Controls for specificity of the anti-IP5K peptide antibody

S1 Western blot analysis of specific detection of endogenous and over-expressed IP5K by anti-IP5K peptide antibody

Methods:

For Western blot analysis cells were plated at a density of $3x10^5$ cells/ml in 6-well plates and transfected with EGFP/IP5K or IP5K/EGFP as described (Brehm, M. A., Schreiber, I., Bertsch, U., Wegner, A. and Mayr, G. W. (2004) Biochem J **382**, 353-362). After 48h cells were harvested, lysis buffer (8M Urea, 15mM EDTA, 30mM Tris-HCl, pH 7.4) was added and after freezing and thawing in liquid nitrogen and centrifugation (13,000g; 15min; 4°C), the supernatant was removed and protein concentration was determined by a Bradford assay. In other experiments, cells were lysed in MPER® (Pierce) and the fusion proteins were purified using anti-GFP beads as described in Experimental. Next, 50µg of protein or 10µg beads were applied onto SDS-PAGE gels. After gel electrophoresis, protein was transferred onto PVDF membranes followed by blocking non-specific binding sites with 3% BSA in TTBS (10mM Tris-HCl, pH 8.0, 150mM NaCl, and 0.05% Tween20) for 20min. The membranes were washed three times with TTBS for 10min each and incubated with anti-IP5K overnight at 4°C. After washing with TTBS under the same conditions, the membranes were incubated with secondary antibody conjugated with horseradish peroxidase for 1h. Membranes were washed again as above and specific immune complexes were visualized by employing the ECL™ Advance Western Blotting Detection Kit.

Results:

To examine the specificity of the anti-IP5K peptide antibody we performed Western blot analysis of lysates obtained either from untreated H1299 cells or from COS7 cells over-expressing EGFP/IP5K-fusions proteins. The antibody detected endogenous IP5K in all cell lysates (Fig. S1, lanes 1,2,4,6). Only a few non-specific bands were observed, and these were very weak. The EGFP/IP5K fusion protein was detected in the cell lysates (Fig. S1, lane 2) and more strongly after enrichment by pull-down (Fig. S1, lane 3).

The IP5K with C-terminal fusion of EGFP was not detected in the cell lysates (Fig. S1, lane 6) and even after enrichment of the fusion protein by pull-down the detection was about 5-fold weaker than for the N-terminally EGFP fused IP5K. As expected, no endogenous IP5K was detected in the pull-down fractions.

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The reduced ability of the anti-IP5K antibody to detect IP5K when its C-terminus is fused to EGFP is likely due to steric hindrance, since the antibody's epitope is located near the C-terminus of IP5K.

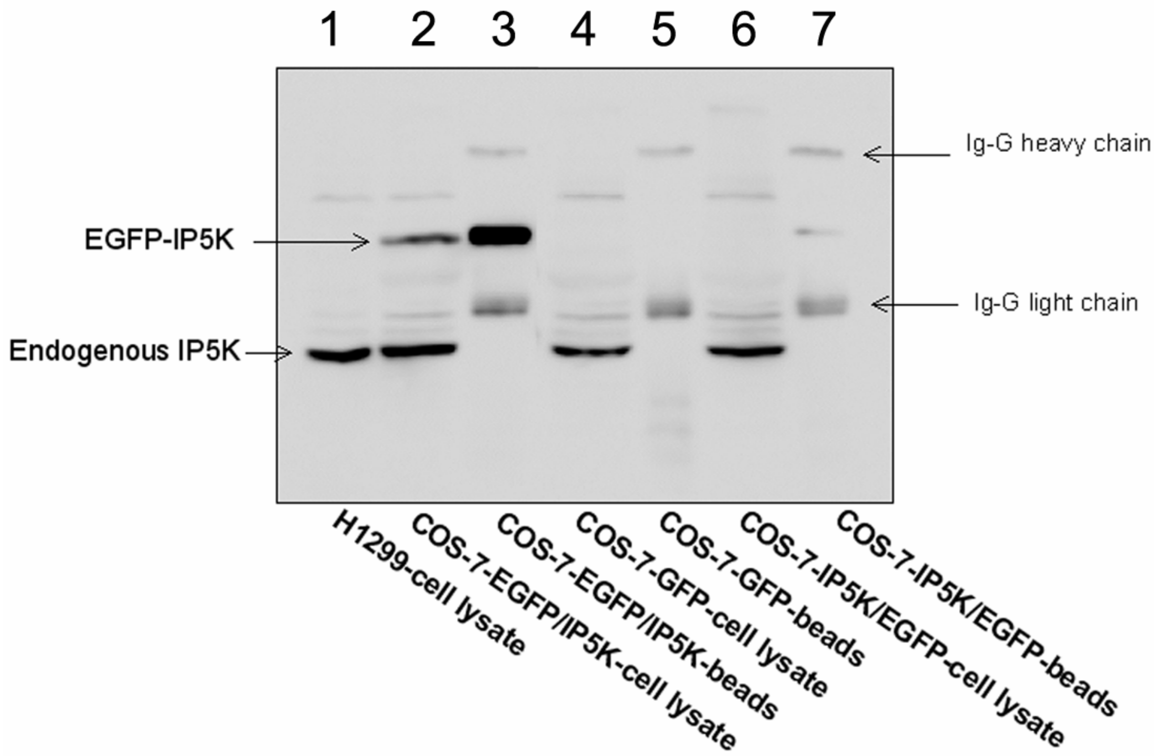


Figure S1: Detection of endogenous and over-expressed IP5K by anti-IP5K antibody in Western blots.

S2 Anti-IP5K specificity demonstrated by using a specific blocking peptide

Methods:

To examine the specificity of anti-IP5K for endogenous IP5K, when employed for indirect immunofluorescence, we pre-incubated the antibody with the antigenic peptide (Invitrogen). A 1:75 dilution of the antibody (in experiments shown in the main manuscript 1:100 dilutions of anti-IP5K were used) was incubated with 5µg of blocking peptide for 3h before the antibody was used for immunofluorescent detection of endogenous IP5K in H1299 cells as described in the Experimental section of the manuscript. In control H1299 cells endogeneous IP5K was detected by anti-IP5K and visualized with a green fluorescent goat anti-rabbit second antibody. All cells were processed under identical conditions and each image was obtained using the same software settings.

Results:

Cells incubated with the the blocking peptide/antibody complex (Fig. S2C,D) showed a weak non-specific staining that was similar in intensity to the signal seen in cells incubated only with secondary antibody (Fig. S2E,F). Moreover, the punctuate nuclear IP5K signal (Fig. S2A,B) was abolished when the blocking peptide was used, or when the anti-IP5K antibody was omitted.

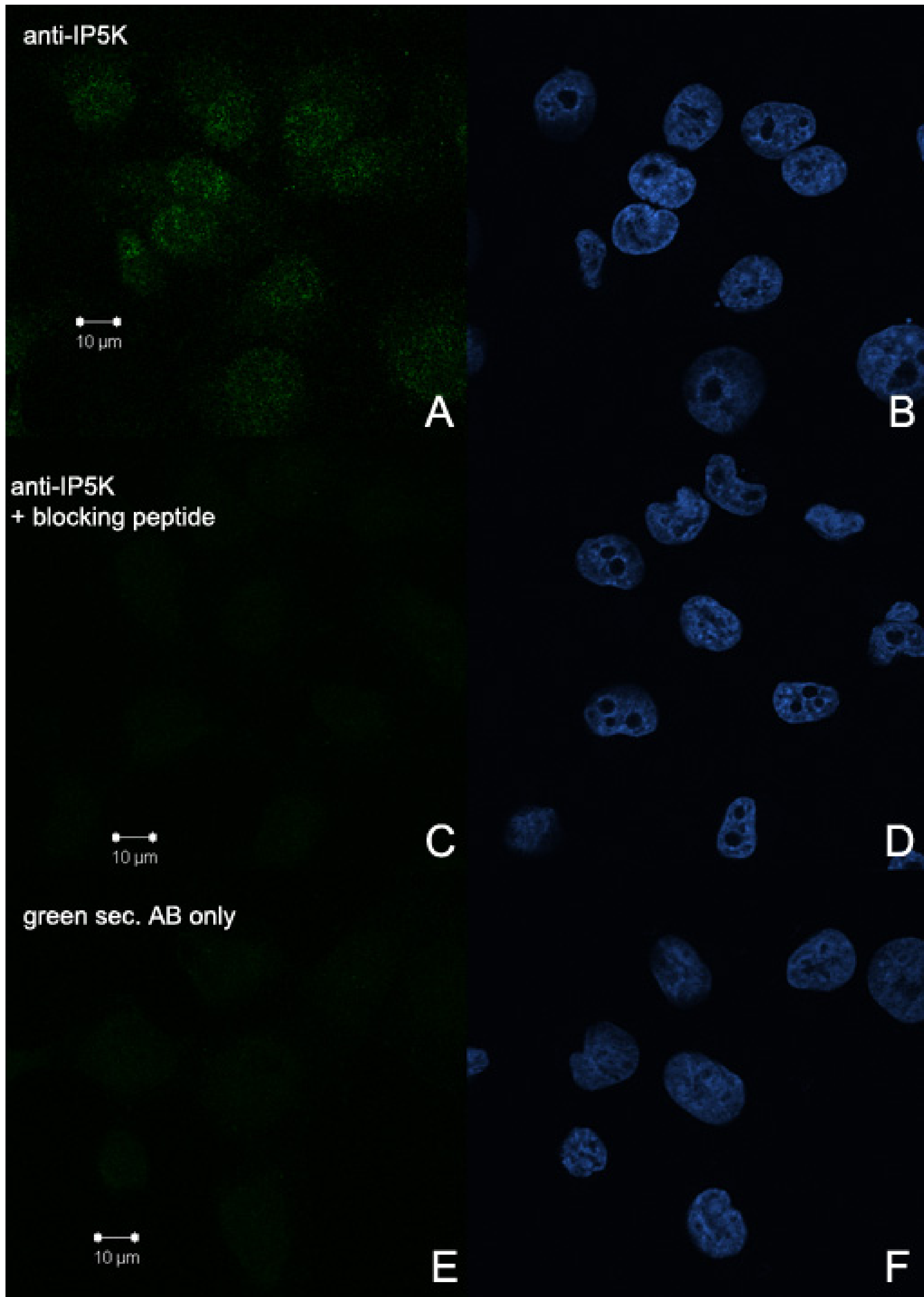


Figure S2. Inhibition of IP5K detection by anti-IP5K with blocking peptide.
(A): Endogenous IP5K was detected in H1299 cells by indirect immunofluorescence employing anti-IP5K peptide antibody and Alexa Fluor 488 conjugated secondary goat anti-rabbit antibody. (C): Cells that were treated with anti-IP5K, pre-incubated with blocking peptide. (E) Cells incubated with secondary antibody only. Nuclei were stained with DAPI (B,D,F). A-F are confocal images recorded at the same exposure time. Scale bars represent 10µm.

S3. Detection of over-expressed untagged IP5K by anti-IP5K

Methods:

To generate a vector coding for untagged IP5K, a stop-codon was introduced behind the C-terminal amino acid of IP5K into the vector coding for IP5K C-terminally fused to EGFP. Therefore the Quikchange^{TM} site-directed mutagenesis kit (Stratagene) was employed according to the manufacturer's instructions with the primer pair: 5'-cattagttctccacaaggtctaatcagatctcgagctc-3' and revers complement primer.

Results:

To investigate the localization of over-expressed untagged IP5K detected with anti-IP5K antibody we transfected H1299 cells with a vector coding for untagged IP5K (see above). Intracellular localization of the untagged IP5K was determined 24h after transfection by immuno-detection by anti-IP5K (Fig. S3A) and nuclei were stained with DAPI (Fig. S3B). The same cytosolic and nuclear localization patterns as for IP5K-EGFP fusion proteins were found. Like observed by immuno-detection of endogenus IP5K, untagged over-expressed IP5K in SG and nucleoli seems not to be fully detected by anti-IP5K. This phenomenon was further investigated in S4.

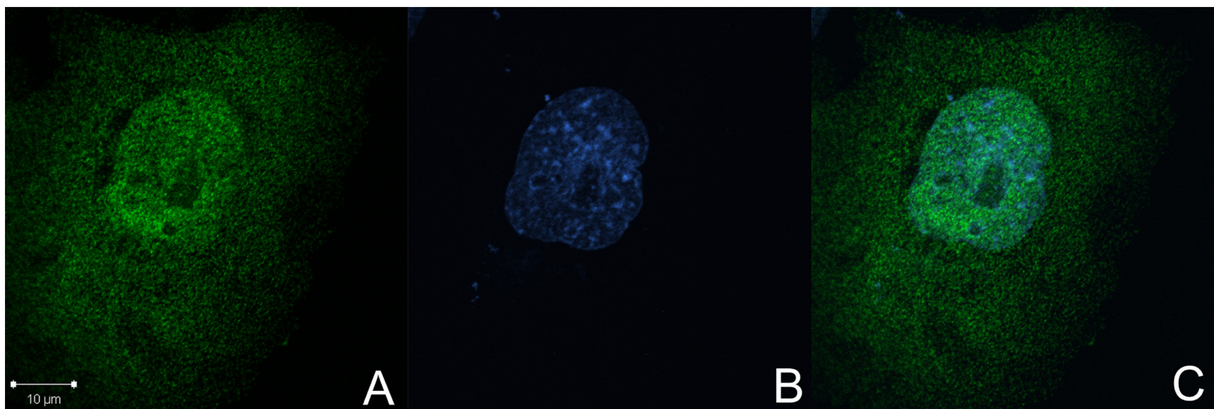


Figure S3. Immunofluorescent staining of untagged over-expressed IP5K by anti-IP5K
H1299 cells over-expressing untagged IP5K for 24h were fixed and IP5K was detected by indirect immunofluorescence employing anti-IP5K peptide antibody and green fluorescent secondary antibody (A). Nuclei were stained with DAPI (B). Oberlay is shown in (C). Scale bar represents 10µm.

S4. Investigation of reduced IP5K-detetcion in nucleoli and stress granules by anti-IP5K

Methods:

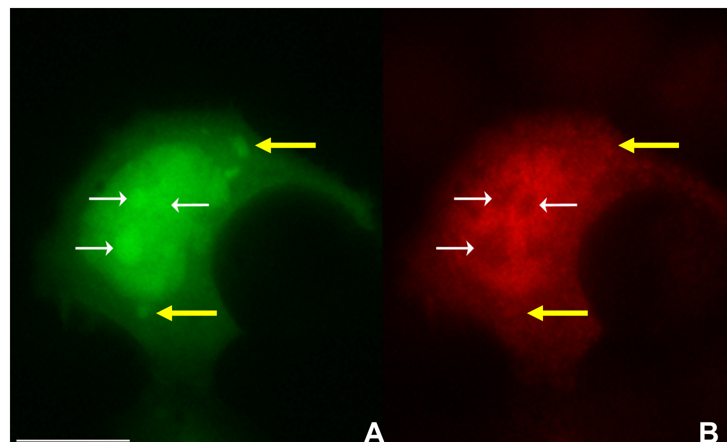
In H1299 cells that over-expressed N-terminally EGFP fused IP5K for 24h, EGFP/IP5K was detected by indirect immunofluorescence with anti-IP5K and Alexa Fluor 546 conjugated secondary antibody. Images of both fluorescences were taken and the intensities of EGFP/IP5K and the red secondary antibody were measured with ImageJ software at one pixel intervals along two 3µm lines through the cytoplasm, stress granules and nucleoli in 25 cells and the average curves were calculated.

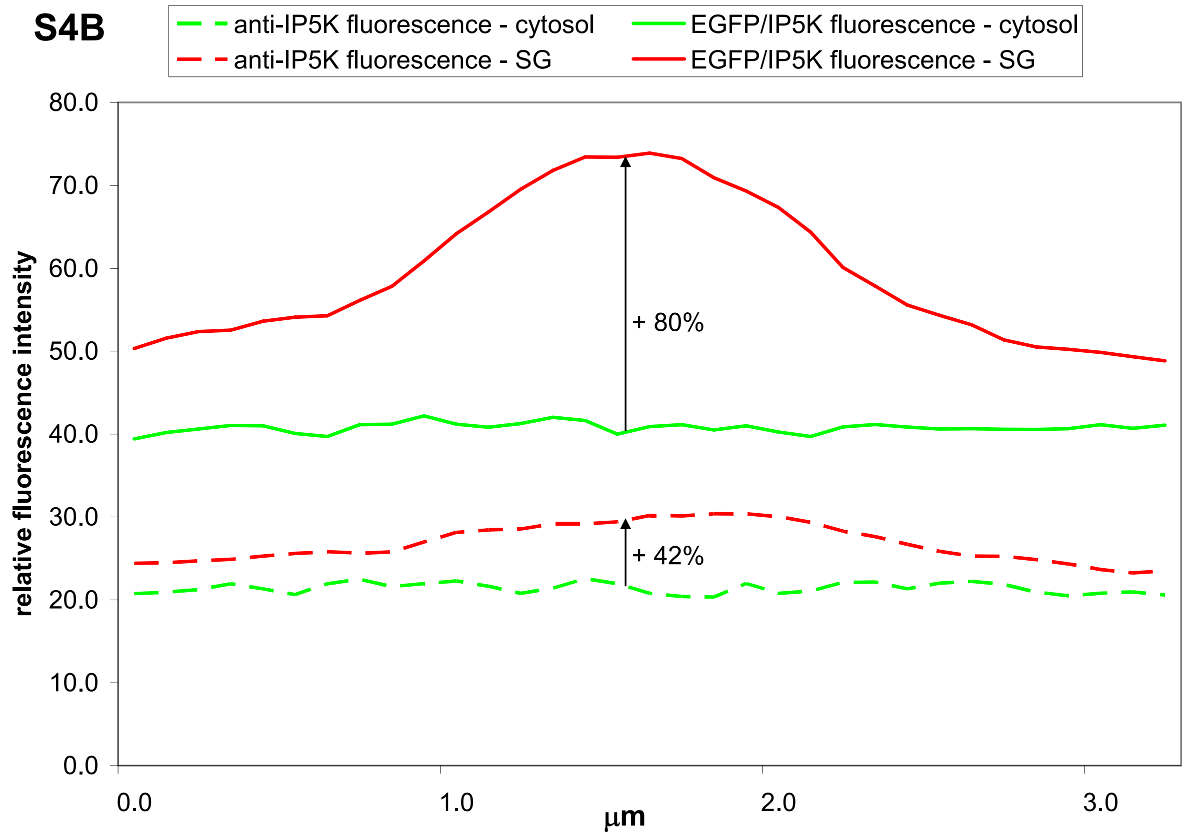
Results:

To investigate if anti-IP5K is able to fully detect IP5K in nucleoli and SG, we determined antibody detection of over-expressed EGFP/IP5K. A representative cell is shown in Fig. S4A visualizing an enrichment of EGFP/IP5K in nucleoli (white arrows) and SG (yellow arrows) that is significantly underestimated using the anti-IP5K antibody (Fig. S4B).

Quantifications of the fluorescence intensities in the cytoplasm, SG and nucleoli are shown in Figs. S4B and C. Graph S4B shows that SG fluorescence intensity detected by anti-IP5K is 42% higher (red dotted line) than the cytosolic level, whereas EGFP fluorescence is 80% (red line) higher than the basic cytosolic level. These data reveal that IP5K is detected about 48% more weakly by anti-IP5K when the enzyme is a component of SG. Graph S4C shows that the nucleolar fluorescence intensity detected by anti-IP5K is 67% higher (blue dotted line) than the cytosolic level, whereas EGFP fluorescence is 130% (blue line) higher than the basic cytosolic level. Thus, IP5K is detected about 51% more weakly by anti-IP5K when the enzyme is located in nuceoli (see white arrows in Fig. S4C). We presume that components of stress granules and nucleoli mask the epitope within IP5K that is normally recognized by the anti-peptide antibody. This situation could arise if IP5K were part of a multiprotein complex.

S4A




S4B
anti-IP5K fluorescence - cytosol
EGFP/IP5K fluorescence - cytosol
anti-IP5K fluorescence - SG
EGFP/IP5K fluorescence - SG
relative fluorescence intensity
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
+ 80%
+ 42%
0.0
1.0
2.0
3.0
µm

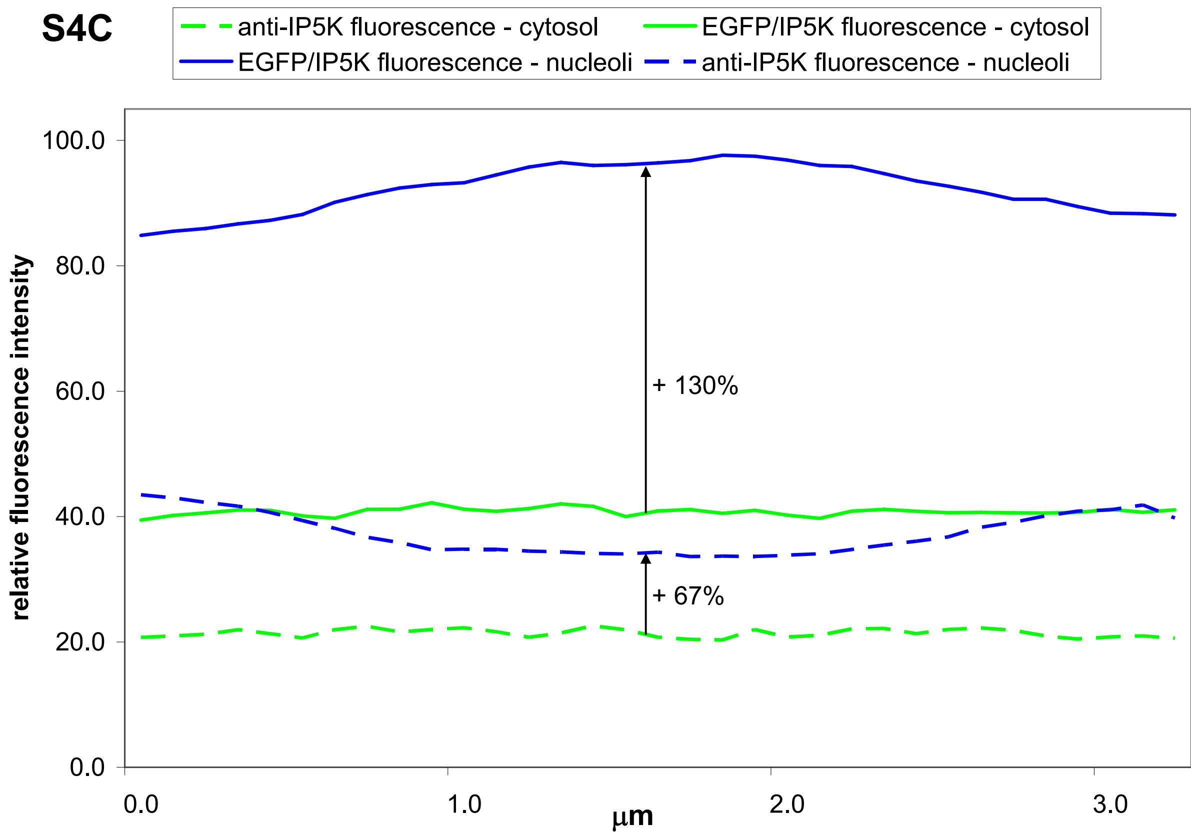

S4C
anti-IP5K fluorescence - cytosol
EGFP/IP5K fluorescence - cytosol
EGFP/IP5K fluorescence - nucleoli
anti-IP5K fluorescence - nucleoli
relative fluorescence intensity
100.0
80.0
60.0
40.0
20.0
0.0
+ 130%
+ 67%
0.0
1.0
2.0
3.0
µm

Figure S4. Detection of over-expressed N-terminally EGFP-fused IP5K by anti-IP5K antibody

Panel (S4A) shows the EGFP fluorescence of EGFP/IP5K (A) and the fusion protein detected by anti-IP5K (B) of a representative H1299 cell. Nucleoli are indicated by white arrows, SG by yellow arrows, scale bar represents 10µm. Panels S4B and C: In H1299 cells over-expressed EGFP/IP5K was detected by indirect immunfluorescence employing anti-IP5K and Alexa Fluor 546 labeled secondary antibody, 24h after transfection. Both fluorescences were measured along 3µm lines in the cytosol (graphs S4 B and C, green), SG (graph S4B, red), and nucleoli (graph S4C, blue) with ImageJ software. The avaraged fluorescence insentities from 25 cells are shown. The percentages show increase in fluorescence intensity in relation to fluorescence intensity in the cytoplasm.