Published in final edited form as:

Methods. 2017 April 15; 118-119: 101–110. doi:10.1016/j.ymeth.2016.09.010.

Simultaneous detection of the subcellular localization of RNAs and proteins in cultured cells by combined multicolor RNA-FISH and IF

Cindy Meyer, Aitor Garzia, and Thomas Tuschl

Laboratory of RNA Molecular Biology, Howard Hughes Medical Institute, The Rockefeller University, New York, NY 10065

Abstract

Fluorescence *in situ* hybridization (FISH) and immunofluorescence (IF) are sensitive techniques for detecting nucleic acids and proteins in cultured cells. However, these techniques are rarely applied together, and standard protocols are not readily compatible for sequential application on the same specimen. Here, we provide a user-friendly step-by-step protocol to perform multicolor RNA-FISH in combination with IF to simultaneously detect the subcellular localization of distinct RNAs and proteins in cultured cells. We demonstrate the use of our protocol by analyzing changes in the subcellular distribution of RNAs and proteins in cells exposed to a variety of stress conditions.

Keywords

FISH; immunofluorescence; antibody; nucleic acids; GFP; stress granule

1. Introduction

In eukaryotes, RNA maturation and function are compartmentalized, and RNA transport is a critical step in gene expression. An mRNA molecule starts its life cycle as a nascent transcript transcribed by RNA polymerase II in the nucleus. After capping, splicing, editing, and polyadenylation, the mature mRNA is exported into the cytoplasm. The orderly flow of these processes is coordinated by hundreds of mRNA-binding proteins (RBPs) [1]. In the cytoplasm, the composition of the ribonucleoprotein complexes (RNPs) is extensively remodeled. Specific cytoplasmic RBPs ensure correct mRNA localization, translational regulation, and degradation.

Imaging the intracellular distribution of RNAs and interacting proteins has become of considerable interest and has established links between mRNA localization and protein targeting [2]. The two most widely used techniques to monitor the subcellular localization of

Correspondence to: Thomas Tuschl.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

nucleic acids and proteins are *in situ* hybridization (ISH) and immunofluorescence (IF), respectively. In 1969, Joseph Gall and Mary Pardue reported the first ISH experiment, in which they hybridized radioactive ribosomal RNA (rRNA) to extra-chromosomal ribosomal DNA (rDNA) in *Xenopus tropicalis* oocytes. The RNA/DNA hybrids were visualized in the cytological preparations by tritium autoradiography [3]. ISH is a technique in which labeled single-stranded antisense RNA, DNA, or locked nucleic acid (LNA) probes are hybridized to immobilized RNA or DNA molecules in paraformaldehyde fixed cells or tissues [4,5]. The probes are designed with sequence complementarity to the target nucleic acid to ensure specific base pairing. If the target nucleic acids are double-stranded, they must be denatured prior or during probe hybridization, and fixative nucleobase-modifications interfering with base pairing must be reverted. The nucleic acid probes are typically conjugated to suitable fluorescent dyes or small molecule haptens to allow for subsequent direct or signal-amplification-system-dependent visualization by microscopy, respectively [3,6]. Whereas ISH was originally limited to only abundantly expressed RNAs, improvements in microscopy and fluorescence signal detection, in the stability and quantum yield of fluorophores, and the access to genome-wide transcriptome sequence information impacting specific probe design have allowed for the visualization of single molecules like rare mRNAs and non-coding RNAs [7].

Analogous to sequence-complementarity for RNA-FISH, IF relies on the shape-complementarity of antibody-antigen interactions for detection of proteins of interest. IF was first practiced in the late 1940s by Albert Coons and co-workers who used antibodies conjugated to anthracene or fluorescein isocyanate to specifically detect the presence of the pneumococcal antigen in tissue sections [8,9]. For the visualization of proteins by IF, the specific antibody is either directly conjugated to fluorophores or visualized by the use of a fluorophore-labeled secondary antibody specific to the constant region of the first antibody. The success of immunofluorescence is dependent on the specificity of the antibody and its compatibility to the immunofluorescence conditions. To circumvent the time-demanding development of highly specific antibodies for every new protein of interest, the protein can be directly expressed as a fusion construct with a short peptide tag, such as FLAG- and HA-tag, for which highly specific monoclonal antibodies have been commercialized for detection [10]. Alternatively, the protein of interest can be fused to a green fluorescent protein (GFP), a widely used marker protein in molecular and cell biology due to its strong intrinsic visible fluorescence. GFP, along with the luminescent protein aequorin, was first discovered and purified from the jellyfish *Aequorea victoria* by Osamu Shimomura in 1962 [11]. After successful cloning of the GFP cDNA by Douglas Prasher [12], Martin Chalfie used the gene to monitor GFP expression and protein localization in *E. coli* and *C. elegans* [13]. Shortly after, Tulle Hazelrigg was able to genetically link GFP to other proteins and follow the localization and movement of the fusion protein in living cells [14]. The GFP isolated from jellyfish has been engineered to create a variety of blue, cyan, and yellow fluorescent proteins (FPs). Additionally, a variety of FPs from other species have been identified, expanding the available color palette of the FPs to the orange, red and far-red spectral regions [15-17]. Using GFP or its derivatives as a fusion protein has the advantage of directly labeling the protein of interest and skipping all preparatory steps for immunofluorescence. However, a large globular tag may affect the function and subcellular

localization of such fusion proteins. Finally, a new generation of protein tags with high affinity to bright and stable fluorophores, such as the SNAP-, CLIP-, or HALO-tag, can be fused to the protein of interest and the fluorophores soaked into the cells prior to imaging [18,19].

Combining multicolor RNA-FISH with multicolor IF allows for the simultaneous detection of multiple RNA and protein molecules in cells or tissues. Our laboratory combined these techniques to simultaneously capture the localization of cellular RNAs as well as RBPs to study the consequences of various stress conditions affecting mRNA translation initiation and halting protein synthesis. Arrest of translation initiation often leads to the formation of cytoplasmic RNA-protein complexes referred to as stress granules (SGs) [20]. It is suggested that SGs accumulate mRNAs stalled in the process of translation initiation [20]. SGs typically consist of polyadenylated mRNAs and components of the small 40S ribosomal subunit, several translation initiation factors (including eIF4E, eIF4A, and eIF4G), poly(A)-binding protein, and a number of RBPs involved in mRNA transport, translation, and stability, such as G3BP1 (Fig. 1A) [21,22]. SGs are distinct from processing bodies (PBs). PBs are cytoplasmic RNP granules involved in mRNA decay. Even though PBs and SGs share some of its constituents, PBs contain many protein components of the RNA decay machinery, such as the mRNA decapping enzymes DCP1a, DCP2, and DDX6 [23]. PBs are present in actively growing, unstressed cells, as well as in cells exposed to environmental stressors. Accumulation of SGs is a hallmark of many viral infections [24], cancer [25], and multiple neurological disorders [26]. Thus, the visualization and compositional characterization of SGs are of considerable interest for the functional study of physiological and cellular stress conditions that lead to human disease.

2. Experimental design

2.1. Flexibility of the procedure

In this protocol we describe the combination of RNA-FISH and IF to simultaneously detect RNAs and proteins in cultured and subsequently fixed cells. Figure 1B outlines the protocol in a workflow diagram. First, RNA-FISH is performed, which includes cell growth and sample preparation, fixation of the cells with formaldehyde, and probe hybridization. Further steps enable the detection of the proteins of interest by immunofluorescence staining prior to the visualization of RNAs and proteins by fluorescence microscopy (Fig. 1C). If the protein of interest is genetically fused to a fluorescent protein, IF might not need to be performed.

2.2. Cell lines

The following RNA-FISH and IF protocol is optimized for HEK293 cells inducibly producing FLAG/HA- or eGFP-tagged proteins. HEK293 cell lines inducibly expressing tagged proteins were generated using the Gateway Recombination Cloning Technology and Flp-In T-REx HEK293 cell lines (Invitrogen) as previously described [27]. Additionally, we tested the applicability of our RNA-FISH and IF protocol using human HeLa and THP-1 cell lines. Since THP-1 cells grow in suspension, it is necessary to attach the cells to microscopy slides before performing FISH. Suspension cells were treated with the lectin Concanavalin A (30 min, 25 µg/ml) to facilitate their adhesion to the slide surface in a monolayer [28].

2.3. Preparation of cell samples and application of stress agents

RNA-FISH and IF are widely used to analyze the RNP composition of SGs and PBs [29,30]. SGs were first observed by exposing cells to the oxidizing reagents, such as sodium arsenite [31]. A variety of drugs and cellular stress conditions have been described to trigger SG formation and translational arrest. Table 1 lists different stress assays routinely performed in our laboratory for HEK293 cells (Fig. 2). If a different cell line is used, the concentration and incubation time of the stress reagents may need to be revised.

2.4. Fixation

For optimal concurrent detection of RNAs and proteins in cells, it is important to fix the cellular morphology, immobilize RNA, preserve protein antigenicity, and obtain sufficient permeability for probes and antibody penetration. In our procedure, we perform fixation using formaldehyde. Formaldehyde shows broad specificity for most cellular targets since it reacts with primary amine residues on proteins and nucleic acids forming partially reversible methylene bridges [35,36]. The fixation reaction strongly stabilizes the cellular architecture, and it is partially reversed during or prior to hybridization or antibody binding to an extent that allows for the detection of target RNAs and proteins.

2.5. Probe design and hybridization conditions

We use LNA-modified DNA (LNA/DNA) oligonucleotide probes (Table 2) that target human polyadenylated mRNAs and 18S or 28S rRNAs [37]. Hybridizing the LNA/DNA probes at the proper temperature is crucial for preventing mis-hybridization. For the probes and hybridization solution described in this protocol, the temperature (40 °C) at which hybridization is performed and the hybridization time of 12–16 h result in strong fluorescence signals with low background signal.

2.6. Antibody staining

FISH is performed prior to IF since the high temperatures necessary for FISH would denature antigen-antibody interactions. Oligonucleotide probe hybridization is also performed in the presence of 50% formamide, which acts as denaturant. It is therefore important to reduce the level of formamide after FISH by performing a series of wash steps before starting the antibody incubation. Furthermore, we include 0.1% (v/v) Tween 20 in all wash and incubation buffers to reduce non-specific binding of probes and antibodies.

Antibodies suitable for IF can be purchased from commercial sources. Otherwise, monoclonal or polyclonal antibodies may be raised in the laboratory or obtained commercially through specialized services. The choice of antibodies is also important. Polyclonal antibodies tend to be less suitable as they often recognize unrelated antigens, while the generation and screening of monoclonal antibodies for suitability in IF can become very time consuming, generally requiring a minimum of one year. As an alternative, we tag our proteins of interest with small polypeptides, such as FLAG-, polyhistidine-, or HA-tag, for which suitable antibodies are available [10]. Fluorescently labeled secondary antibodies detect the primary antibody, simultaneously increasing the sensitivity through signal amplification. Tables 3 and 4 list primary and secondary antibodies suitable for IF that we routinely use in our laboratory.

3. Material

3.1. Reagents

Sodium (meta)arsenite	Sigma, S7400-100G
Aurin (p-rosolic acid)	Sigma, 861324-25G
Baker's yeast tRNA	Sigma, Cat# R8759-2KU
Blasticidin	Invivogen, Cat# ant-bl-5
Chaps	Sigma, Cat# C3023-1G
Cover glass 24×50	ThermoFisher Scientific, Cat# 12450S
DMEM high glucose (1X)	ThermoFisher Scientific, Cat# 11965092
50X Denhardt's	Applichem, Cat# A3792
4',6-Diamidino-2-phenylindole dihydrochloride	DAPI, Sigma, Cat# D8417, MW 350.25 g/mol
Doxycycline	Sigma, Cat# D1822-500MG
Fetal bovine serum	Clontech, Cat# 631101
Fluorescence microscope	VS 110 fluorescence virtual slide scanning system (Olympus)
Formamide	Sigma, Cat# F7503, MW 45.04 g/mol, 1.132 g/ml
Goat serum	Sigma, Cat# G9023
L-Glutamine	ThermoFisher Scientific, Cat# 25030081
Glycerol	ThermoFisher Scientific, Cat# G31, Stock# 222,000, MW 92.1 g/mol
Hydrochloric acid	ThermoFisher Scientific, Cat# A144S, Stock# 114,000, 37.3%, 12.1 M
Hydrogen peroxide solution	Sigma, H1009-5ML
Hygromycin	Invivogen, Cat# ant-hg-1
Fluorescently labeled probes	A detailed protocol for the oligonucleotide probe design and preparation for fluorescent detection routinely used in the laboratory was previously described [37].
MOWIOL	Polysciences Inc., Cat# 17951
Paraformaldehyde	PFA, Electron Microscopy Sciences, Cat# 15710, MW 30.03 g/mol, 16% w/v
Penicillin-Streptomycin	ThermoFisher Scientific, Cat# 15140163
Salmon sperm DNA	Applichem, Cat# A2159
Sodium chloride	NaCl, ThermoFisher Scientific, Cat# S271-3, Stock# 300,000, MW 58.44 g/mol
Sodium hydroxide	NaOH, ThermoFisher Scientific, Cat# S318, Stock# 307000, MW 40.00 g/mol
Thapsigargin	Sigma, T9033-1MG
Tris-HCl	Sigma, Cat# T3253, MW 157.60 g/mol
Tris base	ThermoFisher Scientific, Cat# BP152, Stock# 336,000, MW 121.14 g/mol
Tween 20	Sigma, Cat# p1379
4-well-chamber slides	ThermoFisher Scientific* Nunc* Lab-Tek* II CC2* Chamber Slide System, 154917

3.2. Buffers and solutions

1 M Tris-HCl—In a 1000 ml glass bottle equipped with a magnetic stir bar, weigh out 157.6 g of Tris-HCl. Add approximately 800 ml MilliQ water and dissolve the powder. Transfer the solution to a 1000 ml graduated cylinder, fill it up to 1000 ml with MilliQ water and return the solution to a 1000 ml bottle. Store at RT.

1 M Tris base—In a 1000 ml glass bottle equipped with a magnetic stir bar, weigh out 121.1 g of Tris base. Add approximately 800 ml MilliQ water and dissolve the powder. Transfer the solution to 1000 ml graduated cylinder, fill it up to 1000 ml with MilliQ water and return the solution to a 1000 ml bottle. Store at RT.

1 M Tris buffer (pH 7.4)—Combine 820 ml of 1 M Tris-HCl and 180 ml of 1 M Tris base in a 1000 ml glass bottle. $\text{Volume (Tris base)} = (10^{(\text{pH}-\text{pKa})} / (1+10^{(\text{pH}-\text{pKa})})) \times 1000$ ml; pKa = 8.06 at RT. Store at RT.

1 M Tris buffer (pH 8.5)—Combine 266 ml of 1 M Tris-HCl and 734 ml of 1 M Tris base in a 1000 ml glass bottle. $\text{Volume (Tris base)} = (10^{(\text{pH}-\text{pKa})} / (1+10^{(\text{pH}-\text{pKa})})) \times 1000$ ml; pKa = 8.06 at RT. Store at RT.

3 M NaCl solution—Weigh out 175.3 g of NaCl and transfer it to a 1000 ml bottle equipped with a magnetic stir bar. Add 800 ml MilliQ water measuring with a graduated cylinder. Transfer the solution to a 1000 ml graduated cylinder, fill it up to 1000 ml with MilliQ water and return the solution to a 1000 ml bottle. Store at RT.

1 M NaOH solution—Weigh out 4.0 g of NaOH and transfer it to a 100 ml bottle equipped with a magnetic stir bar. Add 80 ml MilliQ water measuring with a graduated cylinder. Transfer solution to 100 ml graduated cylinder, fill it up to 100 ml with MilliQ water and return the solution to a 100 ml bottle. Store at RT.

1X TBS (large volume)—In an 8 l plastic bottle, combine:

Reagent or solution	Final concentration (M)	Volume (ml)
3 M NaCl solution	0.10	266.7
1 M Tris buffer (pH 7.4)	0.01	80

Fill it up to 8 l with MilliQ water and shake vigorously. Store at RT.

4% PFA (10% formalin)—In a 50 ml falcon tube, combine 10 ml of commercial 16% paraformaldehyde with 30 ml 1X TBS. The total volume is 40 ml. Prepare fresh formaldehyde for each experiment. Discard unused reagent.

Hybridization buffer (50% FA)—In a 50 ml Falcon tube, combine:

Reagent or solution	Final concentration	Volume (ml)	Mass (mg)
Formamide	12.6 M (50% (v/v))	25.0	
3 M NaCl solution	1.0 M	16.7	
1 M Tris buffer (pH 8.5)	75 mM	3.75	
50X Denhardt's	1X Denhardt's	1.00	
Baker's yeast tRNA (20 mg/ml)	250 µg/ml	0.625	
Salmon sperm DNA	500 µg/ml		25
Chaps	2.5 mM		77
Tween 20	0.5% (v/v)	0.25	
MilliQ water		2.675	

The total volume is 50 ml. The hybridization buffer can be stored at -20°C for a few weeks.

Hybridization solution—In a 1.5 ml siliconized Eppendorf tube, combine:

Reagent or solution	Final concentration (µM)	Volume (µl)
Hybridization buffer		500 µl per well
LNA probe	Depending on the probe	Depending on the probe

A total volume of 500 µl will be used for one well of the 4-well-chamber. Only prepare the amount of hybridization solution that is necessary for your experiment.

Wash buffer 1 (50% FA)—In a 1000 ml glass bottle equipped with a magnetic stir bar, combine:

Reagent or solution	Final concentration	Volume (ml)
Formamide	12.6 M (50% (v/v))	500
3 M NaCl solution	0.250 M	83.3
1 M Tris buffer (pH 8.5)	0.075 M	75.0
Tween 20	0.1% (v/v)	1.0

Fill it up with MilliQ water to 1 l in a graduated cylinder. Wash buffer 1 can be stored at RT.

Wash buffer 2—In a 1000 ml glass bottle equipped with a magnetic stir bar, combine:

Reagent or solution	Final concentration	Volume (ml)
3 M NaCl solution	0.050 M	16.7
1 M Tris buffer (pH 8.5)	0.075 M	75.0

Reagent or solution	Final concentration	Volume (ml)
Tween 20	0.1% (v/v)	1.0

Fill it up with MilliQ water to 1 l in a graduated cylinder. Wash buffer 2 can be stored at RT.

1X TBS-T—In a 1000 ml glass bottle equipped with a magnetic stir bar, combine:

Reagent or solution	Final concentration	Volume (ml)
1X TBS	10 mM Tris (pH 7.4) 100 mM NaCl	999
Tween 20	0.1% (v/v)	1.0

The total volume is 1000 ml. Store at RT.

Antibody blocking solution—In a 15 ml falcon tube, combine:

Reagent or solution	Final concentration	Volume (ml)
Goat serum	5%	0.5
1X TBS-T buffer		9.5

The total volume is 10 ml. Store goat serum in 0.5 ml aliquots at -20°C . Thaw one aliquot of goat serum to prepare the antibody blocking solution. Discard unused solution.

Note: The blocking solution used in our protocol contains 5% goat serum since the secondary antibodies used in our example were raised in goat. Depending on the species of the secondary antibody alternative blocking solutions may need to be used. As a guideline, use 10% serum from the species the secondary antibody was raised in.

Primary antibody solution (per well)—In a 1.5 ml reaction tube, combine:

Reagent or solution	Final concentration	Volume (μl)
Primary antibody (Table 3, e.g. Monoclonal Anti-CELF1 antibody produced in mouse; Santa Cruz)	1:500 (can vary)	0.5
Antibody-blocking solution		499.5

The total volume is 500 μl . Note: it is possible to combine two or more primary antibodies depending on your need.

Secondary antibody solution (per well)—In a 1.5 ml reaction tube, combine:

Reagent or solution	Final concentration	Volume (μl)
Fluorescently labeled secondary antibody (Table 4, e. g. Alexa Fluor® 488 Goat Anti-Mouse IgG)	1:1,000 (can vary)	0.5
Antibody-blocking solution		499
DAPI (5 mg/ml)	5 μg/ml	0.5

The total volume for one well is 500 μl. Note: it is possible to combine two or more secondary antibodies depending on the primary antibodies used for IF.

DAPI stock solution—Dissolve 5 mg DAPI in 1 ml MilliQ water to a final concentration of 5 mg/ml. The DAPI stock solution can be safely stored at 2-8 °C for at least one year.

DAPI working solution (for wells that are not stained with antibodies)—In a 15 ml Falcon tube, combine:

Reagent or solution	Final concentration	Volume (μl)
DAPI (5 mg/ml)	5 μg/ml	0.5
1X TBS-T buffer		499.5

A 1:1000 dilution is recommended. Discard unused solution.

Mounting solution—In a 50 ml glass bottle equipped with a magnetic stir bar, combine:

Reagent or solution	Final concentration	Volume (ml)	Mass (g)
Glycerol	25% (w/v)		6.0
MOWIOL	N/A		2.4
MilliQ water		6	
0.2 M Tris (pH 6.8)	0.1 M	12	

Prepare 0.2 M Tris (pH 6.8, final volume 100 ml) by mixing 1 ml 1 M Tris base, 19 ml 1 M Tris-HCl and 80 ml MilliQ water.

Volume (Tris base) = $10^{(\text{pH}-\text{pKa})} / (1+10^{(\text{pH}-\text{pKa})}) \times 1000$ ml; pKa = 8.06 at RT. To 6 g of glycerol, slowly add 2.4 g of MOWIOL while mixing. Add 6 ml of MilliQ water and incubate for overnight at RT. Add 12 ml of 0.2 M Tris (pH 6.8) and heat for 10 min at 50 °C with occasional mixing. Stir until all MOWIOL is dissolved. Clear by centrifugation at 5000xg for 15 min at 4 °C. MOWIOL solution can be safely stored at RT for several months.

3.3. Probes

3.4. Antibodies

When using cell lines stably expressing FLAG/HA-tagged proteins, we recommend using an anti-HA antibody as a primary antibody. For immunofluorescence of endogenous proteins it is necessary to check for the suitability of your antibody for this application. Here we list antibodies that we routinely use for IF.

4. Multicolor RNA-FISH and IF: Step-by-step protocol

Day 1: Growing cells on microscope slides

Seed about 50,000 HEK293 cells per well of a 4-well-chamber slide in appropriate growth medium. The number of cells might vary depending on their size and growth rate. HEK293 cells were grown in DMEM high glucose (1x) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml hygromycin, and 15 µg/ml blasticidin.

Day 2 (optional): Induction of tagged protein production

24 h after cells were seeded, induce the expression of FLAG/HA-tagged or eGFP-tagged proteins by adding 1 ng/µl doxycycline directly to the cell culture media. We recommend inducing protein expression for at least 24 h. Depending on the protein of interest, it may be necessary to perform a time course experiment of doxycycline treatment to determine optimal protein expression levels.

Note: If endogenous proteins will be detected by IF, it is not necessary to induce protein expression with doxycycline. In this case, the researcher can seed 100,000 HEK293 cells per well of a 4-well-chamber slide and continue after 24 h with the induction of SG formation, fixation, and RNA-FISH.

Day 3: Induction of SG formation by sodium arsenite, fixation, RNA-FISH

1. Induction of SG formation by sodium arsenite—24 h after induction of protein expression, treat cells with sodium arsenite to induce SG formation. Add sodium arsenite to a final concentration of 400 µM directly to the cell culture media. Incubate the cells for 30 min at 37 °C.

2. Fixation—After stress administration, wash each well of the 4-well-chamber slide once with 500 µl of 1X TBS-T for 1 min. Make sure that the cells are always fully covered with solution to prevent the samples from drying out. Perform all wash steps carefully, to avoid detaching of the cells from the slide. Remove the 1X TBS-T and immediately add 500 µl precooled 4% PFA. Incubate slides for 90 min at 4 °C with gentle agitation. Alternatively, fixation for 15 min at RT is possible. After fixation, wash the samples once with 500 µl of 1X TBS-T for 1 min at RT.

Note: While handling PFA, wear gloves and work under a fume hood. Dispose solutions containing PFA as hazardous waste according to your laboratory's safety instructions.

3. RNA-FISH—Place slides on a stainless steel slide rack. Pre-hybridize each well by adding 500 µl of freshly prepared hybridization buffer (50% FA) to each well. Make sure all cells are fully covered with solution. Incubate the slides in a sealed metal tray for 15 min at room temperature (RT). Meanwhile, pre-heat the hybridization oven to 40 °C. Select the desired RNA-FISH probes and dilute them in hybridization buffer (50% FA) to the final concentration of 20 nM. Discard the hybridization buffer (50% FA) from each well and add 500 µl of the freshly prepared hybridization solution (50% FA) containing the probes for RNA-FISH. Incubate the slides in a sealed humidified chamber for 16 h or overnight at 40 °C.

Day 4: IF and Imaging

For the following steps, slides should be kept under light protection.

1. Wash steps—After performing RNA-FISH, wash each well once with 500 µl of Wash buffer 1 (3.2.) for 5 min at RT. Subsequently, wash each well with 500 µl of Wash buffer 2 (3.2.) for 5 min at RT, followed by one wash step with 500 µl of 1X TBS-T for 5 min at RT.

2. Blocking the slides for IF staining—Block each well with 500 µl antibody blocking solution (3.2.). Blocking is performed for 20 min at RT.

3. Primary antibody incubation—Incubate wells with 500 µl primary antibody solution (3.2.) for 1 h at RT. Wash cells thoroughly three times with 500 µl 1X TBS-T (3 min each).

4. Secondary antibody incubation—Incubate wells with 500 µl secondary antibody solution (3.2.) for 1 h at RT. To counterstain the nuclear DNA, the secondary antibody solution should contain a 1:1000 dilution of the DAPI stock solution (3.2.).

5. Mounting slides for microscopy—Wash each well three times with 500 µl of 1X TBS-T buffer for 3 min at RT. Remove the chamber material and add two drops (approximately 50 µl) of mounting solution (3.2.) on each section. Carefully mount a glass coverslip on top of the prepared slide. Avoid creating bubbles, which will perturb future imaging. Seal the cover slip with nail polish and air-dry for 10 min. Images can be recorded on the Olympus VS110 and processed using Visiopharm Integrated Systems Inc. software or similar devices. The slides can be stored in a dark slide rack at 4 °C for few weeks.

Note:

If proteins are fused to fluorescent proteins and IF is not performed, skip steps 2 – 4. Counterstain the nuclear DNA by adding 500 µl DAPI working solution (2.1.2) to each well. Incubate for 1 h at RT. Proceed with step 5.

5. Demonstration of FISH and IF for monitoring conditions of cellular stress

5.1. Visualization of the distribution of cellular RNAs under oxidative stress by multicolor RNA-FISH

We used our RNA FISH protocol to visualize the distribution of poly(A)-mRNAs, 18S and 28S rRNAs in HEK293 cells and record changes in localization upon stress. We created a HEK293 cell line inducibly expressing a FLAG/HA-tagged fusion of G3BP1, an RBP known to localize to SGs and therefore used as SG marker [20,22]. We induced oxidative stress by treating cells with sodium arsenite (400 μ M, 30 min at 37 °C). Treated and untreated control cells were fixed with 4% paraformaldehyde and further processed for RNA-FISH using probe sets for poly(A)-mRNAs (labeled with ATTO 647N), 28S rRNA (labeled with Alexa Fluor 750), and 18S rRNA (labeled with ATTO 550). We analyzed the subcellular localization of G3BP1 and the RNAs by fluorescence microscopy (Fig. 3, left panel). In untreated cells, G3BP1 is equally distributed throughout the cytoplasm. Polyadenylated mRNAs are detected in the cytoplasm as well as the nucleus. The 18S and 28S rRNA signals showed overlapping localization in the cytoplasm and nucleoli. Nuclei were visualized using DAPI staining.

Upon exposure to sodium arsenite (Fig. 3, right panel) approximately 90% of all cells formed SGs, as revealed by eGFP-G3BP1 and poly(A)-mRNAs localizing to the granular structures inside the cytoplasm. As expected for SG formation, the 28S rRNA component of the large ribosomal subunit does not accumulate in SGs. However, our studies correctly identified that the 18S rRNA component of the small ribosomal subunit localizes to SGs induced by sodium arsenite.

5.2. Visualization of cellular RNAs and proteins by combined multicolor RNA-FISH and IF

CELF1 is a member of the CELF (CUGBP, Elav-like) family of mRNA-binding proteins localizing to cytoplasmic SGs [38]. To visualize CELF1 containing SGs and to distinguish those from mRNA degrading PBs, we performed multicolor RNA-FISH and IF on HEK293 cells exposed to oxidative stress caused by the treatment with sodium arsenite. First, we performed RNA-FISH to detect the localization of poly(A)-mRNAs and 28S rRNAs. Subsequently we performed IF using primary antibodies for CELF1 and DDX6, a helicase known to specifically localize to PBs [23]. We detected the primary antibodies with the corresponding species-specific fluorophore-conjugated secondary antibodies (Table 4). As shown in Figure 4, DDX6 accumulates in PB bodies readily detectable in unstressed cells. CELF1 mainly localizes to the nucleus. In response to environmental stress, CELF1 is recruited to poly(A)-mRNA-containing cytoplasmic SGs.

6. Conclusions

Here we provide a detailed protocol to perform multicolor RNA-FISH in combination with IF to simultaneously detect the subcellular localization of RNAs and proteins in cultured cells under diverse conditions of cellular stress. We describe conditions and reagents used in our laboratory to image abundant cellular RNAs, such as 18S and 28S ribosomal RNAs, as well as polyadenylated mRNAs, and monitor their characteristic localization using

fluorescently labeled, linear LNA/DNA oligonucleotide probes. The protocol is compatible with subsequent IF application, allowing for the detection of proteins of interest within the same experiment. We further show that the presented RNA-FISH conditions are also compatible with *in vivo* labeled GFP-fusion proteins, allowing endogenous fluorescent tagging and imaging of proteins of interest. In conclusion, our method is a simple, robust, and flexible tool to study the co-localization of RNAs and proteins. It is suitable for studying the impact of cellular stress on RNA expression, transport, and mRNA translation.

Acknowledgements

We thank Pavol Cekan for helpful discussions. We thank Stefanie Gerstberger and Michael Mazzola for carefully reading the manuscript. T.T. is an HHMI investigator and acknowledges support from an award by the US National Institutes of Health (RO1-CA159227). C.M. was supported by the German Academic Exchange Service (DAAD). A.G. held a postdoctoral fellowship from the Basque Government.

9. References

- [1]. Gerstberger S, Hafner M, Tuschl T. A census of human RNA-binding proteins. *Nat. Rev. Genet.* 2014; 15:829–845. DOI: 10.1038/nrg3813 [PubMed: 25365966]
- [2]. Lehmann R, Nüsslein-Volhard C. Abdominal segmentation, pole cell formation, and embryonic polarity require the localized activity of oskar, a maternal gene in *Drosophila*. *Cell.* 1986; 47:141–152. [PubMed: 3093084]
- [3]. Gall JG, Pardue ML. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proc. Natl. Acad. Sci. U.S.A.* 1969; 63:378–383. [PubMed: 4895535]
- [4]. Kloosterman WP, Wienholds E, de Bruijn E, Kauppinen S, Plasterk RHA. In situ detection of miRNAs in animal embryos using LNA-modified oligonucleotide probes. *Nat. Methods.* 2006; 3:27–29. DOI: 10.1038/nmeth843 [PubMed: 16369549]
- [5]. Vester B, Wengel J. LNA (locked nucleic acid): high-affinity targeting of complementary RNA and DNA. *Biochemistry.* 2004; 43:13233–13241. DOI: 10.1021/bi0485732 [PubMed: 15491130]
- [6]. Tautz D, Pfeifle C. A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene hunchback. *Chromosoma.* 1989; 98:81–85. [PubMed: 2476281]
- [7]. Renwick N, Cekan P, Masry PA, McGeary SE, Miller JB, Hafner M, et al. Multicolor microRNA FISH effectively differentiates tumor types. *J. Clin. Invest.* 2013; 123:2694–2702. DOI: 10.1172/JCI68760 [PubMed: 23728175]
- [8]. Coons A, Creech, Hugh, Jones RN, Berliner E. The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. *J. Immunol.* 1942:1–12.
- [9]. Coons AH, Kaplan MH. Localization of antigen in tissue cells; improvements in a method for the detection of antigen by means of fluorescent antibody. *J. Exp. Med.* 1950; 91:1–13. [PubMed: 15395569]
- [10]. Terpe K. Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Appl. Microbiol. Biotechnol.* 2003; 60:523–533. DOI: 10.1007/s00253-002-1158-6 [PubMed: 12536251]
- [11]. Shimomura O, Johnson FH, Saiga Y. Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusa, *Aequorea*. *J Cell Comp Physiol.* 1962; 59:223–239. [PubMed: 13911999]
- [12]. Prasher DC, Eckenrode VK, Ward WW, Prendergast FG, Cormier MJ. Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene.* 1992; 111:229–233. [PubMed: 1347277]
- [13]. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. *Science.* 1994; 263:802–805. [PubMed: 8303295]
- [14]. Hazelrigg T, Liu N, Hong Y, Wang S. GFP expression in *Drosophila* tissues: time requirements for formation of a fluorescent product. *Dev. Biol.* 1998; 199:245–249. DOI: 10.1006/dbio.1998.8922 [PubMed: 9698444]

- [15]. Matz MV, Fradkov AF, Labas YA, Savitsky AP, Zaraisky AG, Markelov ML, et al. Fluorescent proteins from nonbioluminescent Anthozoa species. *Nat. Biotechnol.* 1999; 17:969–973. DOI: 10.1038/13657 [PubMed: 10504696]
- [16]. Shaner NC, Campbell RE, Steinbach PA, Giepmans BNG, Palmer AE, Tsien RY. Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat. Biotechnol.* 2004; 22:1567–1572. DOI: 10.1038/nbt1037 [PubMed: 15558047]
- [17]. Lukyanov KA, Chudakov DM, Lukyanov S, Verkhusha VV. Innovation: Photoactivatable fluorescent proteins. *Nat. Rev. Mol. Cell Biol.* 2005; 6:885–891. DOI: 10.1038/nrm1741 [PubMed: 16167053]
- [18]. Böhme I, Mörl K, Bamming D, Meyer C, Beck-Sickinger AG. Tracking of human Y receptors in living cells—a fluorescence approach. *Peptides.* 2007; 28:226–234. DOI: 10.1016/j.peptides.2006.08.042 [PubMed: 17207557]
- [19]. Crivat G, Taraska JW. Imaging proteins inside cells with fluorescent tags. *Trends Biotechnol.* 2012; 30:8–16. DOI: 10.1016/j.tibtech.2011.08.002 [PubMed: 21924508]
- [20]. Buchan JR, Parker R. Eukaryotic stress granules: the ins and outs of translation. *Mol. Cell.* 2009; 36:932–941. DOI: 10.1016/j.molcel.2009.11.020 [PubMed: 20064460]
- [21]. Anderson P, Kedersha N. Stress granules: the Tao of RNA triage. *Trends Biochem. Sci.* 2008; 33:141–150. DOI: 10.1016/j.tibs.2007.12.003 [PubMed: 18291657]
- [22]. Aulas A, Caron G, Gkogkas CG, Mohamed N-V, Destroismaisons L, Sonenberg N, et al. G3BP1 promotes stress-induced RNA granule interactions to preserve polyadenylated mRNA. *J. Cell Biol.* 2015; 209:73–84. DOI: 10.1083/jcb.201408092 [PubMed: 25847539]
- [23]. Chahar HS, Chen S, Manjunath N. P-body components LSM1, GW182, DDX3, DDX6 and XRN1 are recruited to WNV replication sites and positively regulate viral replication. *Virology.* 2013; 436:1–7. DOI: 10.1016/j.virol.2012.09.041 [PubMed: 23102969]
- [24]. Onomoto K, Yoneyama M, Fung G, Kato H, Fujita T. Antiviral innate immunity and stress granule responses. *Trends Immunol.* 2014; 35:420–428. DOI: 10.1016/j.it.2014.07.006 [PubMed: 25153707]
- [25]. Anderson P, Kedersha N, Ivanov P. Stress granules, P-bodies and cancer. *Biochim. Biophys. Acta.* 2015; 1849:861–870. DOI: 10.1016/j.bbagr.2014.11.009 [PubMed: 25482014]
- [26]. Ash PEA, Vanderweyde TE, Youmans KL, Apicco DJ, Wolozin B. Pathological stress granules in Alzheimer's disease. *Brain Res.* 2014; 1584:52–58. DOI: 10.1016/j.brainres.2014.05.052 [PubMed: 25108040]
- [27]. Spitzer J, Landthaler M, Tuschl T. Rapid creation of stable mammalian cell lines for regulated expression of proteins using the Gateway® recombination cloning technology and Flp-In T-REx® lines. *Meth. Enzymol.* 2013; 529:99–124. DOI: 10.1016/B978-0-12-418687-3.00008-2 [PubMed: 24011039]
- [28]. Mori Y, Akedo H, Tanigaki Y. Changes induced by concanavalin A in morphological and adhesive properties of suspension cells. *Exp. Cell Res.* 1973; 78:360–366. [PubMed: 4633335]
- [29]. Kedersha N, Stoecklin G, Ayodele M, Yacono P, Lykke-Andersen J, Fritzler MJ, et al. Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. *J. Cell Biol.* 2005; 169:871–884. DOI: 10.1083/jcb.200502088 [PubMed: 15967811]
- [30]. Reineke LC, Dougherty JD, Pierre P, Lloyd RE. Large G3BP-induced granules trigger eIF2 α phosphorylation. *Mol. Biol. Cell.* 2012; 23:3499–3510. DOI: 10.1091/mbc.E12-05-0385 [PubMed: 22833567]
- [31]. Kedersha NL, Gupta M, Li W, Miller I, Anderson P. RNA-binding proteins TIA-1 and TIAR link the phosphorylation of eIF-2 α to the assembly of mammalian stress granules. *J. Cell Biol.* 1999; 147:1431–1442. [PubMed: 10613902]
- [32]. Emara MM, Fujimura K, Sciaranghella D, Ivanova V, Ivanov P, Anderson P. Hydrogen peroxide induces stress granule formation independent of eIF2 phosphorylation. *Biochem. Biophys. Res. Commun.* 2012; 423:763–769. DOI: 10.1016/j.bbrc.2012.06.033 [PubMed: 22705549]
- [33]. Thomas MG, Tosar LJ, Martinez, Desbats MA, Leishman CC, Boccaccio GL. Mammalian Staufen 1 is recruited to stress granules and impairs their assembly. *J. Cell. Sci.* 2009; 122:563–573. DOI: 10.1242/jcs.038208 [PubMed: 19193871]

- [34]. Rozelle DK, Filone CM, Kedersha N, Connor JH. Activation of stress response pathways promotes formation of antiviral granules and restricts virus replication. *Mol. Cell. Biol.* 2014; 34:2003–2016. DOI: 10.1128/MCB.01630-13 [PubMed: 24662051]
- [35]. Evers DL, Fowler CB, Cunningham BR, Mason JT, O'Leary TJ. The effect of formaldehyde fixation on RNA: optimization of formaldehyde adduct removal. *J Mol Diagn.* 2011; 13:282–288. DOI: 10.1016/j.jmoldx.2011.01.010 [PubMed: 21497290]
- [36]. Masuda N, Ohnishi T, Kawamoto S, Monden M, Okubo K. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. *Nucleic Acids Res.* 1999; 27:4436–4443. [PubMed: 10536153]
- [37]. Renwick N, Cekan P, Bognanni C, Tuschl T. Multiplexed miRNA fluorescence in situ hybridization for formalin-fixed paraffin-embedded tissues. *Methods Mol. Biol.* 2014; 1211:171–187. DOI: 10.1007/978-1-4939-1459-3_14 [PubMed: 25218385]
- [38]. Fujimura K, Kano F, Murata M. Dual localization of the RNA binding protein CUGBP-1 to stress granule and perinucleolar compartment. *Exp. Cell Res.* 2008; 314:543–553. DOI: 10.1016/j.yexcr.2007.10.024 [PubMed: 18164289]

Figure 1. Combined multicolor RNA-FISH and IF protocol to simultaneously monitor the localization of cellular RNAs and proteins under cellular stress

(A) SGs and P-bodies are mRNA-containing cytoplasmic granules that control mRNA translation and decay. The exposure of cells to diverse stress conditions leads to stalled translation initiation and SGs formation. SGs contain non-translating mRNAs, some translation initiation factors, and shuttling RBPs. P-bodies contain non-translating mRNAs, mRNA-destabilizing RBPs, as well as factors involved mRNA decay. (B) Simultaneous detection of polyadenylated mRNAs and RBPs using fluorophore-linked Oligo(dT) probes and fluorescently labeled antibodies. As an alternative to IF, the RBP of interest can be fused to a fluorescent protein like GFP. (C) Workflow for the detection of cellular RBPs and RNAs using our combined RNA-FISH and IF protocol.

Figure 2. G3BP1 is a marker of SGs

HEK293 cells expressing eGFP-tagged G3BP1 were left untreated or exposed to different kinds of cellular stress. Cells were fixed and eGFP fluorescence was examined by fluorescence microscopy. In unstressed cells, G3BP1 shows a diffuse distribution throughout the cytoplasm. Upon exposure to sodium arsenite, thapsigargin, heat, aurin, or virus infection, G3BP1 re-localizes into large cytoplasmic granules. Scale, 10 μ M

Figure 3. G3BP1, polyadenylated mRNAs and 18S rRNA localize to SG upon exposure to sodium arsenite as revealed by multicolor RNA-FISH

HEK293 cells expressing eGFP-tagged G3BP1 were left untreated or exposed to sodium arsenite. After fixation, 18S and 28S rRNA as well as poly(A) mRNAs were hybridized to fluorescently labeled probes and detected by fluorescence microscopy. G3BP1, 18S rRNA, as well as poly(A) mRNAs localize to SG upon oxidative stress. Nuclei were stained with DAPI. Scale, 10 μ M

Figure 4. Simultaneous detection of SGs and PBs by combined multicolor RNA-FISH and IF
 (A) HEK293 cells were left untreated or exposed to sodium arsenite. After fixation, 28S rRNA as well as poly(A) mRNAs were hybridized to fluorescently labeled probes. CELF1 and DDX6 were stained by IF using primary anti-CELF1 and anti-DDX6 antibodies, and corresponding fluorescently labeled secondary antibodies. Localization of RNAs and proteins was visualized by fluorescence microscopy. Nuclei were stained with DAPI. (B). DDX6 localized to PBs, whereas CELF1 and poly(A)-mRNAs co-localized to SGs. Shown are overlays of magnified color (green, blue and red) images of (A). Scale, 10 μ M

Table 1

Methods for induction of cellular stress in HEK293 Flp-In cells

Stress trigger	Condition	Time	Reference
Sodium arsenite	100 – 500 μ M	5 – 30 min	[31]
Aurin	10 μ M	5 – 30 min	
H ₂ O ₂	100 – 1000 μ M	5 – 60 min	[32]
Thapsigargin	1 – 20 μ M	5 – 60 min	[33]
Heat	43 – 46 °C	5 – 30 min	[31]
Viral infection	VACV- E3L, MOI 20	4 – 8 h	[34]

Table 2

Oligonucleotide sequences of the LNA/DNA probes used for RNA-FISH

Target	LNA/DNA probes (LNA residues in blue lowercase)	Length (nt)	RNA target sequence	Fluorescent label
Poly(A)	TtTtTtTtTtTtTtTtTtTtT	21	AAAAAAAAAAAAAAAAAAAAA	ATTO 647N
28S rRNA	gCTtAaATtCaGCGG	15	CCGCUGAAUUAAGC	Alexa Fluor 750
	GGTCCtAaCaCGtGC	15	GCACGUGUUAGGACC	Alexa Fluor 750
	AGGCaCtCGCaTtCC	15	GGAAUGCGAGUGCCU	Alexa Fluor 750
	CtTCGCGaTGcTtG	15	CAAAGCAUCGCGAAG	Alexa Fluor 750
18S rRNA	GaGaCaAaGCAtAtGC	15	GCAUAUGCUUGUCUC	ATTO 550
	CaAGtAGGaGaGGaG	15	CUCCUCUCCUACUUG	ATTO 550
	GGCaTCaCagACcTg	15	CAGGUCUGUGAUGCC	ATTO 550
	GGaAaCCtTGtTaCG	15	CGUAACAAGGUUCC	ATTO 550

Note: A detailed protocol for the oligonucleotide probe design and preparation for fluorescent detection routinely used in the laboratory was previously described [37].

Table 3

Primary antibodies suitable for combined RNA-FISH and IF

Antigen	Immunogen	Isotype	Mono/polyclonal	Company	Number
HA	HA	Mouse IgG1	Monoclonal, clone 16B12	Covance	901513
FLAG	FLAG	Mouse IgG1	Monoclonal, clone M2	Sigma	F1804-1MG
Human G3BP1	Human G3BP aa. 210-323	Mouse IgG1	Monoclonal, clone 23/G3BP	BD	611126
Human DDX6	Human DDX6 aa. 425-283	Rabbit IgG	Polyclonal	Bethyl Laboratories	A300-461A
Human CELF1	Full length CELF1	Mouse IgG1	Monoclonal, clone 3B1	Santa Cruz	Sc-20003

Table 4

Secondary antibodies suitable for combined RNA-FISH and IF

Host	Reactive species	Isotype	Conjugate	Company	Number
Goat	Mouse	IgG	Alexa Fluor 488	ThermoFisher Scientific	A-11001
Goat	Mouse	IgG	Alexa Fluor 546	ThermoFisher Scientific	A-11030
Goat	Rabbit	IgG	Alexa Fluor 546	ThermoFisher Scientific	A-11035