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Abstract

Tetrazine-bearing fluorescent labels enable site-specific tagging of proteins that are genetically manipulated with dienophile modified noncanonical amino acids. The inverse electron demand Diels-Alder reaction between the tetrazine and the dienophile fulfills the criteria of bioorthogonality allowing fluorescent labeling schemes of live cells. Here, we describe the detailed synthetic and labeling protocols of a near infrared emitting siliconrhodamine-tetrazine probe suitable for super-resolution imaging of residue-specifically engineered proteins in mammalian cells.

Keywords (separated by " - ")

Bioorthogonality - Tetrazine - Fluorogenicity - NIR emission - Super-resolution microscopy

Chapter 22

Fluorogenic Tetrazine-Siliconrhodamine Probe for the Labeling of Noncanonical Amino Acid Tagged Proteins

Eszter Kozma, Giulia Paci, Gemma Estrada Girona, Edward A. Lemke, and Péter Kele

Abstract

Tetrazine-bearing fluorescent labels enable site-specific tagging of proteins that are genetically manipulated with dienophile modified noncanonical amino acids. The inverse electron demand Diels-Alder reaction between the tetrazine and the dienophile fulfills the criteria of bioorthogonality allowing fluorescent labeling schemes of live cells. Here, we describe the detailed synthetic and labeling protocols of a near infrared emitting siliconrhodamine-tetrazine probe suitable for super-resolution imaging of residue-specifically engineered proteins in mammalian cells.

Key words Bioorthogonality, Tetrazine, Fluorogenicity, NIR emission, Super-resolution microscopy

1 Introduction

Emerging super-resolution microscopy (SRM) techniques have brought substantial progress in the exploration of biomolecular processes in the sub-diffraction range [1–3]. Live organisms can now be studied in fine details, yet, improvements can result in further increase of resolution and enable new biological insights. To address current limitations that impede such improvements, small synthetic dyes with suitable spectral characteristics that allow site-specific tagging of intracellular structures even under in vivo conditions are needed [4, 5]. Preferred synthetically tailored, small-sized organic fluorophores are membrane permeant, photostable, brightly fluorescent, and allow minimal background labeling and autofluorescence in order to result in a high signal-to-noise ratio. The means by which such ideal probes are installed onto the biomolecule of interest is also crucial. The applied chemistry should be biocompatible and highly selective. Such chemical transformations are termed

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bioorthogonal [6–8]. Most of the time, fast kinetics are also required. Inverse electron demand Diels-Alder (IEDDA) cycloaddition of tetrazines and strained unsaturated ring systems enable fast and highly selective reactions [9, 10]. Most IEDDA labeling schemes rely on the use of tetrazine bearing fluorescent probes in combination with cyclooctyne or *trans*-cyclooctene modified biomolecules (e.g., by means of genetically encoded noncanonical amino acids, ncAAs). Furthermore, tetrazine scaffolds can efficiently quench fluorescence of dyes giving rise to fluorogenic scaffolds [11–15].

In live cell imaging applications, phototoxicity and autofluorescence can be minimized if the spectral characteristics of the applied probe allow far-red/near-infrared (NIR) excitation/emission. Since labeling schemes often apply large excess of the labeling species, background fluorescence of unreacted probes bound nonspecifically to hydrophobic surfaces is often encountered, and several washing cycles are needed before imaging, which excludes labeling of, e.g., proteins with rapid turnovers. So-called fluorogenic probes efficiently reduce background fluorescence as they are minimally fluorescent when bound nonspecifically but become intensely emitting upon the particular specific chemical reaction [16, 17].

Siliconrhodamines (SiRs) are widely used membrane-permeable NIR dyes suitable for SRM applications [18–23]. Besides their high photostability and brightness, the unique environment-dependent fluorescence of carboxy-SiRs due to a polarity dependent lactone-formation offers an appealing opportunity to distinguish between specific and nonspecific labeling (polarity-based fluorogenicity) [18]. Carboxyl-SiRs exist in a fluorescent zwitterionic form when they are in polar environment such as near protein surfaces. In non-polar microenvironments, however, they isomerize to their respective, non-fluorescent spirolactone form. These characteristics render carboxy-SiRs minimally emitting when bound nonspecifically onto nonpolar surfaces. Despite their wide applications [21], there are only a few examples of SiR-based probes for site-specific labeling of proteins [18, 22, 23].

Herein, we describe the detailed synthesis of a bioorthogonally applicable, NIR-emitting, membrane permeable, double fluorogenic carboxy-SiR suitable for SRM imaging, as well as the labeling scheme for genetically modified proteins in living cells (Fig. 1) [24]. We show the applicability of the method by labeling site-specifically an intracellular skeletal protein, vimentin, engineered with an IEDDA reactive ringstrained ncAA by means of Amber suppression using an orthogonal tRNA/tRNA synthetase system derived from *Methanosarcia mazei* as described previously [18, 25–30].We demonstrate the power of the developed SiR-dye in subsequent site-specific SRM imaging applications.

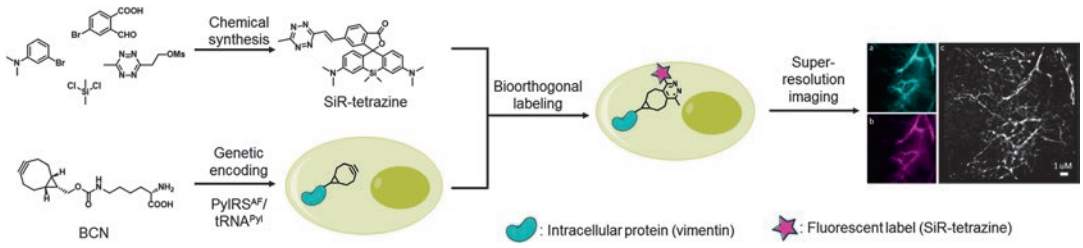


Fig. 1 Illustration of chemical synthesis, biorthogonal labeling, and subsequent imaging steps described in the protocol

2 Materials

The laboratory should be equipped to perform basic chemical synthesis. All synthetic steps shall be performed in a chemical hood equipped with magnetic stirrer, nitrogen-line, and vacuum line. The laboratory should be further equipped with a rotary evaporator (with a high vacuum pump), and a standard S1 cell culture. In this protocol specific examples of cell line, protein of interest, and microscopy setup are given, but the user should keep in mind that the method can easily be applied to other biological systems and that imaging can be performed on any fluorescence microscope.

2.1 Synthesis of 3,3′-(Dimethylsilanediyl)bis(N,N-dimethylaniline) (2)

1. Magnetic stirrer with a stand clamp.
2. 250 mL round bottom flask with a conical joint NS 29/32.
3. 500 mL round bottom flask with a conical joint NS 29/32.
4. 250 mL separatory funnel.
5. 500 mL Erlenmeyer flask.
6. Magnetic stir bar.
7. Bubbler filled with mineral oil.
8. Nitrogen gas.
9. Crystallizing dish.
10. Rotary evaporator.
11. High vacuum pump (like Vacuubrand Vario Pro PC3001 chemistry pumping unit).
12. Automated flash chromatographer (such as Teledyne Isco CombiFlash Rf+).
13. Empty solid load cartridge 65 g with three pieces of frit and a loading rod (like RedisepRf).
14. Silica gel (25–40 μm).
15. Analytical thin-layer chromatography (TLC) plates (such as silica gel 60 F_{254} precoated aluminum TLC plates from Merck).

16. 3-bromo-*N*,*N*-dimetylaniline (**1**, commercially available).
17. *n*-Butyllithium (*n*-BuLi, in 1.6 M solution in hexane, commercially available).
18. Dichlorodimethylsilane ($SiCl_2Me_2$, commercially available).
19. Absolute tetrahydrofurane (THF).
20. Dry ice.
21. Acetone.
22. Water (distilled or purified).
23. Ethyl acetate (EtOAc).
24. Saturated NaCl solution.
25. Anhydrous magnesium sulfate ($MgSO_4$).
26. Dichloromethane (DCM).
27. Celite.
28. Hexane.

2.2 Synthesis of 4-Bromo-2-formylbenzoic acid (3)

1. Magnetic stirrer with stand clamp and oil bath.
2. 250 mL round bottom flask with a conical joint NS 29/32.
3. Reflux condenser for with a conical joint NS 29/32.
4. Magnetic stir bar.
5. Rotary evaporator.
6. High vacuum pump (like Vacuubrand Vario Pro PC3001 chemistry pumping unit).
7. Fritted funnel.
8. Desiccator.
9. 5-bromophtalide (commercially available).
10. *N*-bromosuccinimide (NBS) (commercially available).
11. Azobisisobutyronitrile (AIBN) (commercially available).
12. 1,2-dichloroethane (DCE).
13. Water (distilled or purified).

2.3 Synthesis of 6′-Bromo-3,7-bis(dimethylamino)-5,5-dimethyl-3′H,5H-spiro[dibenzo[b,e]siline-10,1′-isobenzofuran]-3′-one (SiR-Br, 4)

1. Magnetic stirrer with stand clamp and oil bath.
2. 2–6 mL borosilicate microwave vial (like Anton Paar G10) with snap cap and PTFE-coated silicone septa.
3. Magnetic stir bar.
4. Sonicator (such as Selecta ultrasons 6.5 L).
5. Rotary evaporator.
6. High vacuum pump (like Vacuubrand Vario Pro PC3001 chemistry pumping unit).
7. Chromatography column with fused-in-frit and PTFE stopcock.

8. Silica gel (60–200 μM).
9. Preparative TLC plate (glass-backed such as Kiesegel 60 F_{254} 20 × 20 cm, 2 mm).
10. Glass chamber for preparative TLC purification.
11. Analytical TLC plates (such as silica gel 60 F_{254} precoated aluminum TLC plates from Merck).
12. Compound **2** (for preparation *see* Subheading 3.1).
13. Copper(II) bromide ($CuBr_2$) (commercially available).
14. Compound **3** (for preparation *see* Subheading 3.2).
15. DCM.
16. Celite.
17. Hexane.
18. EtOAc.
19. Triethylamine (Et_3N).
20. Methanol (MeOH).

2.4 Synthesis of Ethyl 3-hydroxypropionimidate hydrochloride (9)

1. Magnetic stirrer with stand clamp.
2. 250 mL round bottom flask with a conical joint NS 29/32.
3. 500 mL two-neck round bottom flask.
4. Magnetic stir bar.
5. Dropping funnel.
6. Analytical TLC plates (such as silica gel 60 F_{254} precoated aluminum TLC plates from Merck).
7. Rotary evaporator.
8. High vacuum pump (like Vacuubrand Vario Pro PC3001 chemistry pumping unit).
9. 3-Hydroxypropionitrile (commercially available).
10. Acetyl chloride (commercially available).
11. Absolute ethanol (EtOH).
12. NaCl.
13. cc. H_2SO_4.
14. Diethyl ether (Et_2O).

2.5 Synthesis of 2-(6-Methyl-1,2,4,5-tetrazin-3-yl)ethyl methanesulfonate (OMs-tet, 5)

1. Magnetic stirrer with stand clamp.
2. 250 mL round bottom flask with a conical joint NS 29/32.
3. 100 mL round bottom flask with a conical joint NS 29/32.
4. 500 mL Erlenmeyer flask.
5. 250 mL separatory funnel.

6. Vacuum line (with vacuum pump like Vacuubrand rotary vane pump RZ 2.5).
7. Rotary evaporator.
8. High vacuum pump (like Vacuubrand Vario Pro PC3001 chemistry pumping unit).
9. Magnetic stir bar.
10. Bubbler filled with mineral oil.
11. Nitrogen gas.
12. Crystallizing dish for ice/water bath.
13. Automated flash chromatographer (such as Teledyne Isco CombiFlash Rf+).
14. Empty solid load cartridge 65 g with three pieces of frit and a loading rod (like RedisepRf).
15. Silica gel (25–40 μm).
16. Analytical TLC plates (such as silica gel 60 F_{254} precoated aluminum TLC plates from Merck).
17. Compound **9** (for preparation *see* Subheading 3.4).
18. Acetonitrile (MeCN).
19. Hydrazine hydrate (N_2H_4 50–60%) (commercially available).
20. Sodium nitrite ($NaNO_2$) (commercially available).
21. Distilled water.
22. Ice.
23. cc. HCl.
24. EtOAc.
25. Saturated NaCl solution.
26. Anhydrous $MgSO_4$ (commercially available).
27. DCM.
28. Celite.
29. Hexane.
30. Methanesulfonyl chloride (MsCl, commercially available).
31. Et_3N.

2.6 Synthesis of (E)-3,7-Bis(dimethylamino)-5,5-dimethyl-6′-(2-(6-methyl-1,2,4,5-tetrazin-3-yl) vinyl)-3′H,5H-spiro[dibenzo[b,e] siline-10,1′-isobenzofuran]-3′-one (SiR-tetrazine, 6)

1. 2–6 mL borosilicate microwave vial (like Anton Paar G10) with snap cap and PTFE-coated silicone septa.
2. 50 mL round bottom flask with conical joint NS 29/32.
3. Magnetic stir bar.
4. Microwave reactor (such as Anton Paar Monowave 300).
5. Analytical TLC plates (such as silica gel 60 F_{254} precoated aluminum TLC plates from Merck).

6. Rotary evaporator.
7. High vacuum pump (like Vacuubrand Vario Pro PC3001 chemistry pumping unit).
8. Preparative TLC plate (such as glass-backed Kiesegel 60 F_{254} 20 × 20 cm, 2 mm).
9. Glass chamber for preparative TLC.
10. Preparative HPLC system.
11. Preparative C18 column (such as Gemini 5 μm C18 110 Å LC column 150 × 21.2 mm).
12. Round bottom flask freeze-drier for lyophilization.
13. SiR-Br (compound **4**, for preparation *see* Subheading 3.3).
14. OMs-tet (compound **5**, for preparation *see* Subheading 3.5).
15. Tris(dibenzylideneacetone)dipalladium(0) ($Pd_2(dba)_3$, commercially available).
16. 1,2,3,4,5-Pentaphenyl-1′-(di-tert-butylphosphino)ferrocene (QPhos, commercially available).
17. Anhydrous dimethylformamide (DMF).
18. *N*,*N*-dicyclohexylmethylamine ($(Cy)_2NMe$, commercially available).
19. DCM.
20. Hexane.
21. EtOAc.
22. MeCN.
23. Distilled water.

2.7 Site-Specific Protein Engineering with the Ringstrained ncAA Bicyclo[6.1.0] nonyne-Lysine (BCN^{endo}): Cell Culture and Transfections

Note that many steps are similar, if not even identical to procedures described in Chapter 18, by Nikic et al. [31].

1. COS-7 (Sigma 87021302) cell line or any other cell line of interest.
2. 1× PBS (phosphate-buffered saline).
3. Dulbeccos's modified Eagle medium (DMEM, Gibco 41965039) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma F7524), 1% (v/v) penicillin-streptomycin 10,000 U/mL (Gibco 15140122), 1% (v/v) 100 mM sodium pyruvate (Gibco 11360070), and 1% (v/v) 200 mM L-glutamine (Sigma G7513). Store at 4 °C (*see* **Note 1**). Other manufacturers providing similar formulations could also be used.
4. Trypsin-EDTA (Gibco 25300054 or similar). Long-term storage at −20 °C. Once thawed, keep at 4 °C.
5. Hemocytometer.

6. Cell culture incubator at 37 °C with a humidified 5% CO_2 atmosphere.
7. Cell culture hood.
8. Water bath at 37 °C.
9. Sterile plastic ware (Falcon and microcentrifuge tubes, 100 mm cell culture dishes, serological pipettes).
10. 4-well Lab-Tek™ II Chambered Coverglass (Nunc™).
11. JetPrime transfection reagent (Polyplus, or other transfection reagents appropriate for the cell line of choice).
12. Eukaryotic expression vector for the Amber suppression machinery. In this protocol a vector with *M. mazei* NESPylRS^{AF}/tRNA^{Pyl} was used (available from Dr. Edward Lemke, EMBL, Heidelberg) (*see* **Note 2**).
13. Eukaryotic expression vector for the Amber mutant of the protein of interest. In this protocol pVimentin^{N116TAG}-PSmOrange was used (*see* **Notes 3** and **4**).
14. Plasmid Maxiprep kit (low or endotoxin-free recommended, e.g., Qiagen 12362 or Invitrogen K210007).
15. Noncanonical amino acid stock: 100 mM endo BCN-L-Lysine (BCN^{endo}, SiChem SC8014) in 15% (v/v) DMSO, 0.2 M NaOH. Store at −20 °C.
16. Sterile 1 M HEPES. Store at 4 °C.
17. Vortex.
18. Benchtop mini-centrifuge.
19. SiR-tetrazine dye of choice. Stock in DMSO at 0.5 mM.
20. 2% PFA: 2% paraformaldehyde in 1× PBS (*see* **Note 5**).

2.8 Localization-Based Super-Resolution Imaging and Analysis

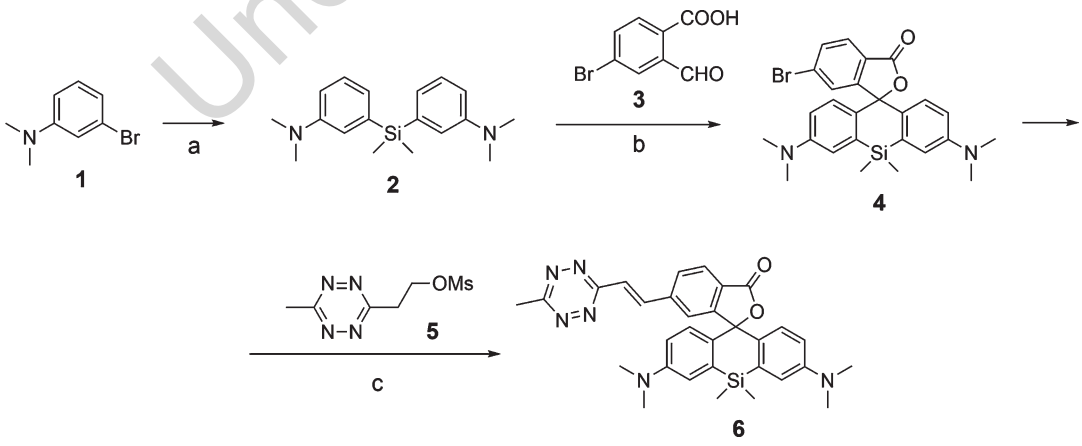
1. 20× TN buffer: 1 M Trizma base, 0.2 M NaCl, adjust pH to 8.0 with HCl and filter. Store at 4 °C.
2. 20% Glucose. Store at 4 °C for maximum 2 weeks.
3. 20× Glucose Oxidase/Catalase (GO/C) stock: 51% glycerol, 50 mM Tris–HCl pH 8.0, 800 µg/mL Catalase (Sigma C3155), 10 mg/mL Glucose Oxidase (Sigma G0543). Store at −20 °C.
4. MEA (Sigma 411000). Store at −20 °C, aliquotes can be reused if kept cold all the time.
5. GLOX-MEA buffer: 1× TN buffer, 10% glucose, 10 mM MEA, and 1× GO/C mix) in water (*see* **Notes 6** and **7**).
6. TIRF-microscope (e.g., Leica GSDIM) with appropriate laser and filter cube for dyes of choice (*see* **Note 8**) and software for data analysis (*see* **Note 9**).

3 Methods

Perform all synthesis steps in a chemical hood (for safety instructions, *see* **Note 10**) (*see* Scheme 1).

3.1 Synthesis of 3,3′-(Dimethylsilanediyl)bis(N,N-dimethylaniline) (2)

1. Weigh 5.0 g 3-bromo-*N*,*N*-dimethylaniline (**1**) with a glass pipette into a well-dried 250 mL round bottom flask with conical joint NS 29/32 equipped with a magnetic stir bar (*see* **Note 11**).
2. Close the flask with a fold over rubber septum, mount it above a magnetic stirrer using a stand clamp, and flush the flask carefully with nitrogen using a bubbler. Make sure the reaction is kept under nitrogen atmosphere until otherwise stated (*see* **Note 12**).
3. Transfer 100 mL absolute THF to the flask while stirring the solution.
4. Fill a large crystallizing dish with dry ice and add acetone very carefully until the temperature reaches −78 °C. Place the dish under the flask and make sure the temperature is kept constant during the reaction. Refill dry ice if needed.
5. When the temperature of the reaction mixture reaches −78 °C, add 17 mL *n*-butyllithium (in 1.6 M solution in hexane) dropwise to the reaction mixture over the course of 15 min.
6. Stir the reaction at −78 °C under nitrogen atmosphere for 2 h.
7. Add 1.8 mL dichlorodimethylsilane dropwise.
8. Remove the cooling dish, and let the reaction mixture warm to room temperature. Keep stirring the reaction mixture for an additional 16 h (*see* **Note 13**).

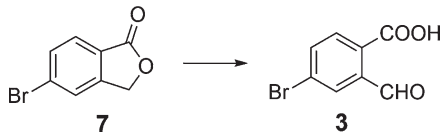


Scheme 1 Synthesis of SiR-tetrazine (**6**). Reaction conditions: (a) (1) *n*-BuLi, THF, −78 °C, 2 h; (2) $SiCl_2Me_2$, RT, 16 h, 71%; (b) $CuBr_2$, 140 °C, 16 h, 21%; (c) $Pd_2(dba)_3$, QPhos, $(Cy)_2NMe$, DMF, 50 °C, 40 min, MW, 48%

9. Remove the septum and very carefully quench the reaction with 30 mL of distilled water. Add only small portions of the water at once and wait until the bubbling stops before adding another portion.
10. Transfer the mixture to a 250 mL separatory funnel. Add 30 mL EtOAc and extract the aqueous phase (*see* **Note 14**). Repeat it two more times with 2 × 30 mL EtOAc. Combine the organic phases and wash it with saturated NaCl solution to remove any remaining water soluble components and impurities. In a 500 mL Erlenmeyer flask add anhydrous $MgSO_4$ to the organic phase to remove water. Filter the solution to remove the drying agent.
11. Transfer the solution to a 500 mL round bottom flask with a conical joint NS 29/32 and remove the solvents under reduced pressure with a rotary evaporator set to 40 °C and 400 mbar. Decrease the pressure until no additional solvent is evaporating.
12. Redissolve the oily residue in 20 mL DCM and add 5 g celite to the solution. Remove the solvent under reduced pressure set to 40 °C and 850 mbar on a rotary evaporator slowly decreasing the pressure until the celite is completely dry (*see* **Note 15**).
13. Fill a 65 g cartridge with silica gel up to 2/3, add a frit using a loading rod, transfer the celite from the previous step onto the top, place a second a frit on top of it, and place the column into an automated flash chromatographer. Start the chromatographic separation using a 1–15% EtOAc gradient in hexane over 25 min. Use 254 and 280 nm detection wavelengths for fraction collection. Check the collected fractions by TLC (*see* **Notes 13** and **16**).
14. Combine the fractions containing purely the product in a round bottom flask, remove the solvent under reduced pressure with a rotary evaporator set to 40 °C and 300 mbar. Further decrease the pressure until no additional solvent is evaporating.
15. Collect the light yellow oil formed and store it at 4 °C until further use (*see* **Note 17**).

3.2 Synthesis of 4-Bromo-2-formylbenzoic acid (3) (Scheme 2)

1. Weigh 4.582 g 5-bromophtalide and add it to a 250 mL round bottom flask with a conical joint NS 29/32 equipped with a magnetic stir bar.
2. Weigh 4.209 g NBS and add it to the 250 mL round bottom flask.
3. Weigh 177 mg AIBN and mix it with the solid compounds in the 250 mL round bottom flask (*see* **Notes 18** and **19**).
4. Dissolve the solid in 100 mL dichloroethane (DCE) and mount the flask using a stand clamp and immerse it in an oil bath on a magnetic stirrer. Adjust a reflux condenser with a conical joint NS 29/32.



7 3

Scheme 2 Synthesis of 4-bromo-2-formylbenzoic acid (**3**). Reaction conditions: (1) NBS, AIBN, DCE, reflux, 2 h; (2) water, reflux, 2 h; 90%

5. While continuously stirring, heat the reaction mixture to 88 °C until the solvent starts to reflux and keep the temperature for an additional 2 h (*see* **Note 20**).
6. Let the reaction mixture cool to room temperature. Close the flask with a fold over rubber septum and keep it at −20 °C for 2 h.
7. Filter the precipitate and collect the filtrate.
8. Using a 250 mL round bottom flask, remove the solvent under reduced pressure with a rotary evaporator set to 40 °C and 200 mbar. Further decrease the pressure until no additional solvent is evaporating. A white crystalline residue forms.
9. Suspend the crystals in 50 mL water in a 250 mL round bottom flask with a conical joint NS 29/32. Adjust a condenser with a conical joint NS 29/32 and mount the flask using a stand clamp and immerse it in an oil bath on a magnetic stirrer. Start stirring the suspension.
10. Adjust the temperature to 100 °C and keep the suspension refluxed for 2 h under continuous stirring (*see* **Note 21**).
11. Let the reaction mixture cool to room temperature. Close the flask with a fold over rubber septum and keep it at 4 °C for 16 h.
12. Collect the precipitate using a fritted funnel by applying vacuum. Wash the precipitate with 2 × 10 mL ice-cold water. Remove as much water as possible from the precipitate and then place the funnel into a desiccator, supplied with a drying agent, under vacuum for 24 h to remove any residual water.
13. Remove the funnel from the desiccator and collect the white crystalline powder. Store it at 4 °C until further use (*see* **Note 17**).

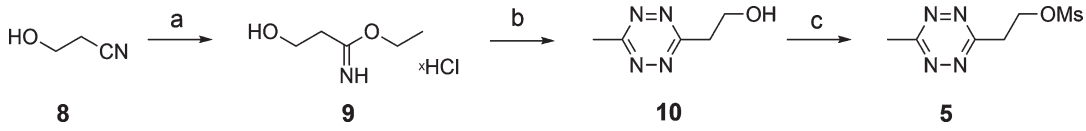
3.3 Synthesis of 6′-Bromo-3,7-bis(dimethylamino)-5,5-dimethyl-3′H,5H-spiro[dibenzo[b,e]siline-10,1′-isobenzofuran]-3′-one (SiR-Br, 4)

1. Weigh 641 mg compound **3** and transfer it to a microwave borosilicate glass vial (2–6 mL) equipped with a magnetic stir bar.
2. Weigh 13 mg $CuBr_2$ and add it to the microwave vial.
3. Weigh 167 mg of compound **2** and add it to the vial using a pipette. Slightly mix the mixture.
4. Close the tube with a snap cap containing a PTFE-coated silicone septum and mount it using a stand clamp and immerse it in an oil bath (covered 2/3 in oil) on a magnetic stirrer. Start stirring.

5. Heat the reaction to 140 °C and keep stirring for 16 h at this temperature (*see* **Note 22**).
6. Let the reaction mixture cool to room temperature.
7. Add 3 mL DCM and dissolve the reaction mixture by applying sonication (*see* **Note 23**).
8. Transfer the solution to a round bottom flask and add 4 g celite. Remove the solvent under reduced pressure with a rotary evaporator set to 40 °C and 850 mbar. Further decrease the pressure until no solvent is evaporating (*see* **Note 15**).
9. Purify the product by column chromatography. For this, suspend silica gel in a 4:1 mixture of hexane and EtOAc with 1% (v/v) Et_3N and transfer to a chromatography column with a fused-in frit and PTFE stopcock. Cover the silica gel with sand and then place the celite on the top. Perform chromatographic purification using a 4:1 mixture of hexane and EtOAc with 1% (v/v) Et_3N and collect the eluting fractions in test tubes. Check the presence of the product by TLC (R_f = 0.4) (*see* **Note 24**).
10. Combine fractions containing the product and remove the solvent under reduced pressure with a rotary evaporator set to 40 °C and 200 mbar. Further decrease the pressure once no additional solvent is evaporating. A yellow solid forms.
11. Perform preparative thin-layer chromatography purification (*see* **Note 25**). For this, preincubate a large glass chamber with 50:1 mixture of DCM and MeOH. Dissolve the yellow residue in 2 mL of DCM and slowly apply the solution onto glass-backed preparative TLC plates using a pipette near one edge of the plate. Let the DCM evaporate before placing the plate into the chamber to develop (*see* **Note 26**).
12. Remove the plate and scrape the silica gel containing the blue product. Suspend the silica gel in DCM to dissolve the product from the silica. Filter the silica gel and collect the solution. In order to dissolve as much product from the silica as possible, repeat this step twice.
13. Collect the filtrates in a round bottom flask and remove the solvent under reduced pressure with a rotary evaporator set to 40 °C and 850 mbar. A white crystalline residue forms. Store at 4 °C until further use (*see* **Note 17**).

3.4 Synthesis of Ethyl 3-hydroxypropionimidate hydrochloride (9) (Scheme 3)

1. To synthesize OMs-tet (**5**), start preparing compound **9** from commercially available 3-hydroxypropionitrile (**8**).
2. For this, weigh 1.4 mL 3-hydroxypropionitrile (**8**) and transfer it to a 250 mL round bottom flask with a conical joint NS 29/32 charged with a magnetic stirring bar. Dissolve the compound in 14 mL abs. EtOH and mount the round bottom flask above a magnetic stirrer using a stand clamp.



Scheme 3 Synthesis of mesyl-tetrazine (OMs-tet) (**5**). Reaction conditions: (a) EtOH, HCl gas, acetyl chloride, RT, 2 h, 83%; (b) (1) MeCN, hydrazine hydrate, N_2, RT, 2 h; (2) $NaNO_2$, cc. HCl; 30%; (c) MsCl, Et_3N, DCM, 91%

3. While continuously stirring, add 3 mL acetyl chloride dropwise.
4. Prepare HCl gas in situ. To do this, fill a 500 mL two-necked round bottom flask with NaCl and a magnetic stir bar. Adjust a dropping funnel onto one neck and fill it with cc. H_2SO_4. Adjust a teflon tube to the other neck using a connecting adapter. Insert a glass pipette at the other end of the teflon tube and insert it into the carefully stirred EtOH solution. Start dropping the cc. H_2SO_4 while continuously stirring the solution. (For safety instructions during HCl gas evolution *see* **Note 27**).
5. Continue purging the reaction with HCl gas for 2 h at room temperature. Check for completion (disappearance of starting material) by TLC (starting material R_f = 0.3 in DCM:MeOH 30:1, the product is at R_f = 0).
6. Purge the solution with nitrogen gas to remove residual HCl gas and remove the solvent under reduced pressure with a rotary evaporator set to 40 °C and 150 mbar. Further decrease the pressure until no additional solvent is evaporating. Close the flask with a fold over rubber septum and keep it at −20 °C for 16 h.
7. Collect the formed off-white crystals on a vacuum-filter using a fritted funnel and wash the crystals with Et_2O (2 × 10 mL). Store at 4 °C until further use (*see* **Note 17**).

3.5 Synthesis of 2-(6-Methyl-1,2,4,5-tetrazin-3-yl)ethyl methanesulfonate (OMs-tet, 5)

1. First, prepare the OH-tetrazine (**10**). For this, weigh 6.14 g compound **9** and transfer it to a 250 mL round bottom flask with a conical joint NS 29/32.
2. Suspend compound **9** in 24 mL acetonitrile (MeCN) and 40 mL hydrazine hydrate and start stirring.
3. Close the flask with a fold over rubber septum, mount it above a magnetic stirrer using a stand clamp and flush the flask carefully with nitrogen using a bubbler. Keep the nitrogen flow throughout the reaction (*see* **Note 12**).
4. Keep stirring the solution for 2 h at room temperature (*see* **Note 28**).
5. Remove the septum. Weigh 34.5 g $NaNO_2$ and dissolve it in 50 mL water. Add the solution to the reaction mixture carefully while continuously stirring.
6. Prepare nitrous gases (NO_x) in situ to oxidize the dihydrotetrazine to tetrazine in the reaction mixture: Cool the reaction

flask with an ice/water bath (*see* **Note 29**). While vigorously stirring, very carefully add cc. HCl dropwise. Wait until the gas evolution stops and add more cc. HCl dropwise. Repeat it until pH reaches 3 (check it with universal pH paper) and no more gas is evolved (*see* **Notes 27** and **30**).

7. Add 50 mL EtOAc to the mixture and transfer to a separatory funnel. Separate the organic phase. Add 50 mL EtOAc to the aqueous phase (*see* **Note 14**) and extract it. Repeat the extraction of the aqueous phase three more times each time with 50 mL EtOAc.
8. Combine the organic phases and extract it with 50 mL saturated NaCl solution to further remove water soluble components and impurities. Place the organic phase in an Erlenmeyer flask and add anhydrous $MgSO_4$ to the organic phase to remove residual water. Filter the solution to remove the drying agent.
9. Transfer the solution to a round bottom flask and remove solvents under reduced pressure with a rotary evaporator set to 40 °C and 240 mbar. Further decrease the pressure until no additional solvent is evaporating (*see* **Note 31**).
10. To remove the volatile pink 3,6-dimethyl-1,2,4,5-tetrazine side-product, place the round bottom flask to a high vacuum line and apply vacuum for at least 10 h, or until no further pink residue is removed.
11. Dissolve the remaining pink product in 20 mL DCM and add 5 g celite. Remove the solvent under reduced pressure with a rotary evaporator set to 40 °C and 850 mbar. Further decrease the pressure until no solvent is evaporating (*see* **Note 15**).
12. Fill a 65 g cartridge with silica gel up to 2/3, add a frit using a loading rod, transfer the celite from the previous step to the top, place a second frit on top of it, and place the column into an automated flash chromatographer. Start the chromatography separation using a 0–70% EtOAc gradient in hexane over 25 min. Use 280 and 524 nm for fraction collection. Check the collected fractions using TLC (*see* **Notes 16** and **32**).
13. Combine the fractions containing the product and remove the solvent under reduced pressure with a rotary evaporator set to 40 °C and 300 mbar. Further decrease the pressure until no additional solvent is evaporating. Collect the resulting OH-tetrazine as pink oil. Keep it at 4 °C until further use.
14. Dissolve the pink oil in 20 mL DCM and transfer to a 100 mL round bottom flask with a conical joint NS 29/32 equipped with a magnetic stir bar. Mount the reaction flask above a magnetic stirrer using a stand clamp and cool it with an ice/water bath. Start stirring the reaction mixture.

15. Measure 1.81 mL MsCl and add it to the reaction mixture dropwise.
16. Measure 3.26 mL Et_3N and add it to the reaction mixture dropwise.
17. Keep stirring the solution for 10 min at room temperature. Check for completion by TLC (*see* **Note 33**).
18. Transfer the reaction mixture into a separatory funnel and wash it with 50 mL water. Collect the organic phase. Extract the aqueous phase with an additional 3 × 30 mL DCM (*see* **Note 34**). Combine the organic phases in an Erlenmeyer flask and add anhydrous $MgSO_4$ to the organic phase to remove residual water. Filter the solution to remove the drying agent.
19. Transfer the solution to a round bottom flask, add 3 g celite, and remove solvents under reduced pressure with a rotary evaporator set to 40 °C and 850 mbar. Further decrease the pressure until no additional solvent is evaporating.
20. Perform flash chromatography purification. Fill a 65 g cartridge with silica gel up to 2/3, add a frit using a loading rod, transfer the celite from the previous step to the top, add a second frit on top of it and place the column into an automated flash chromatographer. Start chromatography using a 5–55% EtOAc gradient in hexane over 20 min. Use 280 and 524 nm for fraction collection. Check the collected fractions using TLC (*see* **Notes 16** and **33**).
21. Combine the fractions containing the product in a round bottom flask and remove the solvent under reduced pressure with a rotary evaporator set to 40 °C and 300 mbar. Further decrease the pressure until no more solvent is evaporating. Collect the resulting OMs-tetrazine as pink crystals. Keep it at −20 °C until further use (*see* **Notes 17** and **35**).

3.6 Synthesis of (E)-3,7-Bis(dimethylamino)-5,5-dimethyl-6′-(2-(6-methyl-1,2,4,5-tetrazin-3-yl) vinyl)-3′H,5H-spiro[dibenzo[b,e] siline-10,1′-isobenzofuran]-3′-one (SiR-tetrazine, 6)

1. Weigh 61 mg SiR-Br (**4**) and transfer it to a dry microwave borosilicate glass vial (2–6 mL) equipped with a magnetic stir bar.
2. Weigh 24 mg OMs-tet (**5**) and add it to the vial.
3. Weigh 10 mg $Pd_2(dba)_3$ and transfer it to the vial.
4. Weigh 32 mg QPhos and add it to the solids in the vial.
5. Mix all the solids and dissolve them in 3 mL anhydrous DMF.
6. Add 94 μL $(Cy)_2NMe$ to the solution, purge the vial with nitrogen, and close it with a snap cap containing a PTFE-coated silicon septum.
7. Transfer the vial to a microwave reactor and start the reaction at 50 °C for 40 min. Check for completion by TLC (*see* **Note 36**).
8. Remove the reaction mixture from the vial and transfer it to a 50 mL round bottom flask. Remove the solvent under reduced

pressure with a rotary evaporator set to 45 °C and 30 mbar. Further decrease the pressure until no additional solvent is evaporating.

9. Dissolve the residue in 2 mL DCM and perform preparative TLC purification. For this, preincubate a large glass chamber with 2:1 mixture of hexane and EtOAc. Slowly apply the solution onto glass-backed preparative TLC plates using a pipette near one edge of the plate. Let the DCM evaporate before placing the plate into the chamber to develop.
10. Remove the plate and scrape the silica gel containing the product (*see* **Note 36**). Suspend the silica gel in MeCN to dissolve the product (*see* **Note 37**). Filter the silica gel and collect the solution. In order to dissolve as much product from the silica as possible, repeat this step two more times with fresh MeCN portions.
11. Collect the filtrates in a round bottom flask and remove the solvent under reduced pressure with a rotary evaporator set to 40 °C and 200 mbar.
12. Perform preparative HPLC purification (*see* **Note 25**). For this, dissolve the residue in MeCN:H_2O 1:1. Purify the product using a C18 column with the following gradient: A = H_2O B = MeCN, flow rate: 15 mL/min, 0 min 70% A, 70 min 0% A. Use 220, 296, 520 and 620 nm detection wavelengths. Analyze the fractions with MS (or LC-MS). Combine pure fractions and remove the solvent using a round bottom flask freeze-drier for lyophylization. Store the blue crystalline product at −20 °C until further use (*see* **Note 17**).

3.7 Cell Seeding for Labeling Experiments

Note that many steps are similar, if not even identical to procedures described in Chapter 18, by Nikic et al. [31].

Per culture hood.

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1. Warm up PBS and growth medium in a water bath at 37 °C.
2. Warm up trypsin-EDTA to room temperature (RT).
3. Take cells out of the cell culture incubator.
4. Aspirate off growth medium.
5. Rinse the 100 mm cell culture plate with 5–10 mL of PBS.
6. Aspirate off PBS.
7. Add 2 mL of trypsin-EDTA to one 100 mm plate.
8. Put the plate back to the incubator for 3–5 min.
9. Check if the cells are detached from the plate surface (*see* **Note 38**). When detached, inactivate trypsin-EDTA by adding 8 mL of growth medium.

10. Pipette up and down a few times, rinsing the entire plate and homogeneously resuspend the trypsinized cells.
11. Transfer the cell suspension to a 15 mL falcon tube.
12. Count the number of cells with a hemocytometer.
13. Seed the appropriate number of cells required for the chosen culture surface. In this case, COS-7 cells are seeded in 4-well Lab-Teks at a density of 35,000 cells/well (*see* **Note 39**). Add the required volume of cell suspension to each well and add fresh medium to a total of 500 µL per well. Rock the Lab-Tek to distribute evenly. For multiple well seeding, prepare a master mix.
14. Incubate the cells in the cell culture incubator overnight.

3.8 Transfections of the Amber Suppression System and the Protein of Interest

Perform transfections under aseptic conditions in a cell culture hood. Note that many steps are similar, if not even identical to procedures described in Chapter 18, by Nikic et al.) [31].

1. Transfections are performed on the following day (15–20 h after the seeding).
2. Prepare the transfection mix according to the manufacturer's recommendations. In this protocol, we used 0.5 µg of plasmid coding for NESPylRS^{AF}/tRNA^{Pyl} and 0.5 µg of plasmid coding for vimentin Amber mutant per well (*see* **Note 40**). For each well 50 µL of JetPrime buffer are mixed with the DNAs in a microcentrifuge tube. Please note that you can prepare a master mix by multiplying this amount with the number of wells that you want to transfect.
3. Vortex the tube for 10 s at maximum speed and then briefly spin it down using a mini-centrifuge.
4. Add JetPrime reagent to the tube using a 1:2 DNA to JetPrime ratio (w/v). Each well contains a total of 1 µg of the total DNA and therefore 2 µL of JetPrime reagent are added to the tube.
5. Vortex the tube for 10 s at maximum speed and then briefly spin it down using a mini-centrifuge.
6. Incubate the transfection mix for 10 min at RT.
7. After the incubation time is over, take the Lab-Tek with cells out of the cell culture incubator.
8. Add the transfection mix dropwise to the well.
9. Return the Lab-Tek to the incubator.
10. After 4–6 h the ncAA is added. First, prepare the ncAA working solution. For each well of the Lab-Tek, mix 1.25 µL of ncAA stock and 3.75 µL of 1 M HEPES in a tube (*see* **Note 41**). Prepare a master mix if working with several wells at the same time.

11. Aspirate off the medium containing the transfection mix from the Lab-Tek.
12. Add 500 µL of fresh, pre-warmed (37 °C) growth medium to the well.
13. Add 5 µL of the ncAA working solution per well.
14. Gently rock the Lab-Tek back and forth and from side to side.
15. Return the cells to the incubator and keep for 24 h.
16. After 24 h aspirate off the growth medium and add fresh pre-warmed medium to the Lab-Tek.
17. Incubate the cells in fresh medium without ncAA overnight in the cell culture incubator (*see* **Note 42**).

3.9 IEDDA-Click Chemistry-Based Live Cell Labeling

Perform the labeling under aseptic conditions in a cell culture hood. When handling the dye stock and solution, it is recommended to turn off the light of the cell culture hood and not expose the labeled sample to light. Please note that the steps during and after cell fixation do not require aseptic conditions. Note that many steps are similar, if not even identical to procedures described in Chapter 18, by Nikic et al. [31].

1. On the following day, proceed to label the transfected cells.
2. Pre-warm growth medium at 37 °C.
3. Take the cells out of the cell culture incubator.
4. Aspirate off the medium.
5. Rinse with growth medium once.
6. Prepare the dye solution by diluting the dye stock to 3 µM in growth medium.
7. Add 500 µL of dye solution to each well.
8. Return the Lab-Tek to the incubator and keep for 10 min (*see* **Note 43**).
9. Aspirate off the dye solution.
10. Rinse the well twice with fresh growth medium.
11. Return the Lab-Tek to the incubator and keep for 2 h for additional washing and better image contrast (*see* **Note 43**).
12. Before imaging, fix the cells.
13. Aspirate off the medium and rinse with PBS.
14. Add 500 µL of 2% PFA per well and incubate for 10 min at RT.
15. Aspirate off PFA.
16. Rinse with PBS twice.
17. Leave the cells in 500 µL/well PBS.

18. Proceed with imaging or keep the cells in the fridge (up to 2 days prior to imaging).

3.10 Super-Resolution Imaging and Data Processing

1. Once you are ready to image the cells, change the medium of the well you want to image to freshly prepared GLOX-MEA buffer (*see* **Note 44**).
2. Take the cells to the microscope.
3. Use mOrange laser and excitation/emission filters to identify transfected cells. For optimal results, look for bright cells showing high expression levels and characteristic expression pattern of the target protein (*see* **Note 45**, Figs. 2 and 3).
4. Change to the laser of the dye used for the labeling. Select the appropriate filter set and check that the labeling has been successful (you should see signal colocalizing with the reference mOrange image).
5. Adjust the TIRF illumination angle (*see* **Note 46**).
6. Switch the laser to maximum power to bring the fluorophores to a dark state. You should see the signal becoming very bright at first and gradually bleach until individual blinking molecules appear (*see* **Note 47**).
7. Lower the laser power to an appropriate value (*see* **Note 48**), set the exposure time to 30 ms, and start the acquisition.
8. Acquire 10,000–30,000 frames. The optimal length depends on the sample quality, but recognizable features should already appear around 10,000 frames. With longer imaging a better signal-to-noise ratio could be achieved (*see* **Notes 49** and **50**).
9. Do further image processing in appropriate software (*see* **Note 9**).
10. To localize the spots, apply a threshold based on the maximum likelihood ratio and perform fitting with a symmetrical 2D Gaussian function.
11. If desired, consolidate identical emitters (falling within one standard deviation of the spot fit) into a single intensity-weighed localization.
12. Reconstruct a final super-resolved image from binning all the detected events and convolving the resulting image with a Gaussian width according to the resolution determined by the Fourier ring correlation criterion [32] (Fig. 3).

4 Notes

1. Once the growth medium is prepared with all supplements, we do not recommend using it for longer than a month.

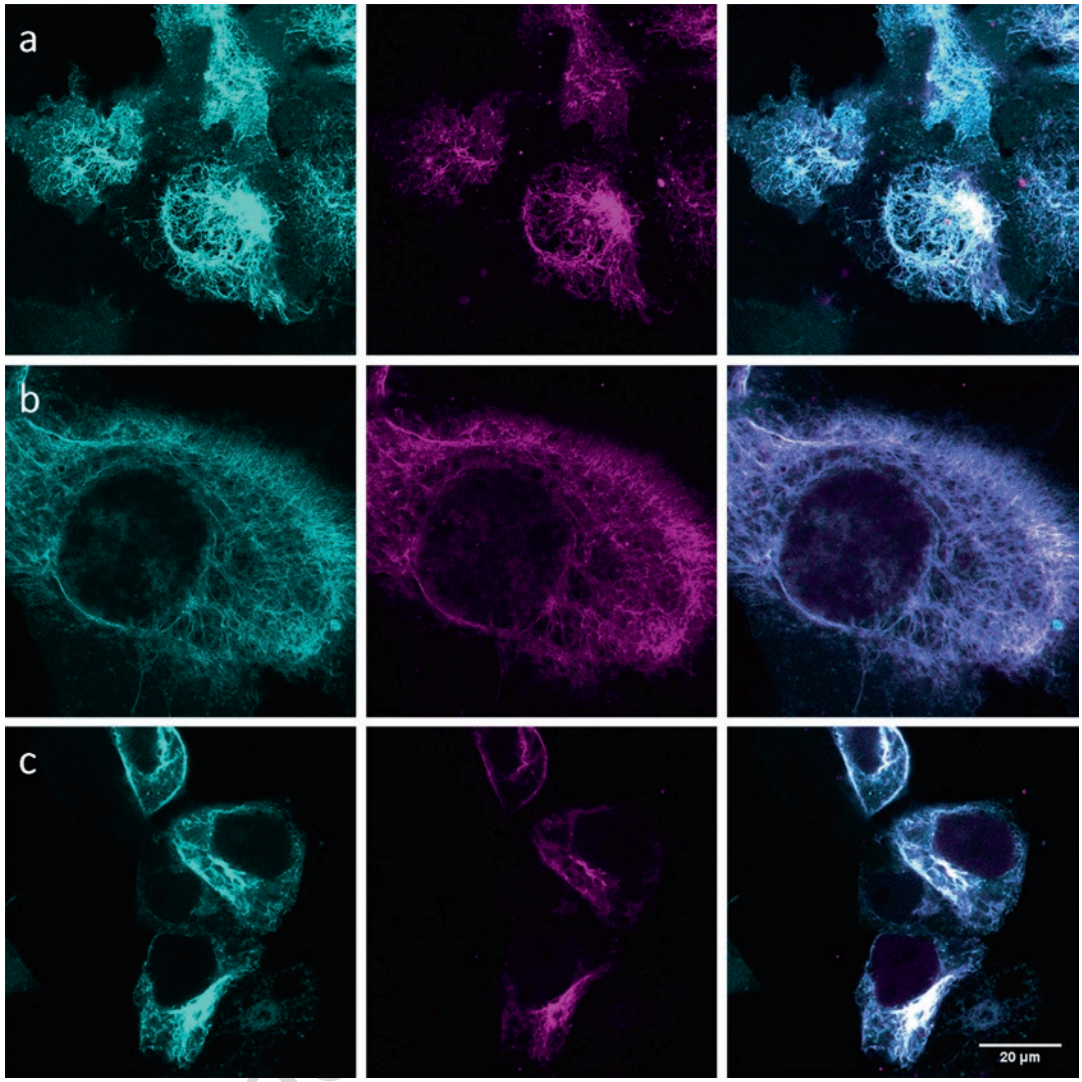

Fig. 2 Representative confocal images of live cell SiR labeling of vimentin^{BCNendo}–mOrange with SiR-tetrazine (dye **6**). Left to right: reference channel (mOrange, in cyan), labeling channel (SiR, in magenta), and merge. The labeling was performed in all cases at 37 °C with a dye concentration and reaction time of 1.5 μM for 10 min (**a**), 3 μM for 10 min (**b**), and 3 μM for 30 min (**c**—images scaled differently). Reprinted with permission from [24]

2. Several Amber suppression expression systems for eukaryotes exist. We use the NESPylRS^{AF}/tRNA^{Pyl} system because of its enhanced efficiency and reduced background in imaging experiments [25].
3. When testing new reagents or labeling methods/conditions, we recommend using target proteins with very characteristic features, e.g., cytoskeletal proteins. However, any other protein of interest can be used.

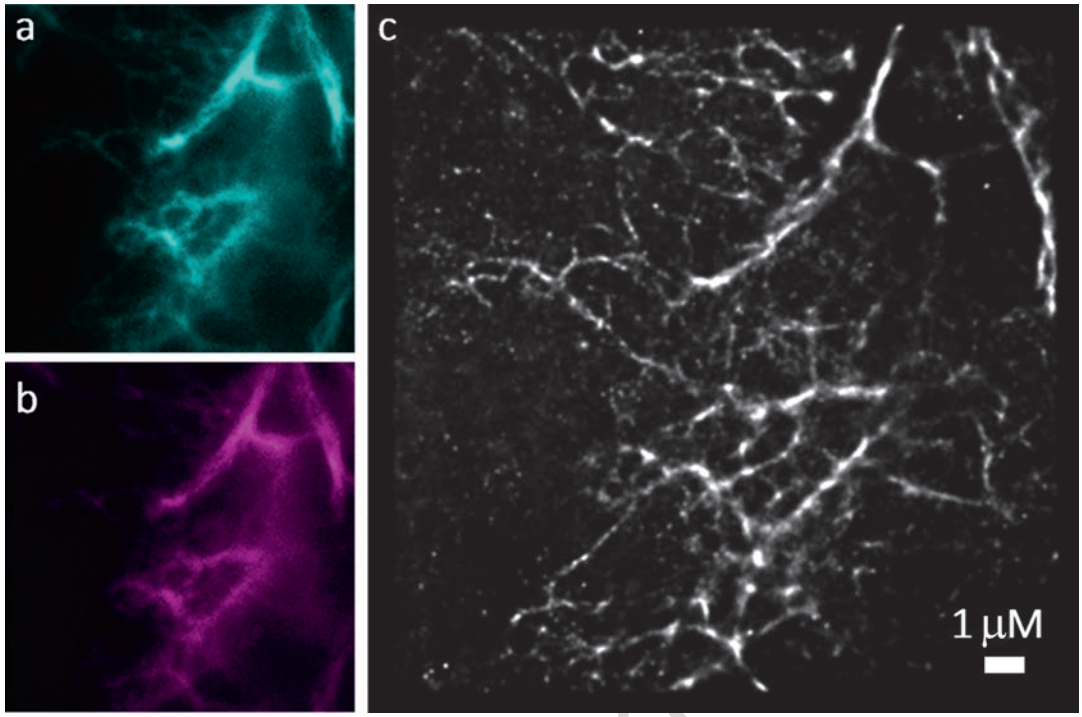

Fig. 3 TIRF SRM imaging of vimentin^{BCNendo}-mOrange labeled with SiR-tetrazine (dye **6**). Panels **a** (mOrange, cyan) and **b** (SiR labeling, magenta) are used as a reference for protein expression and expected structure/pattern. Corresponding SRM image from dye **6** labeling (3 µM for 30 min at 37 °C) is shown in panel **c**, with a resolution of 35 nm as determined by Fourier ring correlation (FRC) [32]. Reprinted with permission from [24]

4. When testing new reagents or labeling methods/conditions, we recommend using a fusion of the target protein with a C-terminally installed fluorescent protein. This provides a direct readout obtained after a successful transfection and ncAA incorporation (only with successful Amber suppression full-length protein will be generated) that can be later on used as a reference for labeling.
5. PFA is a toxic reagent. Avoid inhalation or contact with skin and eyes. Wear protective gear while handling and follow the relevant institutional rules for using chemicals and discarding waste material.
6. The buffer composition is based on [33] and frequently used for blinking (localization-based) super-resolution microscopy.
7. We recommend always preparing the buffer freshly before starting the imaging experiment.
8. We used a commercial Leica GSDIM microscope (based on ground-state depletion and single molecule localization [34]) but any other TIRF microscope with appropriate lasers, cameras,

and filter cubes can be used. Other localization-based microscopy techniques (such as STORM) would also be suitable [2].

9. We used the Localizer package for IgorPro but any other software for localization-based microscopy, such as Leica's GSDIM tools and various ImageJ plugins can be used. The following webpage (http://bigwww.epfl.ch/smlm) provides a benchmarking tool for developers to test different localization-based image analysis algorithms and provides an extensive list of tools available.
10. While performing chemical synthesis, follow general and institutional safety rules. Perform all steps in a well-ventilating chemical hood. Always wear safety glasses, lab coat, protective gloves, and proper clothing. The laboratory has to be equipped with a fire extinguisher, safety shower, and eye wash device. If you do get a chemical in your eye rinse immediately with large quantities of water using the eye-wash station. If possible, collect halogenated and non-halogenated chemical waste separately. Specific instructions on highly hazardous steps are specified at each step.
11. Dry the 250 mL round bottom flask in an oven at 110 °C and let it cool to room temperature before reaction. Make sure that there is no water remaining in the flask before performing the reaction as it can destroy *n*-butyllithium.
12. Turn on the nitrogen flow so that a reasonably rapid stream of bubbles passes through the mineral oil in the bubbler. Flush the apparatus with a gentle flow of nitrogen delivered through a needle; another needle in the top serves as the gas outlet during purging. When adding reagents to the mixture under inert atmosphere, use a syringe and a needle and add it through the septum. Argon can be used instead of nitrogen if needed.
13. The reaction can be followed using thin-layer chromatography. In hexane:EtOAc 10:1 R_f(starting material) = 0.7, R_f(product) = 0.4.
14. The organic phase is the upper phase.
15. When transferring compound mixtures onto celite for chromatography purification, make sure that the mixture is uniformly distributed on the celite powder. If the celite is still oily or cannot be dried completely, resuspend it in an organic solvent (DCM or EtOAc for example), add more celite and remove the solvent under reduced pressure.
16. Here, flash chromatography is used to enhance separation by enabling gradient elution. Alternatively, you can use the classic column chromatography technique.
17. Compounds can be checked by nuclear magnetic resonance (NMR) or MS. For reference spectra *see* ref. 24.

18. AIBN is an explosive compound; handle with care, use an eye protector and protective gloves.
19. The reaction can be followed by thin-layer chromatography. In hexane:EtOAc 3:1 R_f(starting material) = 0.55, R_f(first step product) = 0.76. In hexane:EtOAc 1:1 R_f(second step product) = 0.3.
20. The suspension transforms into a brown solution over the course of 2 h.
21. The white suspension becomes thick after 10–15 min when stirring may be challenging, and then a smooth white suspension again. Check the reaction frequently and make sure that the stirring is continuous.
22. Once it reached 140 °C, the mixture starts to turn blue and it develops a dark blue color by the end of the reaction.
23. The mixture can be challenging to remove from the vial. Use prolonged (15–30 min) sonication on a high-performance sonicator to dissolve the blue residue. Methanol can be used as a co-solvent.
24. The product is blue when on silica gel (column and TLC), but colorless in solution (hexane:EtOAc 4:1 with 1% (v/v) Et_3N) and forms white crystals as a solid.
25. The second purification step is optional. If the compound is pure after the first purification, omit this step.
26. The side-product to be separated is colorless on silica gel and runs just above the product.
27. Take all safety precautions for this step: wear gloves, safety glasses and the reaction must be performed in a ventilation hood. Concentrated H_2SO_4 is seriously corrosive. HCl is a pungent, irritating gas that can cause severe damage to the eyes, skin, lungs, and upper respiratory tract. NO_x is harmful for the lung when inhaled.
28. The reaction can be exothermic. In that case, cool the reaction flask with ice/water bath.
29. Use fresh ice/water bath if the ice melted completely.
30. The orange suspension will turn to magenta.
31. The removed solvent may contain pink 3,6-dimethyl-1,2,4,5-tetrazine side product.
32. R_f(product) = 0.25 in hexane:EtOAc 1:1.
33. R_f(product) = 0.38 in hexane:EtOAc 1:1.
34. The organic phase is the bottom phase under the aqueous solution.
35. The pink crystals can be kept at −20 °C without any degradation up to 9 months.

36. In hexane:EtOAc 1:1 R_f(SiR-Br) = 0.9, R_f(product) = 0.7, R_f(OMs-tet) = 0.4.
37. The product has a blue color on silica gel, but turns light rose in MeCN solution.
38. Under the microscope, check for cell detachment. When detachment is observed, it is important to not leave the cells in trypsin for much longer since this can have toxic effects. Note that trypsinization time is dependent on the cell line and that proper PBS rinse before the addition of the trypsin is required in order to avoid inactivation by any remaining medium.
39. For imaging, very high confluency is not usually desired, but too low confluency might be insufficient for proper transfection. Appropriate densities might need to be optimized first, given that the number of seeded cells will depend on the cell line, the seeding surface, and the transfection reagent.
40. Transfection conditions might need to be optimized for each protein, cell line, and transfection reagent.
41. HEPES is used to buffer the ncAA stock. This step is not necessary but recommended since it helps in maintaining the pH of the medium and will avoid the impact that the direct addition of the basic ncAA stock into the well has on the cell monolayer. Dilution of the ncAA stock with HEPES is always done fresh prior to addition to the medium.
42. Total expression time will depend on the protein of interest as well as the cell line. Longer incubation without ncAA will help in reducing the background during the labeling.
43. A good labeling efficiency is observed when using 3 μM dye for 10 min. However, dye concentration and labeling time can be adapted according to the user/experiment needs. Similar labeling efficiencies (Fig. 2) were observed for lower concentrations (1.5 μM) as well as longer labeling times (up to 30 min). In addition, the user can also adapt the washing time after the labeling reaction: a low background signal was observed with washes as short as 45 min, however, the longer the wash, the better the final contrast on the image becomes. Conditions where the sample was only quickly rinsed showed also specific labeling; nonetheless, here one might suffer from higher background and might need to optimize further the labeling reaction conditions.
44. Leave cells in the GLOX-MEA buffer only when necessary during the SRM imaging: we have observed a detrimental effect of the buffer on the cells; we recommend always changing the buffer back to PBS if you plan to reuse the same cells in a further experiment.
45. Before performing super-resolution imaging, we recommend a first round of imaging experiments at a confocal microscope (for

example Leica SP8, Fig. 2) to establish the method, in order to optimize both expression levels and labeling conditions.

46. E.g. TIRF or HILO [35] can be used and it needs to be adapted to the needs of your experiment, according to the cells used and the protein being imaged. The illumination angle needs to be adjusted to optimize the signal-to-background ratio (Fig. 3).
47. In this step it is quite critical to obtain good blinking in order to achieve an optimal super-resolution image. Issues with the blinking (for example, too long waiting time before the blinking appears or overall insufficient blinking) might be caused among other reasons by: unsuccessful labeling, unsuitable conditions for the dye being used, and old GLOX-MEA buffer. After approximately 30 min we recommend changing the GLOX-MEA buffer with fresh one, to ensure optimal blinking.
48. Laser power should be adjusted to optimize the blinking: if too many events are detected (overlapping blinking particles), try increasing the laser power.
49. If the blinking decreases after some time, back-pumping can be applied by switching on the 405 laser to increase the number of blinking events.
50. Note that longer acquisition time might result in a worse image quality in case of significant drift in the microscopy setup used.

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