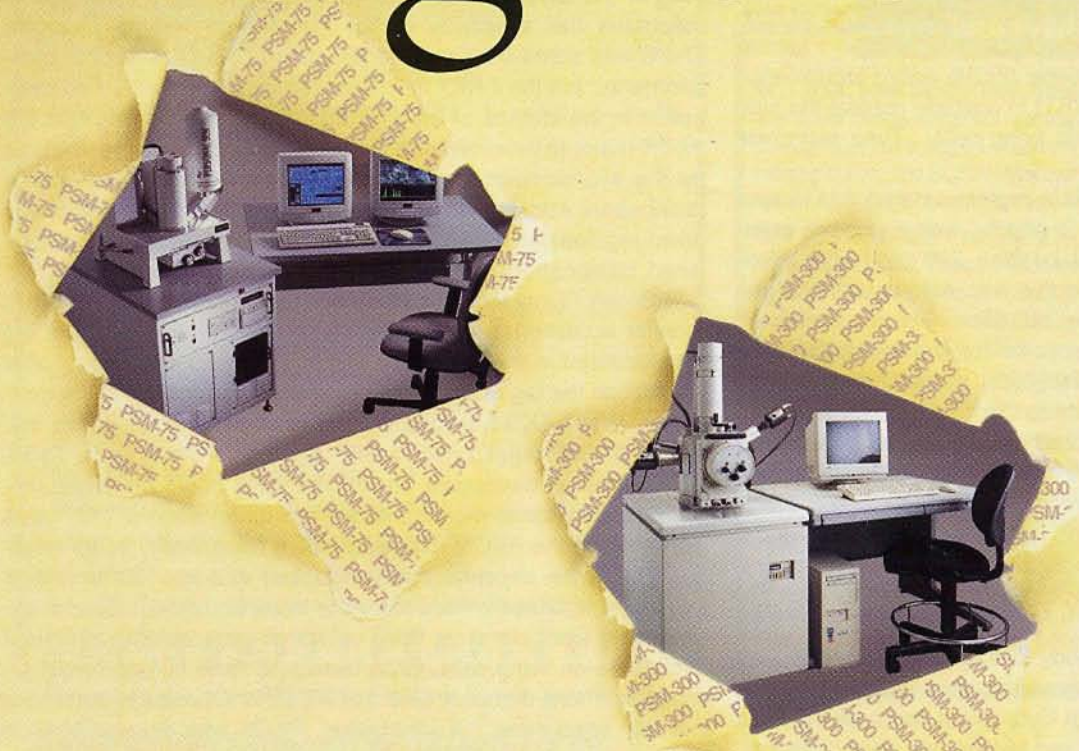
This new technique for imaging the activation of proteins within cells holds much promise. It will be interesting to see how this will be applied to basic problems of cell biology and to the study of human disease.

1. The author gratefully acknowledges Professor Peter Parker for reviewing this article.

2. Ng, T., A. Squire, G. Hansra, F. Bornancin, C. Prevostel, A. Hanby, W. Harris, D. Barnes, S. Schmidt, H. Mellor, P.I.H. Bastiaens, and P.J. Parker, Imaging protein kinase CK activation in cells, *Science,* 283:2085-2089, 1999.

## INDEX OF ARTICLES

# Insightful



## The Right Tools for a Broad Spectrum of Applications

### Better insights, better analysis

Looking for better, more insightful analysis? Look no further. The PSM-300 and the PSM-75 are simple-to-use analytical instruments that give you multiple, valuable insights related to *your specific application*. Better insights for a more comprehensive analysis. Better insights for critical information. Better insights to make the right decisions. Better insights for more effective solutions. Better insights to cost-saving solutions. And the list of insights goes on and on.

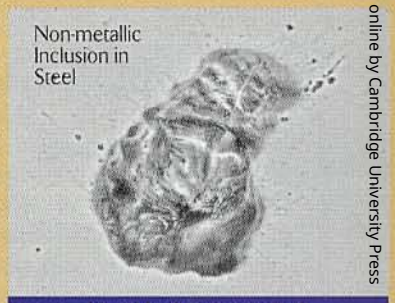
### Customized for your specific application

The PSM-300 or the PSM-75 can be easily customized to your specific application, some of which include:

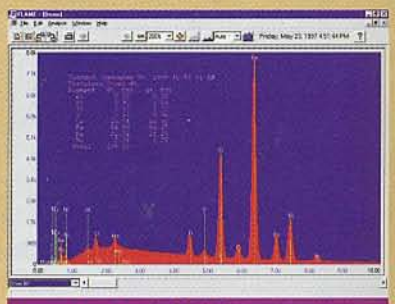
- Automated Gun Shot Residue Analysis
- Automated Inclusion Analysis
- Automated Wear Debris Analysis
- Automated Particle Analysis
- Automated Fiber Analysis

To get more insights into easy, more effective micro-analysis at a truly affordable price call 1-800-538-6850.

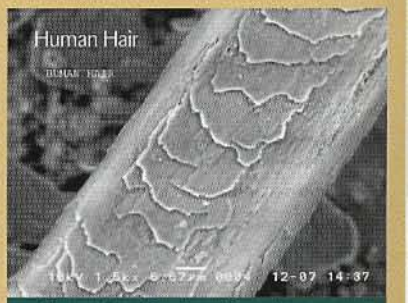

GSR/Forensics/Particle Analysis



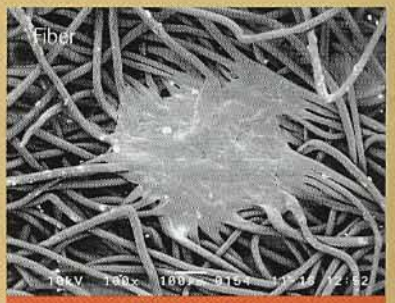

Metal Inclusion Analysis



Inclusion EDX Spectrum




Forensics/Product Development




Fiber Analysis

Microanalysis

THE APPLICATIONS SPECIALISTS

R J Lee Instruments Limited