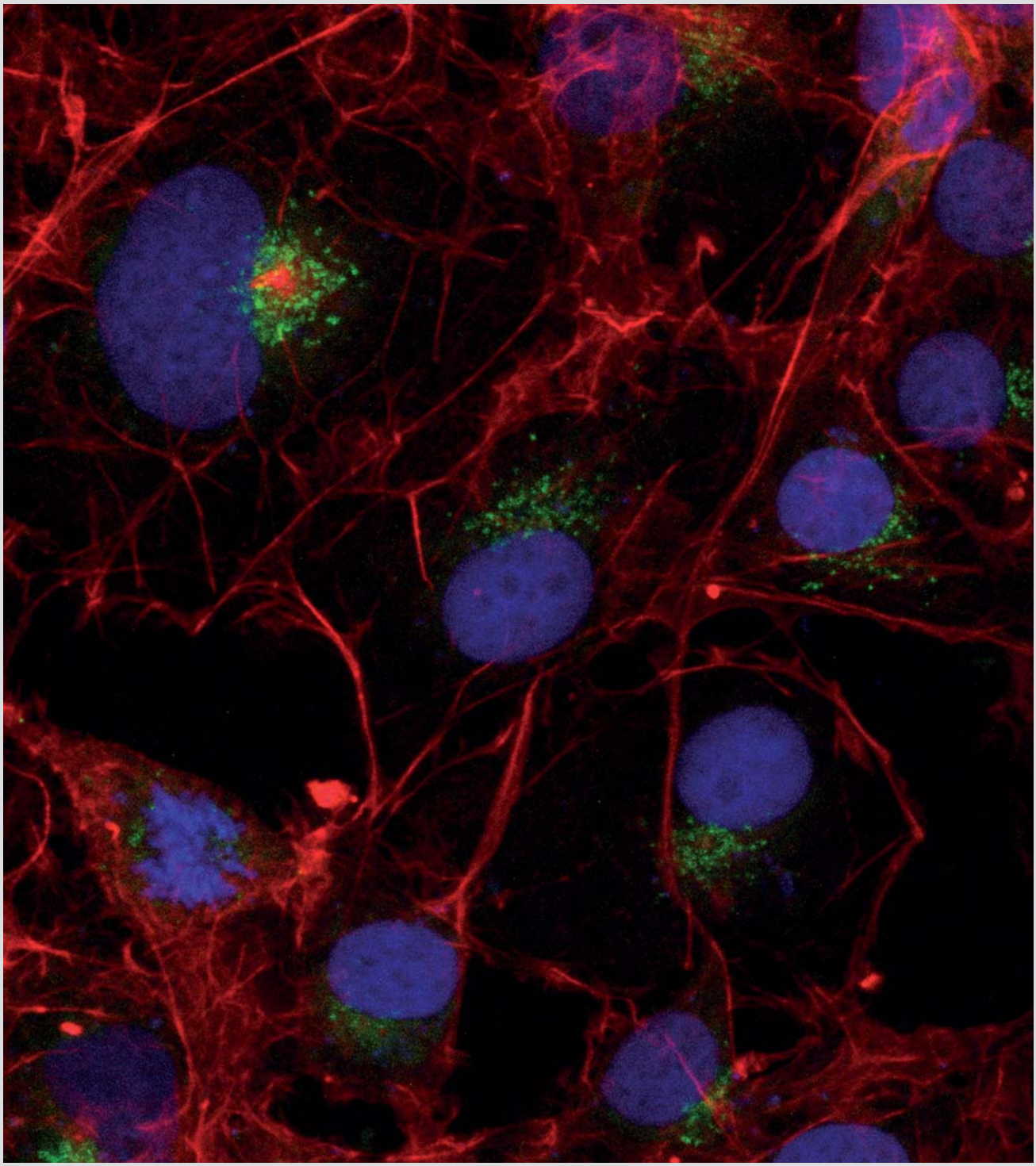
Application Note

Protein Localisation Using Confocal Laser Scanning Microscopy



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Introduction

Over the past ten years, confocal microscopy has developed from a technique limited to specialists in microscopy into a standard research tool in cell biology. Initiated by the discovery of green fluorescent protein (GFP) in the early 1960s, which enabled investigators to apply molecular cloning methods, fusing the fluorophore to various proteins in order to monitor cellular processes in living systems, confocal microscopy gained importance to become an essential technique (**Fig. 1**). The most common application of confocal microscopy is to compare the localisation and behaviour of relevant signalling molecules in one single cell using either fixated cells or live cell imaging. Such studies have been made possible by the development of confocal microscopes that are capable of efficiently collecting multiple colours of fluorescence and developing new dyes beside GFP that have extended the useful spectrum of fluorescence microscopy.

Confocal microscopy offers several advantages over conventional optical microscopy. In the latter, the entire specimen is flooded in light from a light source. Therefore all parts of a specimen throughout the optical path will be excited and the overall fluorescence is detected by a photo detector or a camera. In contrast, a confocal microscope uses point illumination and a pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus information. Only the light within the focal plane can be detected, improving the image quality and resolution compared to wide-field images and reducing bleaching of fluorescent dyes and specimens to improve lifetime and stability of either of them. Additionally a confocal microscope offers the possibility to obtain serial optical sections of the cell or specimen. Using digital image processing techniques, these serial images can be reassembled to form 2D and 3D representations of the structures being studied.

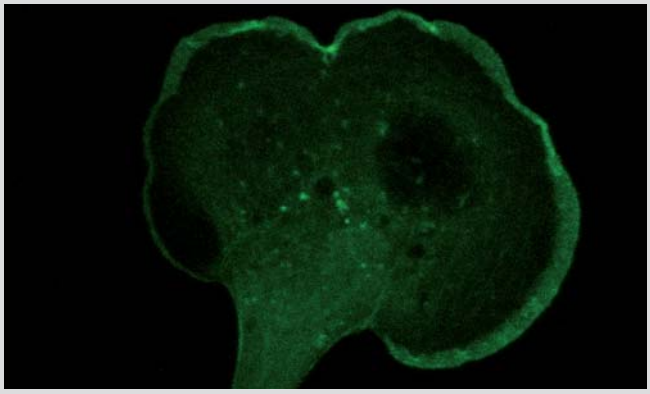


Figure 1: Localisation of green fluorescent protein (GFP) tagged actin at the lamellipodium of a migrating melanoma cell.

The enclosed application note offers a basic protocol for identification of protein localisation by confocal laser scanning microscopy using protein kinase D (PKD) as molecule of interest. The diacylglycerol (DAG) receptor PKD is a family of serine/threonine protein kinases comprising 3 structurally related members: PKD1/PKCμ, PKD2 and PKD3/PKC. PKD contains 2 cysteine-rich motifs that bind DAG, a pleckstrin homology (PH) and a kinase domain. At basal state, PKD isoforms localises to the cytosol, nucleus, plasma membrane and trans Golgi network (TGN), where they are involved in the regulation of diverse cellular processes including vesicle trafficking. The localisation of PKD to the TGN is mediated by the first cysteine-rich motif of the kinase. PKD activation at the TGN requires G proteins and the Golgi-associated PKC. The levels of DAG in the TGN are essential for the localisation and function of PKD at this organelle. At the TGN, PKD is critically involved in the fission of

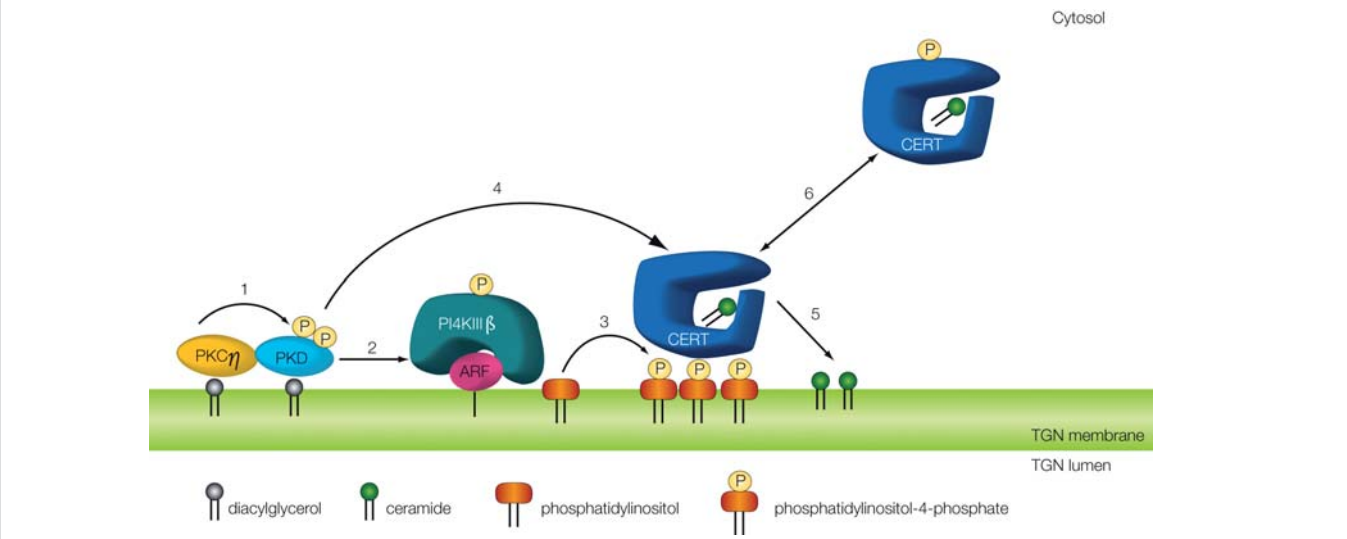


Figure 2: PKD signalling at the Golgi compartment
PKCeta and PKD are recruited to the TGN by DAG. PKCeta activates PKD by direct phosphorylation [1]. PKD then phosphorylates and activates PI4KIIIb [2] , thus increasing PI(4)P levels at the TGN. This, in turn, recruits the CERT protein to the Golgi complex [3] where it contributes to PKD activation and vesicular transport processes by providing ceramide as a precursor for further DAG production. The system is tightly regulated by a negative feedback loop: Active PKD phosphorylates CERT [4], thus decreasing the affinity of CERT towards its lipid target PI(4)P to ensure continuous rounds of lipid transfer from the ER to the Golgi compartment.

Material and Methods

Material, reagents and culture media

Item	Manufacturer	Cat.-No.
RPMI 1640 medium	Invitrogen	21875
Trypsin/EDTA solution	Invitrogen	15400
Opti-MEM	Invitrogen	31985
CELLSTAR®, cell culture flask, 75 cm^2 growth area	Greiner Bio-One GmbH	658 175
CELLSTAR® 24 well cell culture plate	Greiner Bio-One GmbH	662 160
Fetal bovine serum	PAA	A15-101
Lipofectamine 2000	Invitrogen	11668-019
Anti-p230 trans Golgi mouse IgG	BD Biosciences	611280
Alexa Fluor 546 goat anti-mouse IgG	Invitrogen	A-11003
Goat serum	Invitrogen	16210-064
Paraformaldehyde	Electron Microscopy Sciences	19210
Fluormount G	Southern Biotechnology	0100-01

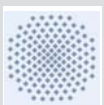
transport carriers en route to the cell surface. This process is mediated by downstream targets of PKD, such as phosphatidylinositol 4-kinase III (PtdIns(4)KIII) and the ceramide transporter CERT [1-3] (**Fig. 2**).
For the identification of PKD and trans Golgi network (TGN) co-localisation Hela cells were transfected with GFP-tagged fusion proteins for PKD1 and PKD2, fixed and stained for a specific TGN marker p230.

Cell culture and transfection

Cell cultures were prepared and maintained according to standard cell culture procedures. Hela cells were maintained in RPMI 1640 medium supplemented with 5% fetal calf serum using CELLSTAR® T75 cell culture flasks. For transient transfection, cells were harvested by Trypsin/EDTA digestion, seeded on coverslips ($1x10^5$ per coverslip) and incubated for 24h in a cell culture incubator at 37°C and 5 % CO_2. Transfection of plasmid DNA for GFP-tagged PKD1 and PKD2 was performed with Lipofectamine 2000 according to the manufacturer's instructions. In brief, cells were incubated with 500 ng plasmid DNA and 1.25 µl Lipofectamine 2000 in Opti-MEM over night before being analysed.

Immunofluorescence and confocal laser scanning microscopy

Transfected Hela cells were washed with PBS, fixed in 4% paraformaldehyde at room temperature for 15 minutes, washed again with PBS, permeabilised with 0.1% Triton-X 100 (2 min at room temperature) and blocked with blocking buffer (5% normal goat serum and 0.05% Tween 20 in PBS) for 30 min. The cells were then incubated with the p230 specific antibody diluted in blocking buffer (1 µg/ml) for 2 hours at room temperature, washed with PBS, incubated with the Alexa Fluor 546 goat anti-mouse secondary antibody diluted in blocking buffer for 1 hour (2 µg/ml) washed with PBS and mounted in Fluormount G. Samples were analysed with a Confocal Laser Scanning Microscope (TCS SP2, Leica, Germany). GFP was excited at 488 nm with an argon laser and fluorescence was detected at 500-540 nm. Alexa546 was excited with the 543nm line of the helium laser and fluorescence was detected at 555-700 nm.
Cells were imaged with a 40.0x/1.25 HCX PL APO objective lens. Images were processed with Adobe Photoshop. Images shown are stacks of several confocal sections.



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Acknowledgment
Experiments have been performed in closed collaboration with Dr. Angelika Hausser at the Institute of Cell Biology and Immunology, University Stuttgart (Germany). Dr. Angelika Hausser leads the group working on molecular characterisation and in vivo function of protein kinase D (PKD) concentrating on intracellular localisation and functional consequences of PKD as well as in vivo analysis of PKD expression profiles in various transgenic mouse models. We want to thank her and her colleagues for the profound introduction in PKD signalling and the excellent experimental setup.

Results and Discussion

Confocal laser scanning microscopy of GFP-tagged PKD1 and PKD2 in Hela cells revealed that both fusion proteins are distributed throughout the cytosol and partly enriched at a perinuclear compartment (**Fig. 3**, green images). To visualise the trans Golgi network cells were stained with the specific marker protein p230 using a specific antibody in combination with the Alexa Fluor 546 goat anti-mouse secondary antibody (**Fig. 3**, red images). Co-localisation of PKD-GFP and p230 at the trans Golgi network is demonstrated by a yellow colour in the overlay image (**Fig. 3**; overlay).

The adjoining described experiment reveals functional distribution of PKD1 and PKD2 in the expected cellular loci indicating that usage of GFP-tagged fusion proteins as well as the applied staining protocol is a useful tool to identify molecules of interest which can be applied to other signalling mediators in a similar way.

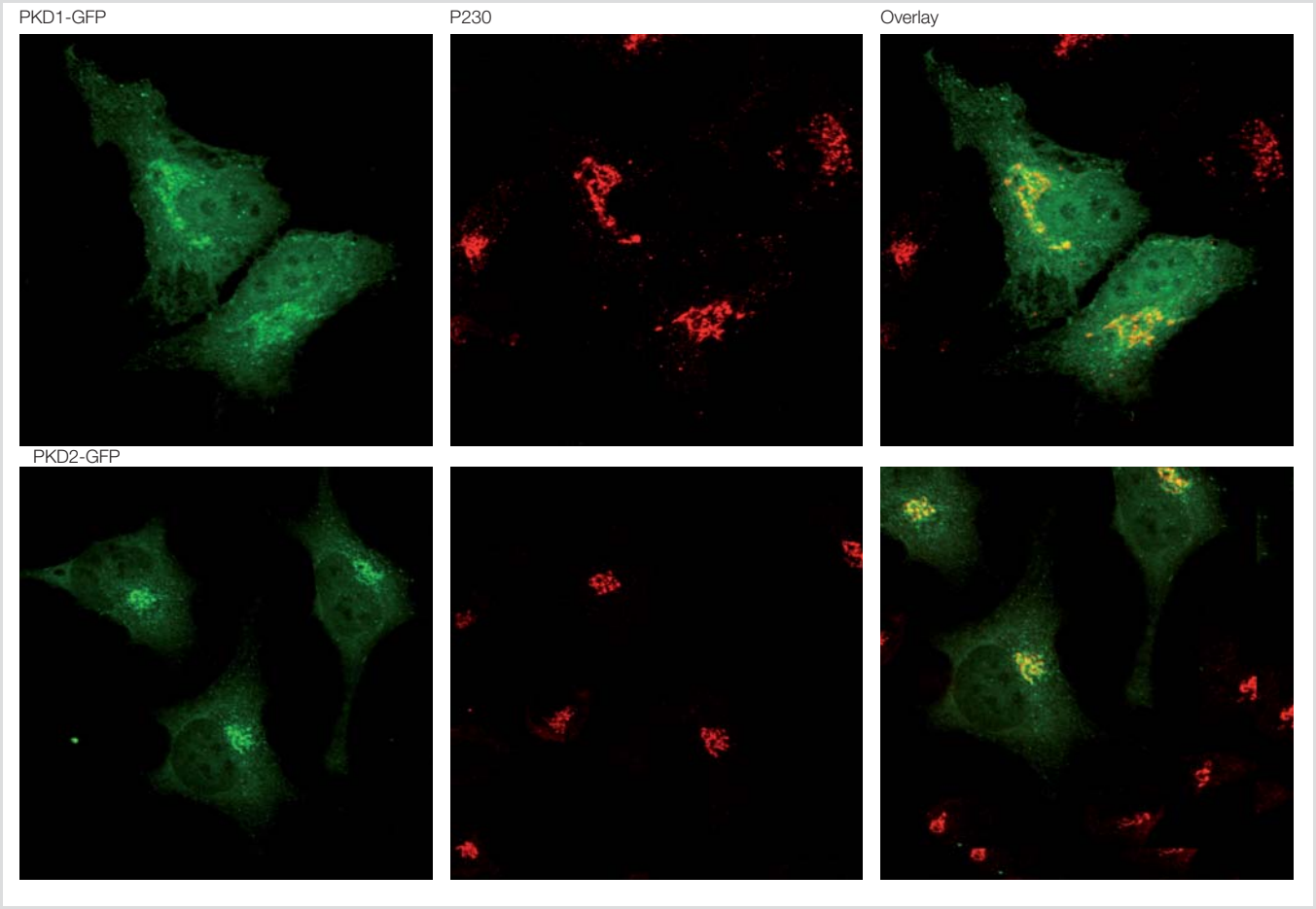

Figure 3: PKD1-GFP and PKD2-GFP localisation to the trans Golgi network
Hela cells were transiently transfected with the indicated expression plasmids. Cells were fixed and stained with p230-specific antibodies as described in material and methods part. Localisation of PKD-GFP fusion proteins is shown in green, p230 in red. Co-localisation of both proteins at the trans Golgi network is indicated by a yellow colour in the overlay image.

References

[1] Wang Q.J. (2006) PKD at the crossroads of DAG and PKC signaling. Trends Pharmacol.Sci. 27, 317-323.

[2] Bard F. and Malhotra 2006) The formation of TGN-to-plasma-membrane transport carriers. Annu.Rev.Cell Dev.Biol. 22, 439-455.

[3] Fugmann T., Hausser A., Schoffler P., Schmid S., Pfizenmaier K. and Olayioye M.A. (2007) Regulation of secretory transport by protein kinase D-mediated phosphorylation of the ceramide transfer protein. J.Cell Biol. 178, 15-22

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