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Immunofluorescence Applications



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The Need for Subcellular Localization Studies

One major rationale for investigating the subcellular location of a specific protein is that location is often tightly connected to function. For example, proteins locating to the nucleus are frequently implicated in gene regulation, proteins in mitochondria with energy production and Golgi-related proteins are often associated with protein modification and sorting. In addition to elucidating functional characteristics of proteins, subcellular location information can also facilitate protein interaction studies. For two proteins to interact, it is a prerequisite that they locate to the same defined site. Thus, knowing the subcellular location of a protein is a key step towards understanding function and probable interaction partners of this protein.

The Human Protein Atlas Project

The Human Protein Atlas (HPA) project (proteinatlas.org) was established in 2003 to allow for a systematic genome-based exploration of the human proteome using antibody-based proteomics¹. This is accomplished by combining high-throughput generation of Prestige Antibodies with protein profiling in a multitude of human tissues and cells. In November 2011, Prestige Antibodies have been used to analyze protein expression of more than 10,200 human genes. Due to the difficulty to acquire spatial information on a fine subcellular level using immunohistochemistry, expression profiling has expanded to analysis with confocal microscopy using fluorescently labeled antibodies^{2,3}. At present, 8,847 proteins have been assigned subcellular positions. Each year protein expression and localization data for approximately 2,500 new proteins are added to the portal. By 2015, a first draft of the spatial expression profile of the human proteome will be completed.

Immunofluorescence (IF) Analysis in the Human Protein Atlas

Aiming at the goal for a subcellular protein atlas, confocal microscopy was selected as imaging system to achieve the highest spatial resolution possible at a reasonable throughput. Three human cell lines were selected for the approach; U-251 MG (glioma), U-2 OS (osteosarcoma) and A-431 (epithelial carcinoma), as well as three reference markers; DAPI staining nucleus and antibodies staining microtubules (anti-tubulin) and the endoplasmic reticulum (anti-calreticulin). The markers serve as controls for sample fixation, permeabilization and immunostaining⁴, as well as guides in the image annotation for assignment of subcellular location.

Antibody dilution and immunostaining procedures are automated and confocal microscopy images are acquired and annotated manually and compared to literature. There are 16 different subcellular locations being annotated (Figure 1) and the staining is further analyzed by scoring intensity on a four-graded scale and assigned staining characteristics.

Figure 2 illustrates differences in nuclear staining patterns. The eight different proteins localize into nucleus, nucleoli or nuclear membrane and show varying staining characteristics such as smooth, granular, speckled and clusters of spots.

On the Human Protein Atlas portal, two images, each containing 6-12 cells, are shown for each Prestige Antibody and cell line. The images are clickable for higher resolution. In addition, three different channels are clickable for visualization of the three markers in blue, red and yellow colors. Antibody staining is shown in green (Figure 3).

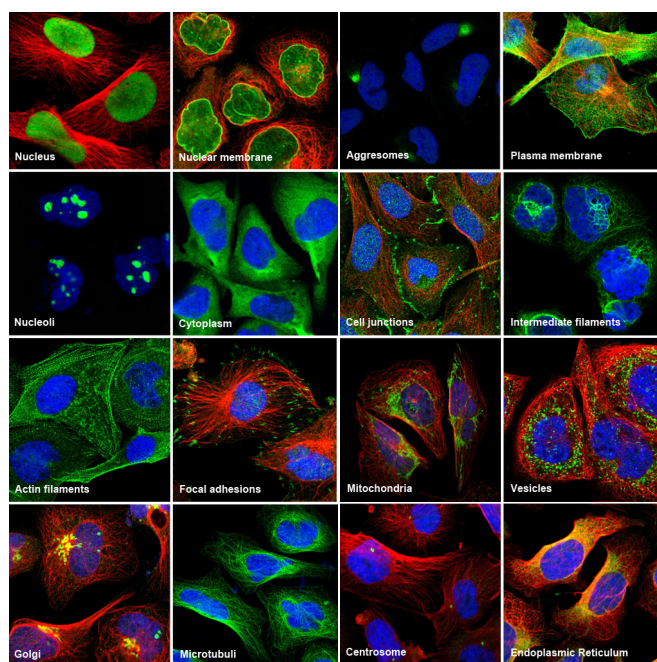


Figure 1: The protein targets on the Human Protein Atlas portal are assigned to one or several of 16 different subcellular compartments; nucleus (HPA002844), nuclear membrane (HPA001209), nucleoli (HPA003436), cytoplasm (HPA001290), actin filaments (HPA006376), focal adhesion sites (HPA001349), Golgi apparatus (HPA000992), microtubuli (HPA006376), aggresomes (HPA027420), plasma membrane (HPA001672), cell junctions (HPA030411), intermediate filaments (HPA000453), mitochondria (HPA000898), vesicles (HPA002290), centrosomes (HPA003647) and endoplasmic reticulum (HPA003906). The HPA product number refers to the Prestige Antibody used in the presented image. Prestige Antibody staining is shown in green, nuclear reference in blue and microtubules reference in red.



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A validation score of the observed staining is assigned for each cell line and is classified as either Supportive, Uncertain or Not Supportive based on concordance with available experimental gene/protein characterization data in the UniProtKB/Swiss-Prot database. Today, 2,485 genes on the HPA portal are represented by antibodies showing results scored as Supportive, i.e. agree with available subcellular localization information. For 6,362 genes with assigned subcellular positions, there is no available literature as reference and the results are thus scored as Uncertain.

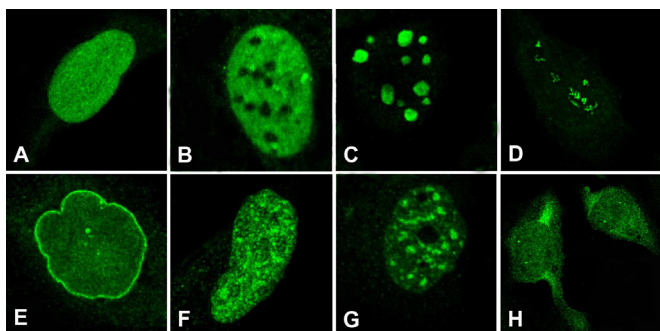


Figure 2: Diversity of nuclear staining patterns shown by eight different antibodies in cell line U2-OS or A-431. **A)** Smooth nuclear staining by anti-MECP2 (HPA000593), **B)** nuclear staining without nucleoli by anti-RBM14 (HPA006628), **C)** nucleoli staining by anti-ZSCAN1 (HPA007938), **D)** cluster staining within nucleoli by anti-MUC4 (HPA005895), **E)** nuclear membrane staining by anti-UNC84B (HPA001209), **F)** nuclear granular staining by anti-BCLAF1 (HPA006669) **G)** nuclear speckled staining by anti-RBM25 (HPA003025), **H)** nuclear body (SMN) staining by anti-DDX20 (HPA005516).

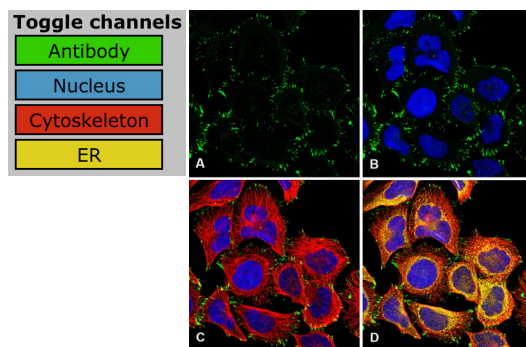


Figure 3: Anti-ZYX (HPA004835) shows staining of focal adhesions in A-431 cells. By clicking on the different channels, the images can be visualized with one or several reference markers. **A)** antibody staining shown in green, **B)** addition of DAPI nuclear reference in blue, **C)** addition of microtubules reference in red and **D)** addition of endoplasmic reticulum (ER) reference in yellow.

HPA Standard Immunofluorescence Protocol

Primary antibodies: Prestige Antibodies® at a working concentration of 1-4 µg/ml. Chicken anti-Calreticulin polyclonal antibody (Abcam) diluted 1000x. Mouse anti-alpha Tubulin monoclonal antibody (Abcam) diluted 1000x.

Secondary antibodies: Alexa® Fluor 555 goat anti-mouse IgG (Invitrogen) diluted 800x. Alexa® Fluor 647 goat anti-chicken IgG (Invitrogen) diluted 800x. Alexa® Fluor 488 goat anti-rabbit IgG (Invitrogen) diluted 800x.

All washes are performed at room temperature (RT).

1. A multiwell, glass-bottomed, plate (Whatman) is coated with fibronectin (conc. 12.5 µg/ml) for 1 h at RT.
2. Cells are seeded (10,000-15,000 cells per well) and incubated at 37 °C in humidified air with 5.2% CO₂ for 4 h.
3. Growth medium is removed and the cells are washed in PBS (8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.2)
4. The cells are fixed for 15 min in ice cold 4% paraformaldehyde pH 7.2-7.3 in growth medium supplemented with 10% fetal bovine serum (FBS).
5. The cells are permeabilized 3x5 min with 0.1% Triton X-100 in PBS.
6. The cells are washed with PBS and incubated overnight at 4 °C with primary antibodies in PBS supplemented with 4% FBS.
7. The following day the cells are washed 4x10 min with PBS and incubated for 1.5 h in RT with secondary antibodies in PBS supplemented with 4% FBS
NOTE: The secondary antibodies are fluorescently labeled and thus light sensitive. The sample should be kept in dim light in this as well as in the following steps.
8. The cells are counterstained for 4 min with the nuclear stain DAPI (Invitrogen) 0.3 µM in PBS.
9. The cells are washed 4x10 min with PBS and mounted in glycerol + 10% 10xPBS.

Summary:

- Subcellular localization studies are a key step for elucidating protein function and interaction. IF images, including annotation of subcellular localization, are freely available via the Human Protein Atlas portal.
- In the Human Protein Atlas project, subcellular localization information of target proteins is gained through confocal microscopy analysis in three different human cell lines. Each target protein is assigned to one or several of 16 individual organelles.
- All IF images shown on the Human Protein Atlas portal are visualized with different organelle probes displayed as different clickable channels in the multicolor images.
- On the Human Protein Atlas portal, IF results are shown for 9,638 proteins, corresponding to 48% of the human proteome. So far, 2,690 Prestige Antibodies are approved for the IF application.

References:

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3. Barbe L. et al. Toward a confocal subcellular atlas of the human proteome. Mol Cell Proteomics 2008 7(3):499-508.
4. Stadler C. et al. A single fixation protocol for proteome-wide immunofluorescence localization studies. J Proteomics 2009 73(6):1067-78.

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