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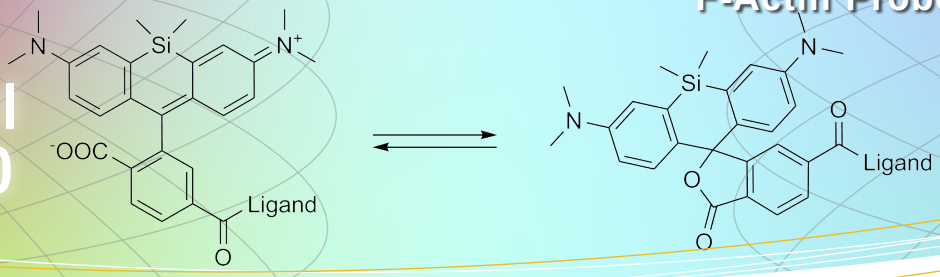

F-Actin Probes in Living Cells

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F-Actin Probes in Living Cells

Dynamic remodeling of the actin cytoskeleton [i.e., rapid cycling between filamentous actin (F-actin) and monomer actin (G-actin)] is required for multiple physiological functions, including intracellular transport, cell growth, morphology, motility, trafficking, polarity, cell-to-cell contacts, and cytokinesis[1,2]. Correspondingly, dysfunctional actin cytoskeletal dynamics are a pathophysiological feature of many human diseases, including those with oncogenic, neurodegenerative, or cardiovascular origins[3-9]. For these reasons, there is continuing interest in F-actin live cell imaging probes to study actin cytoskeletal dynamics in cell culture models of health and disease (Table 1).

SiR and SPY Actin Probes

The ideal actin visualization tool is a small molecule able to bind F-actin in a sensitive and selective manner, while not disrupting actin re-modeling. In addition, introduction directly into the cell culture medium or tissues without need for transfection or electroporation is advantageous[10-13]. The new SiR/SPY actin probes fulfill the needs of an "ideal" actin-binding molecule while surmounting most, if not all, of the concerns and shortcomings associated with existing actin probes (Table 1; Figs. 1,2). Initially characterized by Lukinavicius et al.[12,13] and introduced commercially in 2014, the SiR and SiR700-actin live cell imaging probes label endogenous F-actin and avoid the need for transfections and over-expression of labeled actin proteins or actin-binding proteins[12,13]. SiR/SiR700-actin probes are structurally related to the naturally-occurring F-actin binding molecule jasplakinolide[12,13]. These F-actin probes utilize the proprietary fluorophore silicon rhodamine (SiR), a bright, photostable far-red dye with little, if any, background signal (Figs. 1,2). Because SiR probes exist in a closed, non-fluorescent state (spirolactone), the probes are self-quenching when unbound to F-actin[12,13] (Fig. 3). SiR probes are visualized with standard Cy5 settings (optimal excitation, 650 nm; emission, 670 nm) which confer compatibility with a wide range of genetically-encoded reporter fluorophores (e.g., GFP, m-Cherry)[12,13]. SPY555-actin is the newest addition to Spirochrome's family of F-actin live cell imaging probes. SPY555-actin is an improved version of the SiR-actin probes as a lower concentration can be used which offers robust labeling and reduced cytotoxicity and perturbation of actin cytoskeletal dynamics. SPY555-actin is imaged with a standard TMR or Cy3 channel (optimal excitation, 555 nm; emission, 580 nm) using the same staining protocol as for SiR/SiR700-actins. The key features of SiR and SPY actin probes are their cell permeability, fluorogenic character, minimal cytotoxicity, photostability, and compatibility with both standard fluorescence microscopy (e.g., wide-field, confocal) and super-resolution microscopy (e.g., STED, SIM)[12-18] (Figs. 1,2). The combination of STED and SiR/SPY-actin probes allows for unparalleled fluorescent visualization of subcellular F-actin structures and their physical characterization in living cells[14-18] (Figs. 1,2). SiR-actin probes have been used to examine F-actin in tissue[19] and a wide variety of cell types, including (but not limited to) human-induced pluripotent stem cell lines, cardiac cells, endothelial cells, epithelial cells, muscle cells, multiple cancer cell lines, and primary neurons[14,16-18,20-22] (Figs. 1,2).

Fluorescent actin and fluorescent actin-binding domains

The first studies of live cell actin dynamics were performed with fluorescent derivatives of actin protein whch were microinjected into cells[50,51]. This was a highly effective procedure but the apparatus took a while to setup. Thus overtime, transfections of GFP-actin conjugates became more popular. Fluorescently labeled actin protein or GFP/eGFP-actins worked well with fluorescence recovery after photobleaching (FRAP) microscopy[11,23-25].which indicates the dynamic nature of actin cytoskeleton rearrangements. However, GFP/eGFP-actin has several drawbacks[10]. First, the size of GFP (~28 kDa) can impair polymerization[26] and GFP-actin can differentially label F-actin structures[10,24]. Second, some actin-binding proteins (e.g., formin family nucleators) might sterically hinder incorporation of GFP-actin into actin seeds or growing polymers[27,28]. Third, there is a relatively high background signal from non-filamentous fluorescent actin[29]. Finally, expression of eGFP-actin can affect cell behavior[30,31].

Another method for actin live cell imaging utilizes yeast- or human-derived actin binding domains fused to GFP, eGFP, or m-Cherry fluorophores[10,11,32]. The most common genetically-encoded F-actin probes are Lifeact, utrophin (UtrCH), and F-tractin[10]. Lifeact is a 17 amino acid peptide from yeast Abp140[33,34] used for live cell imaging in mammalian and non-mammalian cells [24,34-36]. Lifeact has several disadvantages, including the possibility of affecting actin dynamics (so-called Lifeact-induced artifacts) and inhibiting the binding of actin-associated proteins such as cofilin[32,37-40]. Although Lifeact-GFP binds strongly to F-actin (Kd, 2.2 ± 0.3 µM), its binding affinity for G-actin is 10-fold higher[33], resulting in high background fluorescence. Lifeact does not bind all actin-containing structures[10,38]. Lifeact is introduced into the cell through transfection rather than simply adding it into the medium as is done for the SiR/SiR700/SPY probes. UtrCH is based on the tandem calponin homology domains (CH1 and CH2) of utrophin[41] and consists of the first 261 amino acid residues of human utrophin, an actin binding protein[42]. The CH domains bind to actin with a Kd of ~18 µM[43]. Utrophin-based probes have been used successfully across a wide range of cell types and species[10,32]. Similar to Lifeact, at high concentrations,

Continued from Page 1

utrophin-based probes can exert deleterious effects on actin cytoskeletal dynamics[32,37,44]. F-tractin is a 43 amino acid peptide derived from the rat actin-binding inositol 1,4,5-triphosphate 3-kinase A which binds F-actin with a Kd of ~10 µM[45,46]. Due to its larger size (in comparison to other probes), F-tractin might sterically hinder binding of actin-binding proteins that regulate and/or facilitate polymerization[10] and can modify actin-based cellular structures[32].

Actin-directed nanobodies and affimer proteins for F-actin

Two new technologies for monitoring actin dynamics in living cells are 1. single-domain antibodies, so-called nanobodies[52], and 2. actin "affimers" - synthetic, actin-binding proteins isolated from phage library screens[47-49]. If not developed correctly, nanobodies can exhibit a high background signal due to G-actin binding[10]. Recently, three eGFP-fusion actin affimers were described with low micromolar binding affinities for F-actin[47], but FRAP microscopy suggests that the eGFP-affimers may preferentially bind to a subset of actin filaments and alter actin organization in the cell[47].

Summary

Despite multiple options for visualizing F-actin-based structures in living cells, there is no perfect live cell probe (Table 1). Ideally, the best F-actin probe will be sensitive, selective, fluorogenic, produce a very low background signal, non-toxic, and easily introduced into a wide range of cells across multiple species. It is of paramount importance to confirm that changes in actin cytoskeleton dynamics/structural organization are physiologically relevant and not artifacts of the probe itself. To assist researchers in F-actin live cell imaging studies, Cytoskeleton offers the SiR and SPY actin live cell imaging probes.

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Figures and Table on Page 3

Figures and Table Appendix

Figures and Table Referenced in Text

Table 1. Comparision Table of F-actin probes

Table 1. Actin-binding probes for live cell imaging.

	SiR/SPY-actins	Lifeact GFP tagged	Actin fluorescently labeled	Actin GFP tagged	Utrophin GFP tagged	F-Tractin GFP tagged	Nanobody GFP tagged	Affimers GFP tagged
Source	*Jaspis johnstoni* SiR or SPY probes with desbromodes-methyl-jasplakinolide	*Saccharomyces cerevisiae* amino-acids 1-17 of ABP140	Skeletal muscle (rabbit) or non-muscle (beta actin human platelet)	Beta-actin fusion protein with GFP	*Homo sapiens* Amino acids 1-261 of utrophin	*Rattus norvegicus* Amino acids 10-52 of ITPKA	*Vicugna pacos* anti-actin-nanobody	Synthetic actin-binding probes isolated from phage library screens
Applications	Live cell imaging of endogenous F-actin including wide field confocal Ref. 14-18 Super-resolution microscopy (e.g., STED, SIM) Ref. 13-18	Live cell imaging Ref. 33,34	FRAP Ref. 50 Live cell imaging Ref. 51	FRAP Ref. 23 Live cell imaging of exogenous and endogenous actin Ref. 24,25	Live cell imaging of endogenous F-actin Ref. 41.	Live cell imaging of endogenous F-actin Ref. 45,46	Live cell imaging of endogenous actin Ref. 52	FRAP Ref. 47-49 Live cell imaging of endogenous actin Ref. 47-49
Advantages	Direct application to cells and tissues Fluorogenic Cell Permeability (No transfection required) Photostability Very Low Background Super-resolution Compatibility (STED, SIM) No Cytotoxicity Multiple Colors (e.g., far-red, red and orange) Binds only F-actin Small organic molecule (more stable)	Multiple Colors (e.g., far-red, red, orange, yellow, green)	Very similar conformation to endogenous actin Small fluorophore size.	Labeled actin is incorporated into endogenous filaments Multiple Colors (e.g., far-red, red, orange, yellow, green)	Does not bind actin monomers (G-actin) Multiple Colors (e.g., far-red, red, orange, yellow, green)	Does not bind actin monomers (G-actin) Multiple Colors (e.g., far-red, red, orange, yellow, green)	Small probe size Low probability of affecting actin dynamics Multiple Colors (e.g., far-red, red, orange, yellow, green)	High nanomolar affinity for F-actin Multiple Colors (e.g., far-red, red, orange, yellow, green) F-actin specific with correct screening protocol.
Disadvantages	Possible effects on actin dynamics at high concentrations	Large fluorescent reporter GFP Binds G-actin to produce a high background signaling Possible effects on actin dynamics Requires transfection	Requires injection	Large fluorescent reporter GFP Exogenous actin expression Binds G-actin to produce a high background signal Requires transfection	Large size Large fluorescent reporter GFP Possible effects on actin dynamics Requires transfection	Large fluorescent reporter GFP Possible effects on actin dynamics Requires transfection	Large fluorescent reporter GFP Binds G-actin to produce a high background signal Requires transfection	Large fluorescent reporter GFP Requires transfection
Overall rating	+++++	+++	+++	++	+++	+++	+++	+++

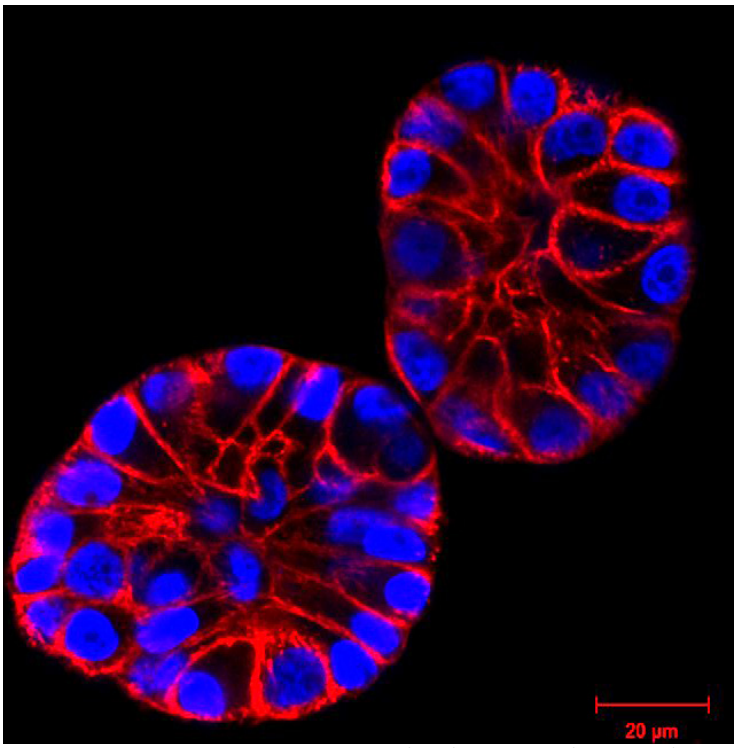

Figure 1. MCF10A cells expressing H2B-GFP (blue) in Matrigel (3D culture) stained with SiR-actin (red). Image taken on an inverted LSM microscope. Courtesy of Christian Conrad and Katharina Jechow, Heidelberg.

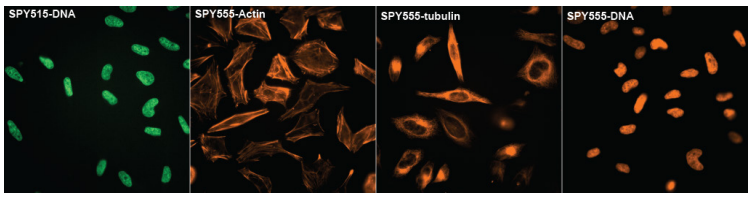

Figure 2. SPY505 and SPY555 staining DNA,Actin,Tubulin, and DNA . Photo comes from front page of Spirochrome's website.

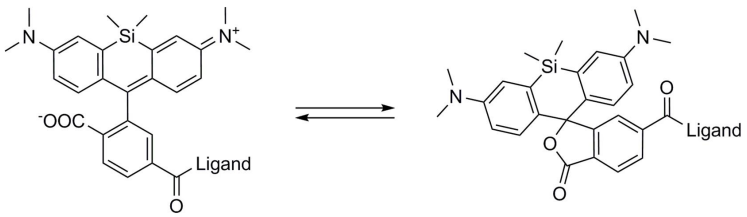

Figure 3. SiR derivatives exist in equilibrium between the fluorescent zwitterionic (open) form (left structure) and the non-fluorescent spiro (closed) form (right structure).

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Live Cell Imaging PRODUCTS

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Live Cell Imaging Products

Product	Ex / Em	Amount	Cat #
SPY555-Actin Includes SPY555-Actin	555 / 580 nm	100 stains	CY-SC202
SPY505-DNA Includes SPY505-DNA	512 / 531 nm	100 stains	CY-SC101
SPY555-DNA Includes SPY555-DNA	555 / 580 nm	100 stains	CY-SC201
SPY555-Tubulin Includes SPY555-Tubulin	555 / 580 nm	100 stains	CY-SC203
SPY595-DNA Includes SPY595-DNA	599 / 615 nm	100 stains	CY-SC301
SPY650-DNA Includes SPY650-DNA	652 / 674 nm	100 stains	CY-SC501
SPY650-Tubulin INcludes SPY650-Tubulin	652 / 674 nm	100 stains	CY-SC503
SPY700-DNA Includes SPY700-DNA	696 / 718 nm	100 stains	CY-SC601
SiR-Actin™ Kit Includes SiR-Actin and Verapamil	630 / 680 nm	50 nmol	CY-SC001
SiR-Tubulin™ Kit Includes SiR-Tubulin, and Verapamil	630 / 680 nm	50 nmol	CY-SC002
Cytoskeleton Kit Includes SiR-Actin, SiR-tubulin and Verapamil	630 / 680 nm	50 nmol each	CY-SC006
SiR-DNA™ Kit Includes SiR-DNA and Verapamil	630 / 680 nm	50 nmol	CY-SC007
SiR700-Actin Kit Includes SiR700-Actin and Verapamil	690 /720 nm	35 nmol	CY-SC013
SiR700-Tubulin Kit Includes SiR700-Tubulin and Verapamil	690 / 720 nm	35 nmol	CY-SC014
SiR700-DNA Kit Includes SiR700-DNA and Verapamil	690 / 720 nm	35 nmol	CY-SC015
Flipper-TR™ Kit For fluorescence cell membrane microscopy	480 / 600 nm	50 nmol	CY-SC020

Featured Papers and Application Notes

"Fluorogenic probes for live-cell imaging of the cytoskeleton"; G. Lukinavičius,et al. *Nature Methods* **11**, 731–733, 2014.

"STED Nanoscopy Reveals the Ubiquity of Subcortical Cytoskeleton Periodicity in Living Neurons"; E. D'Este, et al. *Cell Reports* , Volume 10 , **Issue 8** , 1246 – 1251, 2015.

"A near-infrared fluorophore for live-cell super-resolution microscopy of cellular proteins"; G. Lukinavičius, et al.; *Nature Chemistry* **5**, 132–139, 2013.

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"Red Si–rhodamine drug conjugates enable imaging in GFP cells"; E. Kim, et al.; *Chem. Commun.*, **50**, 4504-4507, 2014.

"A marginal band of microtubules transports and organizes mitochondria in retinal bipolar synaptic terminals"; M. Graffe, et al.; *J. Gen Physiol*. Vol. **146** No.1: 109-117, 2015.

Application Notes

"A Bright Dye for Live-Cell STED Microscopy"; S. Pitsch, I. Köster.

Explore Cytoskeleton's Novel Kits

Acti-Stain™ Phalloidins

Product	Amount	Cat #
Acti-stain 488™ phalloidin	300 Slides	PHDG1-A
Acti-stain 555™ phalloidin	300 Slides	PHDH1-A
Acti-stain 670™ phalloidin	300 Slides	PHDN1-A
Rhodamine Phalloidin	1 x 500 µl	PHDR1

Labeled Actin Proteins

Labeled Actin	Amount	Cat. #
Rhodamine Actin Protein Human platelet, non-muscle	4 x 10 µg 20 x 10 µg	APHR-A APHR-C
Rhodamine Actin Protein Rabbit skeletal muscle	10 x 20 µg 20 x 20 µg	AR05-B AR05-C

Actin Biochem Kits

Product	Assays	Cat. #
Actin Binding Protein Spin-Down Assay Biochem Kit Rabbit skeletal muscle actin	30-100	BK001
Actin Polymerization Biochem Kit (fluorescence format) Measure actin polymerization *in vitro*, contains rabbit skeletal muscle actin.	30-100	BK003
Actin Binding Protein Spin-Down Assay Biochem Kit Human platelet actin	30-100	BK013
G-Actin/F-actin In Vivo Assay Biochem Kit Measure the distribution of monomer and polymer actin	30-100	BK037

G-LISA Activation Assay Kits

Product	Assays	Cat. #
RhoA G-LISA™ Activation Assay (Luminescence format)	96	BK121
RhoA G-LISA™ Activation Assay Kit (Colorimetric format)	96	BK124
Rac1,2,3 G-LISA™ Activation Assay (Colorimetric format)	96	BK125
Rac1 G-LISA™ Activation Assay (Luminescence format)	96	BK126
Rac1 G-LISA™ Activation Assay Kit (Colorimetric Based)	96	BK128
Ras G-LISA™ Activation Assay Kit (Colorimetric Based)	96	BK131
Total RhoA ELISA	96	BK150

Pull Down Activation Assay Kits

Product	Assays	Cat. #
Ras Pull-down Activation Assay Biochem Kit (bead pull-down format)	50	BK008
RhoA / Rac1 / Cdc42 Activation Assay Combo Biochem Kit (bead pull-down format)	3 x 10	BK030
Cdc42 Pull-down Activation Assay Biochem Kit (bead pull-down format)	50	BK034
RhoA Pull-down Activation Assay Biochem Kit (bead pull-down format)	80	BK036