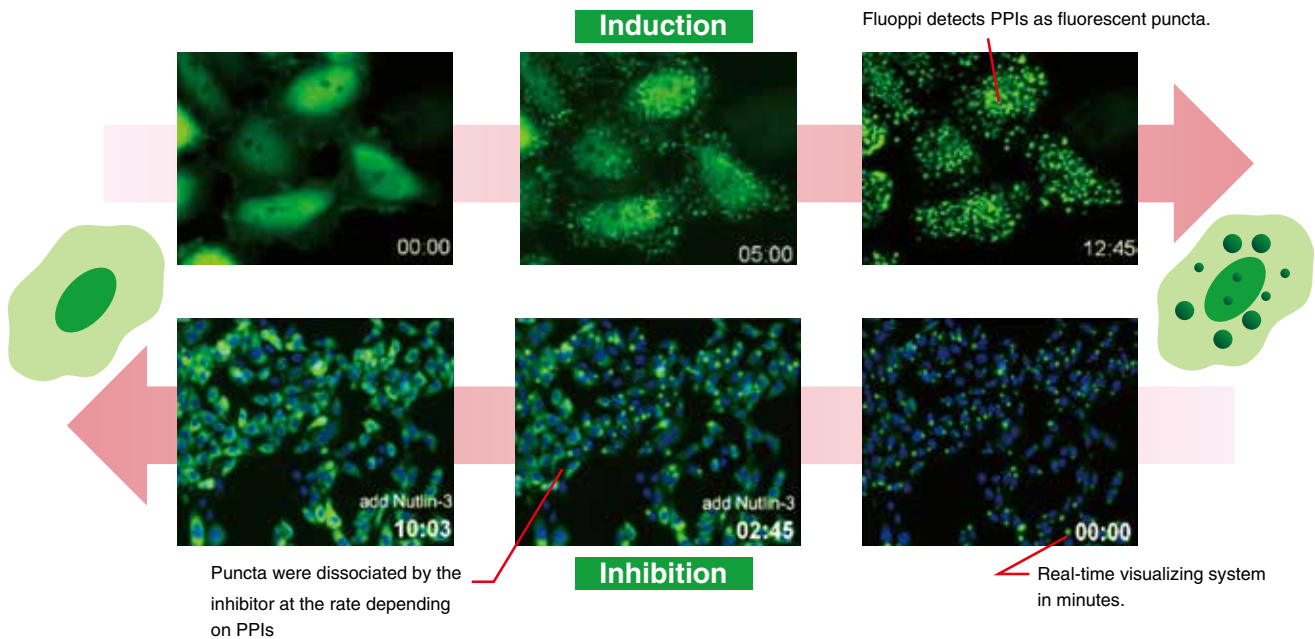


Fluoppi

Image based Protein-Protein
Interaction (PPI) analysis

- Visualize PPI as fluorescent "Puncta" in living cells
- "Reversible" puncta-formation
- Easy to construct an experimental system
- Ideal tool for drug screening

Fluoppi (Fluorescence based technology visualizing protein-protein interactions) is a novel technology to monitor protein-protein interactions (PPIs) in living cells in reversible manner. The most advantage of Fluoppi is establishing assays are very easy and simple, which do not require fine optimizations such as peptide linker sequence or length.



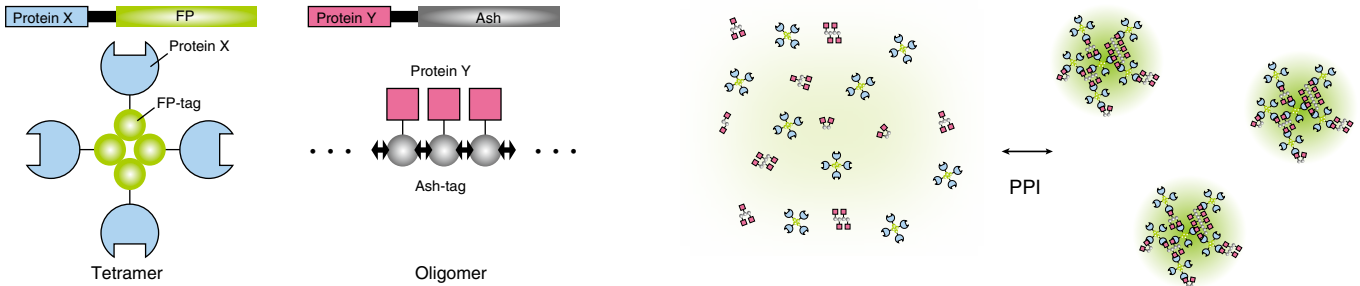
Fluorescent proteins	Code No.	Products	Volume
Monti-Red (Red)	AM-8012M	Fluoppi Ver.2 : Ash-Red (Ash-MNL/MCL + Monti-Red-MNL/MCL)	10 µg each
	AM-VS0802M	Monti-Red for Fluoppi (pMonti-Red-MNL/MCL)	10 µg each
hAG (Green)	AM-8011M	Fluoppi Ver.2 : Ash-hAG (Ash-MNL/MCL + hAG-MNL/MCL)	10 µg each
	AM-8201M	Fluoppi : Ash-hAG [p53-MDM2]	10 µg each
	AM-8202M	Fluoppi : Ash-hAG [mTOR-FKBP12]	10 µg each
	AM-VS0801M	humanized Azami-Green for Fluoppi (phAG-MNL/MCL)	10 µg each

- Fluoppi does not guarantee detection of all Protein-Protein Interactions.
- Any other uses by non-profit research organizations, or any uses by for-profit organizations regardless of commercial or non-commercial purpose, require a license.
- The fluorescent proteins used in product, hAzami-Green and Monti-Red, differ from each other in fluorescence and other properties.

Key components and Mechanism of action

Fluoppi is a tag technology. Tetramer fluorescent protein (FP-tag) and Assembly helper tag (Ash-tag) are genetically fused to Protein X and Y, respectively. For the FP-tag, tetramer Fluorescent protein, humanized Azami-Green (hAG) and Monti-Red can be used.

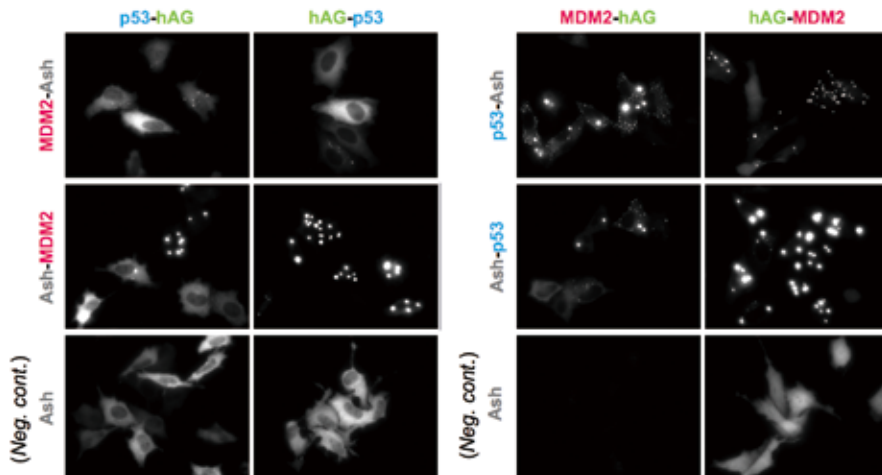
By way of example, genetic fusion of protein X with FP-tag, and Y with Ash-tag creates a tetrameric fluorescent fusion protein X-FP and an oligomeric fusion protein Y-Ash respectively. Because each fusion protein has multiple Xs or Ys, interaction between X and Y causes large lattice like complexes where the fluorescence by X-FP is concentrated and detectable as fluorescent puncta.



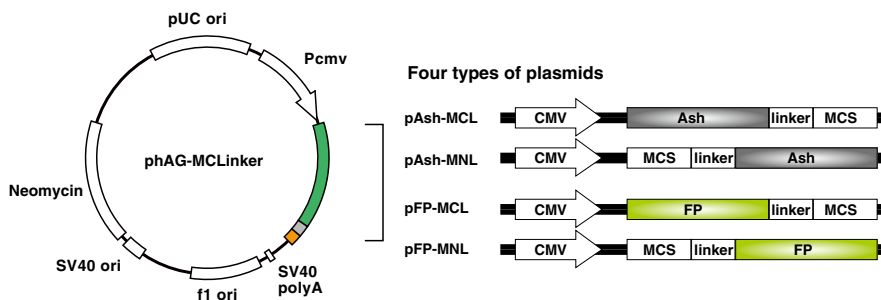
Work Flow of Fluoppi

At first, proteins X & Y of your interest are fused to FP-tag and Ash-tag respectively. We recommend to prepare all the eight possible combinations to identify the best workable combinations.

Because fluorescence signal of Fluoppi is very high, conventional fluorescence microscopy can be used to image the cells. If the proteins interact with each other upon expression, fluorescent puncta will be detected. Formation of puncta is reversible so that they can be dissociated and the fluorescent signal will spread over the cell by PPI inhibitors, and *vice versa* by PPI inducer.

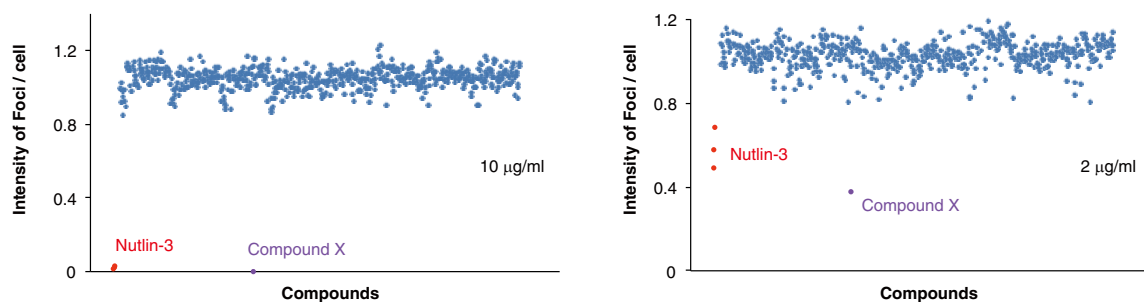


Upper and middle 8 pictures represent 8 possible combinations. (p53-MDM2 protein interaction) Lower 4 panels are results of negative control; hAG tagged protein and only Ash-tag were co-transfected. As shown in the result, fluorescence patterns were different from combination to combination, so that we recommend to try all the 12 experiments represented here.



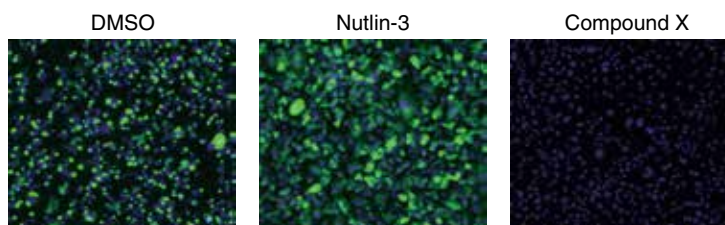
Fluoppi Kit (code: AM-8011M, AM-8012M) includes 4 expression plasmids as pAsh-MNL/MCL, pFP-MNL/MCL. For the FP-tag, tetramer Fluorescent protein, humanized Azami-Green (hAG) (AM-VS0801M) or Monti-Red (AM-VS0802M) can be used.

Pilot screening : p53 – MDM2



A result of pilot screening using commercially available reference library (630 compounds x 2 conc.). High robustness of this screening system is verified.

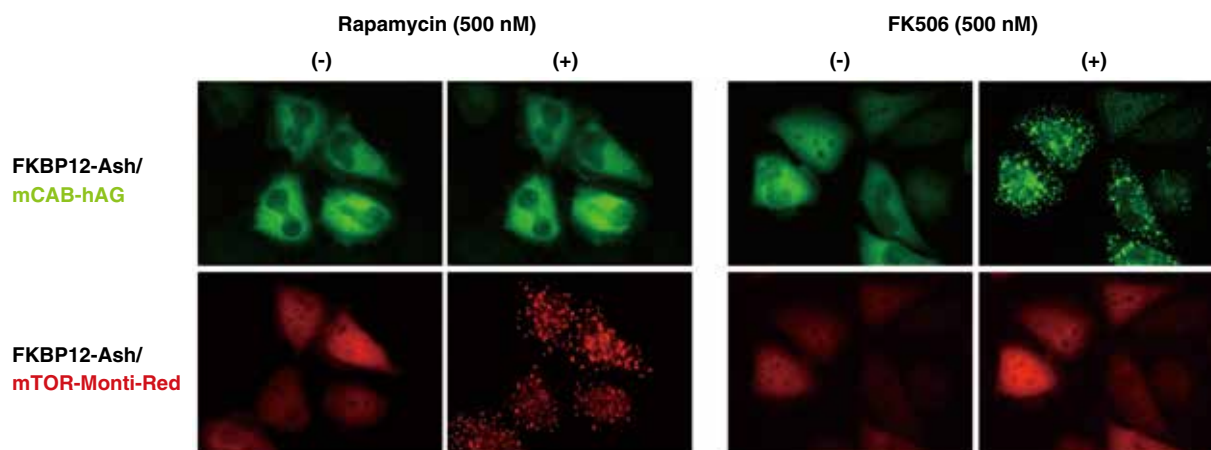
In this screening, compound X was calculated as a HIT compound, however the image represented it was just a quenching compound. Many library contains that kind of compound, therefore Fluoppi can easily exclude pseudo-positive sample without 2nd screening.



Visualizing dual PPIs in a single cell

mTOR (FRB domain) and FKBP12 is a well known PPI whose interaction is induced by Rapamycin. On the other hand, mTOR (FRB domain) and mCAB (the BBH region of Calcineurin A (residues 340-394) fused to Calcineurin B*) interaction is induced by FK506. In this experiment, The mCAB-hAG, mTOR-Monti-Red and Ash-FKBP12 were cotransfected into the cells at the same times. When the cells were exposed to Rapamycin, only the red puncta were formed as shown in the left 4 pictures. On the other hand, in the case of FK506, only the green puncta were detected. The results demonstrate the possibility that multi-color Fluoppi could be applied to visualize branched signaling pathway.

* Reference : P. A. Clemons *et al.* Chem. Biol. (2002)



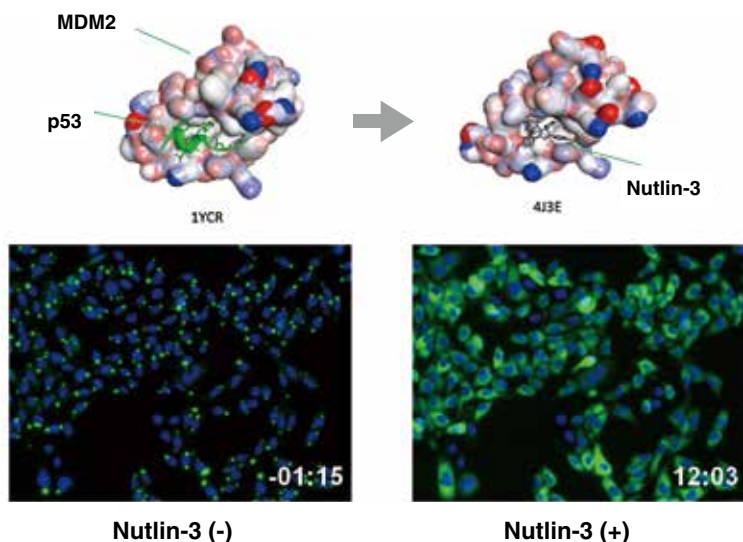
References

1. Saio T *et al.* PCS-based structure determination of protein-protein complexes. *J. Biomol. NMR.* 46, 271-280 (2010) [PMID: 20300805]
2. Banaszynski LA *et al.* Characterization of the FKBP.rapamycin.FRB ternary complex. *J. Am. Chem. Soc.* 127, 4715-21. (2005) [PMID: 15796538]
3. Vassilev LT *et al.* In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science.* 303, 844-848. (2004) [PMID: 14704432]

An example of quantitative analysis for PPI and Screening of PPI modulator

< p53-MDM2 >

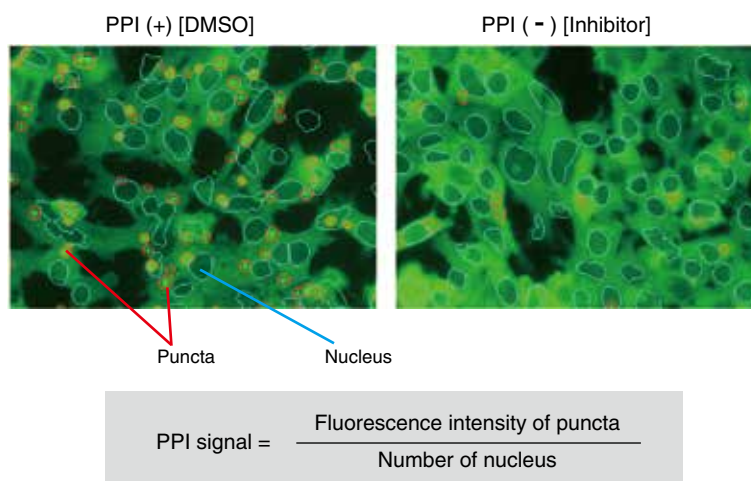
p53-MDM2 is a famous target of PPI modulators in the field of anti cancer drug development. We applied this PPI to Fluoppi for demonstration. First, hAG-MDM2 and Ash-p53 was selected from the 8 pairs as represented above, then stable CHO-K1 cell line was established by using two selection marker; G418 and Hygromycin. Fluoppi plasmids including Hygromycin resistance gene is under released at present.



< Quantitative analysis >

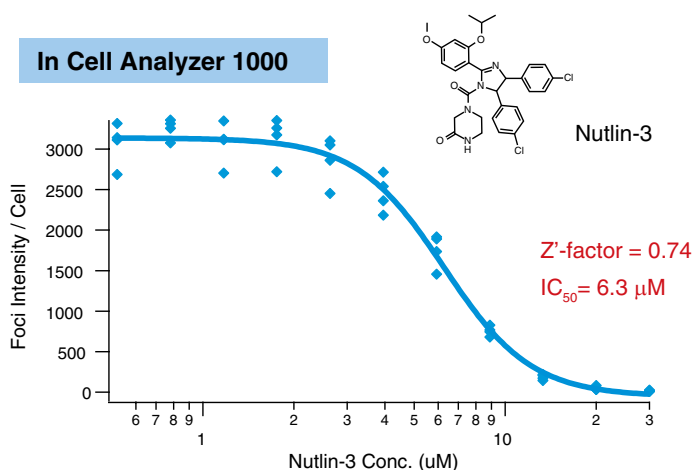
Stable cell lines were seeded on black-wall 96-well plates. PPI modulator was treated for 15 minutes in a room temperature followed by fixation with 4 % PFA. Cells were then stained with Hoechst33342. Imaging was performed with IN Cell Analyzer 1000 (GE Healthcare) using x10 objective lens. Green channel (AG) and blue channel (Hoechst33342) images were used to define puncta and nuclear regions respectively. Puncta or nucleus are segmented with red or blue enclosed lines respectively using IN Cell Investigator software. (If you do not have an In Cell Analyzer and similar equipment, you can use the open sources such as, Spot Detector, provided by Icy: an open community platform for bioimage informatics. <http://icy.bioimageanalysis.org/>)

Puncta Intensity was calculated as fluorescence intensities inside the region of puncta divided by the number of nucleus.



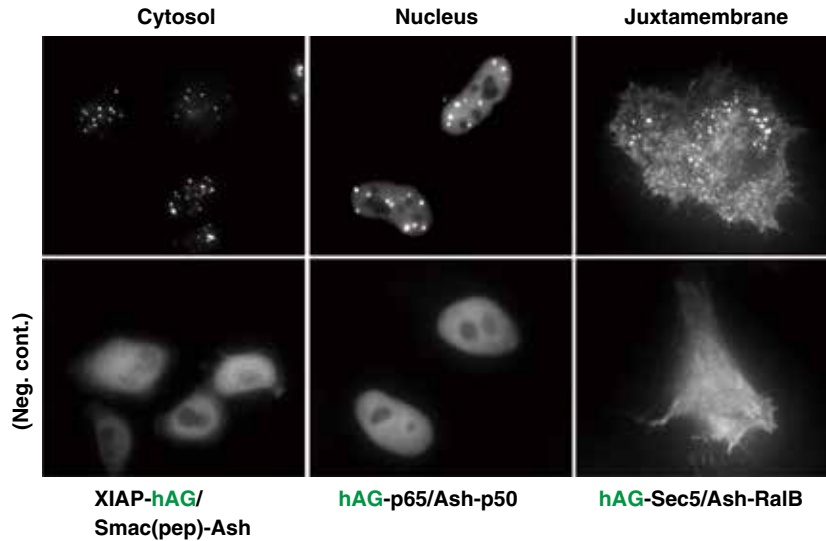
< Data analysis >

In the case of p53-MDM2 experiment, IC_{50} value was determined to be $6.3 \mu\text{M}$. Enough Z'-factor could be obtained, suggesting high reliability of this technology.



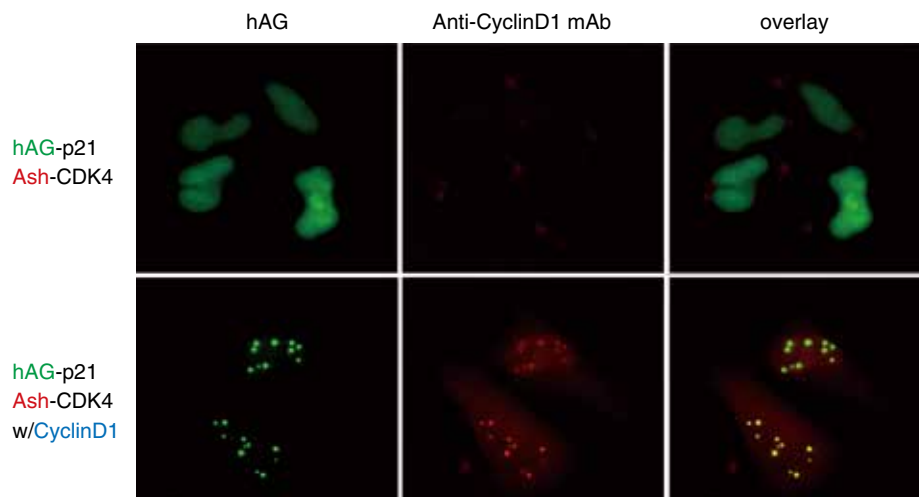
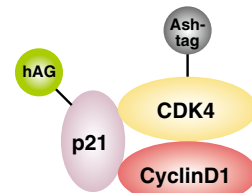
Localization

Because location of puncta is not restricted to specific site inside the cell, Fluoppi can visualize PPIs at several subcellular localizations such as cytosol, nucleus, and juxtamembrane. The upper pictures represent puncta at several subcellular localizations, and the lower pictures are negative controls which express hAG tagged protein and Ash-tag without fusing any proteins. The images of juxtamembrane are taken by Total Internal Reflection Fluorescence Microscopy (TIRFM).



Visualization of protein complex

p21 (CIP/WAF) interacts to and inhibits the activity of cyclin-CDKs complex and thus functions as a regulator of cell cycle progression at G1 and S phase. In this experiment, HeLa cells expressing both AG-p21 and CDK4-Ash displayed disseminated pattern of green fluorescence (upper panels). By contrast, additional cotransfection of cyclin D1 resulted in formation of clear puncta in the nucleus (lower panels). These results indicate CDK4 alone do not interact to p21 and requires additional factor in live cells, which is consistent with previous studies.

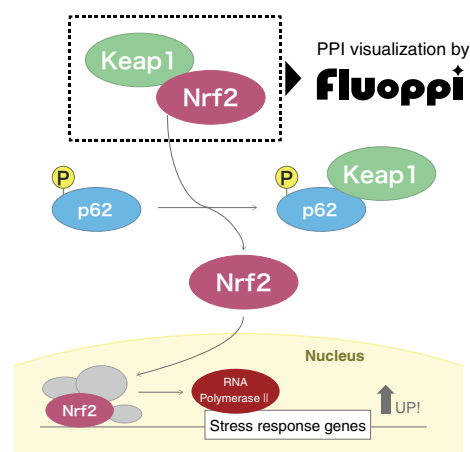


Visualization of protein-protein interactions of p62-Keap1-Nrf2 pathway

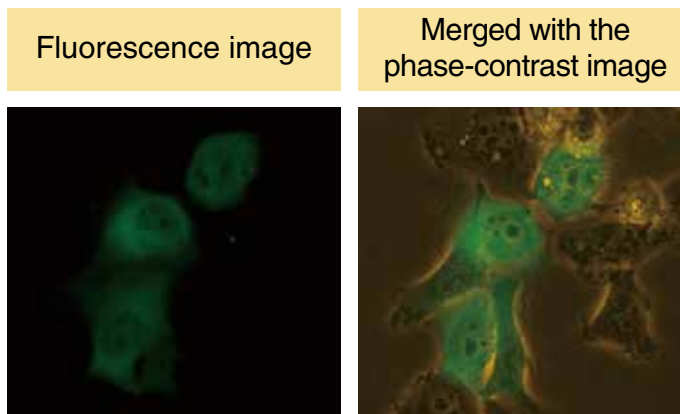
Keap1-Nrf2 interaction is a very important trigger of a variety of cellular stress responses. A recent study demonstrated that Keap1-Nrf2 interaction is regulated through the phosphorylation of an autophagy-related factor, p62.¹⁾ Phosphorylation of p62 increases its affinity for Keap1, and the binding of phosphorylated p62 enables Nrf2 to detach from Keap1 and translocate to the nucleus (the p62-Keap1-Nrf2 pathway). As a result, Nrf2, known as a stress response transcription factor, promotes upregulation of various cytoprotective stress response genes.

Here, using our unique technology known as Fluoppi, we were able to visualize the Keap1-Nrf2 interaction in the cytoplasm. A study in mice revealed that the main intracellular localizations of endogenous Keap1, as detected by anti-Keap1 antibody, were the cytoplasm near the nucleus (81%), endoplasmic reticulum (14%), and nucleus (5%).²⁾

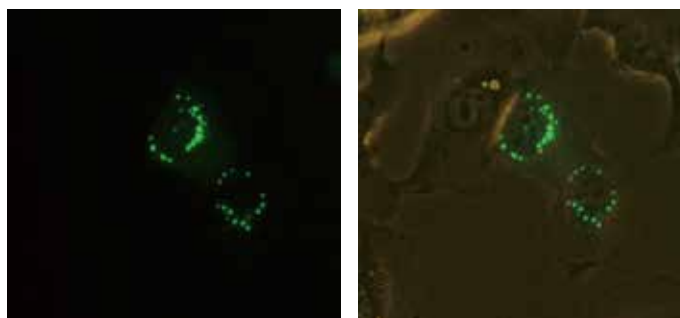
1. Ichimura Y *et al.* Phosphorylation of p62 activates the Keap1-Nrf2 pathway during selective autophagy. *Molecular Cell*. 51. 618-631 (2013)
2. Watai Y *et al.* Subcellular localization and cytoplasmic complex status of endogenous Keap1. *Genes Cells*. 12(10) 1163-78 (2007)



AG-Nrf2 alone
(negative control)



AG-Nrf2
+
Ash-Keap1



Plasmid DNAs for Azami Green (fluorescent protein)-Nrf2 and Ash-Keap1 were constructed and transiently co-transfected into HT1080 cells. Puncta were detected in the periphery of the nucleus.

The data were provided by
Dr. Hiroyuki Suzuki
Laboratory of Experimental Pathology,
Faculty of Medicine, University of Tsukuba

Publications

1. Koyano F *et al.* Ubiquitin is phosphorylated by PINK1 to activate parkin. *Nature*. 510, 162-166 (2014) [PMID: 24784582]
2. Yamano K *et al.* Site-specific Interaction Mapping of Phosphorylated Ubiquitin to Uncover Parkin Activation. *J Biol Chem*. 290, 25199-211 (2015) [PMID: 26260794]
3. Asamitsu K *et al.* Quantification of the HIV transcriptional activator complex in live cells by image-based protein-protein interaction analysis. *Genes Cells*. 21, 706-16 (2016) [PMID: 27193293]
4. Watanabe T *et al.* Genetic visualization of protein interactions harnessing liquid phase transitions. *Sci Rep*. 7, Article number: 46380 (2017) [PMID: 28406179]