Tracking the Activity of mTORC1 in Living Cells Using Genetically Encoded FRET-based Biosensor TORCAR

Xin Zhou,[1] Simin Li,[1] and Jin Zhang[1,2]

[1]Department of Pharmacology, University of California at San Diego, La Jolla, California
[2]Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland

Mechanistic target of rapamycin complex 1 (mTORC1) is a highly conserved serine/threonine protein kinase that responds to multiple distinct signals (e.g., growth factors, amino acids, stress, and energy level) and coordinates cell growth and proliferation. The underlying molecular mechanisms by which these stimuli regulate the activity of mTORC1 are still not fully understood. The spatial compartmentalization of mTORC1 signaling has been suggested as an important mechanism for mTORC1 to achieve the signal specificity and efficiency. To examine the spatial regulation of the activity of mTORC1 in live cells, we describe a protocol using a newly developed molecular tool, a genetically encoded fluorescence resonance energy transfer (FRET)-based mTORC1 activity reporter, TORCAR. When expressed in the cell, TORCAR acts as a surrogate substrate of mTORC1, and exhibits a change in FRET in response to phosphorylation by mTORC1. Genetically targeting TORCAR to specific subcellular locations further allows for the characterization of spatial compartmentalized mTORC1 signaling. © 2016 by John Wiley & Sons, Inc.

Keywords: biosensor • fluorescence • live-cell imaging • mTOR kinase

How to cite this article:
Zhou, X., Li, S., and Zhang, J. 2016. Tracking the activity of mTORC1 in living cells using genetically encoded FRET-based biosensor TORCAR. *Curr. Protoc. Chem. Biol.* 8:225-233. doi: 10.1002/cpch.11

INTRODUCTION

Mechanistic target of rapamycin (mTOR) is a highly conserved serine/threonine protein kinase that constitutes two structurally and functionally distinct complexes, mTOR complex 1 (mTORC1) and complex 2 (mTORC2). mTORC1 is acutely and allosterically inhibited by the antifungal microlide rapamycin, whereas mTORC2 is inhibited by prolonged treatment of rapamycin. mTORC1 consists of mTOR, regulatory associated protein of mTOR (Raptor), and mammalian lethal with SEC13 protein 8 (mLST8), 40-kDa proline-rich Akt substrate (PRAS40), and DEP domain-containing mTOR-interacting protein (DEPTOR) (Laplante and Sabatini, 2012). The interaction of mTOR and Raptor is essential for the specificity of mTORC1 in recognizing and phosphorylating its protein substrates (Hara et al., 2002; Kim et al., 2002; Aylett et al., 2016), such as eukaryotic translation initiation factor 4E binding protein (4EBP1) (Hara et al., 1997), translational regulators ribosomal protein S6 kinase (S6K1) (Burnett et al., 1998), and Unc-51 like autophagy activating kinase 1 (Ulk1) (Kim et al., 2011). Phosphorylation of downstream effectors by mTORC1 regulates a number of cellular anabolic and catabolic processes, including promoting protein synthesis and inhibiting autophagy (Dibble and Manning, 2013).

Current Protocols in Chemical Biology 8:225-233, December 2016
Published online December 2016 in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpch.11

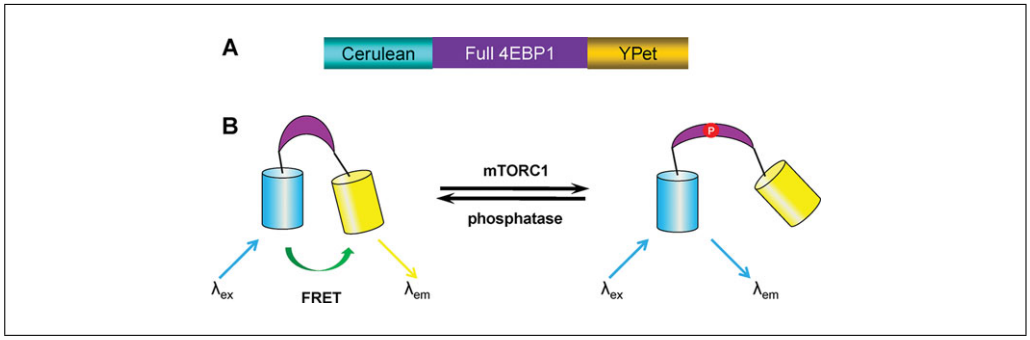

Figure 1 Design of the TORCAR biosensor. (**A**) Domain structure of the TORCAR construct. TORCAR is composed of cyan fluorescent protein (cerulean), full length 4EBP1, and yellow fluorescent protein (YPet) (**B**) General scheme depicting the TORCAR response. Active mTORC1 phosphorylates 4EBP1 in the TORCAR biosensor, which subsequently induces a conformational change that leads to an increase in the cyan-to-yellow emission ratio.

mTORC1 responds to many different extracellular and intracellular cues—e.g., nutrients, growth factors, stress, and cellular energy—to coordinate cell growth and metabolism (Dibble and Cantley, 2015; Goberdhan et al., 2016). As a highly integrated signaling node, the activity of mTORC1 is exquisitely regulated. Dysregulated mTORC1 activity has been associated with pathophysiological conditions, including cancer and type 2 diabetes (Dibble and Manning, 2013; Ilagan and Manning, 2016).

The utilization of a genetically encoded fluorescence resonance energy transfer (FRET)-based mTORC1 activity reporter (TORCAR) enables the dynamic activity of mTORC1 to be quantified in live cells with high spatiotemporal resolution. TORCAR is composed of full-length 4EBP1—a well-studied mTORC1-specific substrate—sandwiched between a cyan fluorescent protein (cerulean) as a FRET donor and a yellow fluorescent protein (YPet) as a FRET acceptor (Fig. 1A). When expressed in cells, TORCAR acts as a surrogate substrate of mTORC1. Phosphorylation of TORCAR by mTORC1 at two specific sites—Thr 37 and Thr 46—in the 4EBP1 region, induces a conformational change within the reporter, resulting in a decrease in FRET. The increase in the cyan-over-yellow (C/Y) emission ratio, which corresponds to a FRET decrease, can be used to indicate an increase in mTORC1 activity (Fig. 1B). Below, we describe a general protocol for using TORCAR to monitor the mTORC1 activity in live cells, including procedures for the maintenance of NIH3T3 fibroblast cells, transfection of cells with TORCAR plasmid DNA, preparation of cells for imaging experiments, preparation of the imaging equipment, imaging of mTORC1 activity in live cells, and analysis of the acquired imaging data to quantify any observed changes in FRET.

BASIC PROTOCOL

MONITORING MTORC1 ACTIVITY USING GENETICALLY ENCODED FRET-BASED BIOSENSOR TORCAR

The following protocol describes the use of the FRET-based biosensor TORCAR to track the activity of mTORC1 in NIH3T3 cells. First, cells are transfected with plasmid DNA encoding the TORCAR biosensor, serum-starved overnight, and then starved for amino acids for 2 hr. While recording a time series of fluorescence intensity images, the cells are stimulated with growth factors or amino acids to activate mTORC1. The images are then analyzed to quantify changes in mTORC1 activity.

Materials

NIH3T3 fibroblast cells plated on sterile 35-mm glass-bottom dishes (see Support Protocol)

NIH3T3 culture medium (see recipe)

Opti-MEM I reduced serum medium (Gibco, cat. no. 31985070)
Lipofectamine 2000 (Thermo Fisher Scientific, cat. no. 11668019)
TORCAR plasmid DNA (Addgene, cat. no. 64927)
Dulbecco's Modified Eagle's Medium, 1 g/liter glucose, no serum (Gibco, cat. no. 11885084)
HBSS* imaging buffer (see recipe)
Immersion oil, e.g., Immersol 518 F fluorescence-free (Carl Zeiss, cat. no. 1262466A)
PDGF stock solution (see recipe)
1 mM torin1 (TOCRIS, cat. no. 4247), in DMSO
3.25 M leucine *O*–methyl ester (LeuOMe; EMD Chemical, cat. no. 8.54071.0005), in water

Inverted fluorescence microscope with appropriate objective, filters/mirrors, and detector, for example:
Zeiss Axiovert 200 M microscope with 40× /1.3-NA oil-immersion objective lens; dichroic mirror, excitation filters (CFP, YFP), and emission filters (CFP, YFP); Lambda 10-2 filter changer (Sutter Instruments); XBO 75 W xenon arc lamp (Carl Zeiss); MicroMAX BFT512 CCD camera (Roper Scientific),
Computer and imaging software (e.g., METAFLUOR 6.2, Universal Imaging) to operate microscope
Spreadsheet application (e.g., Microsoft Excel)

Transfect and starve cells

1. Seed NIH3T3 cells on 35-mm glass-bottom dish, and grow to 40% to 60% confluency at 37°C, 5% CO_2.
2. In a microcentrifuge tube, add 0.8 μg of TORCAR plasmid DNA and Opti-MEM medium to a total volume of 50 μl. Gently mix the plasmid DNA and Opti-MEM, and incubate 5 min at room temperature.

   Opti-MEM should only be handled under sterile conditions, such as in a tissue culture hood.
3. In a separate tube, mix 2 μl Lipofectamine 2000 with 48 μl Opti-MEM, and incubate 5 min at room temperature.
4. Add the 50 μl Lipofectamine solution dropwise to the 50 μl DNA solution, pipet gently to mix, and incubate 20 min at room temperature.

   Mix the Lipofectamine/DNA solution gently and do not vortex.
5. Transfer the 35-mm glass-bottom dish containing NIH3T3 cells to a tissue culture hood, and replace the medium with 2 ml pre-warmed DMEM medium without serum.
6. Add the entire (100 μl) Lipofectamine/DNA mixture dropwise to the dish, and rock the dish gently back and forth to evenly disperse the transfection reagents.

   The volumes are given on a per-dish basis. Prepare tubes for each additional dish to be transfected. Optimize the amounts of components according to the manufacturer's instruction, if necessary. Verify expression of reporter in the transfected cells 18 to 24 hr after transfection. For subcellular targeted TORCAR, it may be necessary to transfect more DNA.
7. Two hours before imaging, replace the serum-free DMEM with 2 ml pre-warmed HBSS* imaging buffer to starve for amino acids, and incubate the dish at 37°C without CO_2.

Prepare microscope for imaging

8. Turn on the lamp, microscope, filter changer, camera, and computer.

9. Load the METAFLUOR imaging software and an appropriate protocol for acquiring a time course of images in the FRET, CFP, and YFP channels. Ensure that the filters are appropriate for imaging the desired fluorophores using control cultures that express the fluorophores (e.g., CFP and YFP). The filter sets for individual channels are:

   FRET: 420DF20 excitation filter, 450DRLP dichroic mirror, 535DF25 emission filter.
   CFP: 420DF20 excitation filter, 450DRLP dichroic mirror, 475DF40 emission filter.
   YFP: 495DF10 excitation filter, 515DRLP dichroic mirror, 535DF25 emission filter.

10. Aspirate the HBSS* imaging buffer from the serum- and amino acid-starved NIH3T3 cells without agitating the cells.

    Aspirate the culture medium from the side of the 35-mm glass-bottom imaging dish.

11. Add 1 ml fresh HBSS* imaging buffer, and gently rock the dish side to side to cover the cells.

    Add the medium to the side of the dish to avoid pipetting directly onto the cells.

12. Place a drop of immersion oil onto the 40× objective, and securely position the imaging dish onto the microscope stage.

13. Focus the cells using the bright field setting, then switch to fluorescence mode and select cells with a healthy morphology and the desired expression level and cellular distribution of reporter.

Begin image acquisition and stimulate mTORC1 activity

14. Use the imaging software to set the time-lapse interval between each set of acquisitions and the excitation exposure time for each fluorescence channel.

    We typically capture images every 25 to 30 seconds for TORCAR, with exposure times of 500 ms for FRET, 500 ms for CFP, and 50 ms for YFP. These parameters can be adjusted based on expression level and microscope configuration.

15. Establish a baseline of mTORC1 activity by acquiring a time series of images for each channel (e.g., CFP, YFP, and FRET)—generally 3 to 5 min, or until the baseline is flat.

16. Remove 300 to 500 μl of imaging buffer from the imaging dish, and add to a microcentrifuge tube containing 1 μl of 50 μg/ml PDGF or 2 μl of 3.25 M leucine *O*–methyl ester (LeuOMe).

17. Gently add the PDGF or LeuOMe mixture back to the imaging dish (resulting in a final concentration of 50 ng/ml PDGF) by pipetting down to the side of the dish, being careful not to disturb the cells. Pipet carefully to evenly mix the drug throughout the imaging buffer without disturbing the cells.

18. Continue acquiring the image series to capture the stimulated response.

19. (Optional) To inhibit mTORC1 activity, repeat steps 16 and 17, but substitute mTORC1 inhibitor—e.g., final concentration of 1 μM torin1—and acquire the image series for several additional minutes to capture the response.

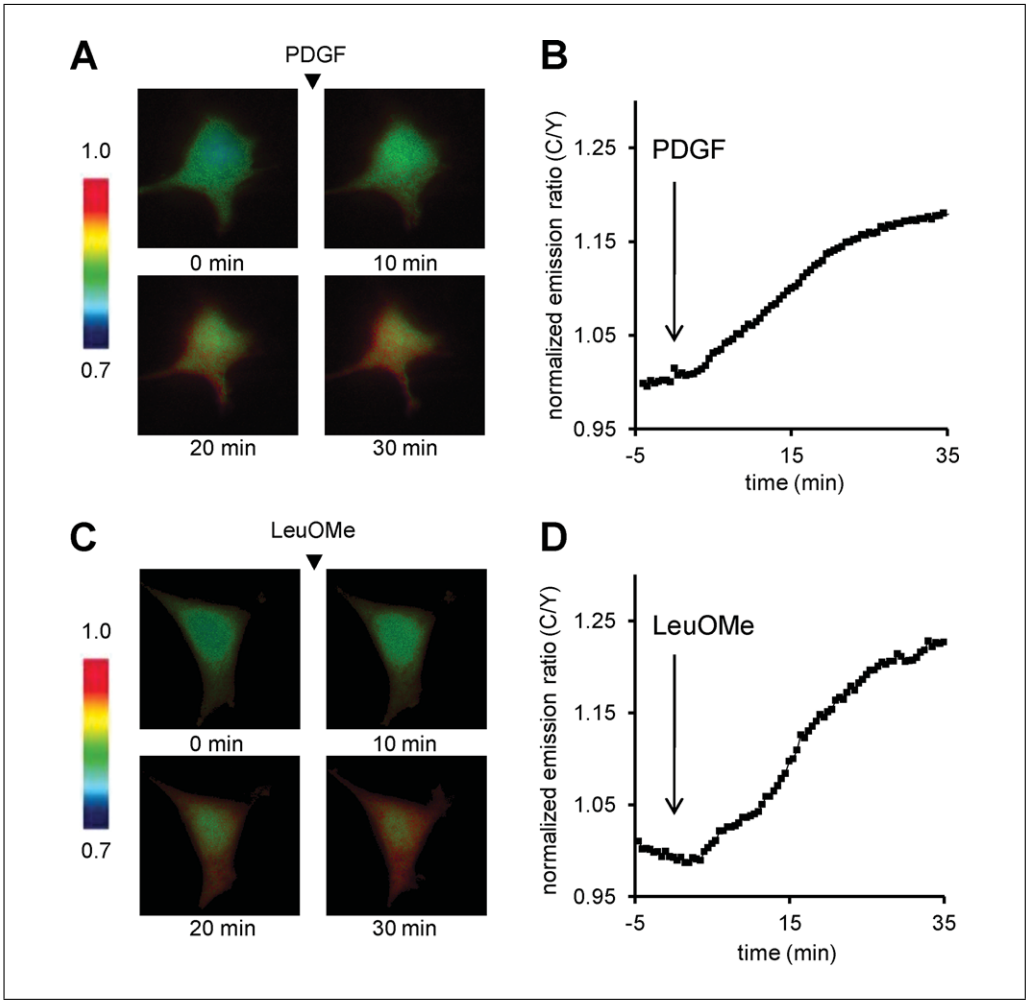

Figure 2 TORCAR responses in live cells. Serum- and amino acid-starved NIH3T3 cells that express TORCAR were stimulated with 50 ng/ml PDGF (**A**, **B**) or 7.5 mM LeuOMe (**C**, **D**). (**A**, **C**) Time courses of the cyan-to-yellow emission ratio of TORCAR upon stimulation. (**B, D**) Pseudo-colored images showing the FRET responses of TORCAR to PDGF (**B**) or LeuOMe (**D**) stimulation.

Analyze images and data

20. Using the imaging software, draw regions of interest (ROIs) for each fluorescent cell in the field. Also choose a region containing non-transfected cells or no cells for background correction.

21. For each channel, log the fluorescence intensities acquired during the experiment.

22. Export the data to an excel file, and calculate the FRET emission ratio (cyan-to-yellow) at each time point using the logged emission intensity data and the following equation:

$$Ratio\ (C/Y) = \frac{CFP\ Intensity\ of\ ROI - CFP\ Intensity\ of\ Background}{FRET\ Intensity\ of\ ROI - FRET\ Intensity\ of\ Background}$$

23. Set the time point immediately after addition of drug as time "0". Calculate the average of emission ratio before addition of drug, and normalize the emission ratio before addition of drug as 1. Generate a plot of the normalized cyan-to-yellow emission ratio versus time (Fig. 2A and 2C).

Pseudocolor images for each acquisition can be generated using the METAFLUOR software to indicate the cyan-to-yellow emission ratio, where warmer colors represent high C/Y emission ratio and cooler colors low C/Y emission ratio (Fig. 2B and 2D).

SUPPORT PROTOCOL

MAINTENANCE OF NIH3T3 CELLS IN CULTURE

This support protocol outlines the procedure for passaging NIH3T3 cells, both for prolonged maintenance and for use in imaging experiments.

Materials

NIH3T3 cells (American Type Culture Collection)
70% (v/v) ethanol
Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+} (DPBS; Gibco, cat. no. 14190144)
NIH3T3 cell culture medium (see recipe)
0.25% trypsin–EDTA (Thermo Fisher Scientific, cat. no. 25200072)

Tissue culture flasks (e.g., T-25 cm^2; BD Falcon)
Tissue culture incubator
Tissue culture hood
35-mm glass-bottom imaging dishes (MatTEK, cat. no. P35G-1.0-14-C)

1. Maintain NIH3T3 cells in NIH3T3 cell culture medium in T-25 cm^2 flasks in a humidified incubator at 37°C, 5% CO_2, and passage cells whenever they reach 50% to 70% confluence based on brief examination using a standard tissue culture microscope.

   NOTE: Do not allow NIH3T3 cells to become completely confluent.

2. Sterilize the tissue culture hood with 70% ethanol, and prepare all the required supplies, including pipets, pipet tips, flasks, media, dishes, etc., so that they are sterile and available in the hood before proceeding.

3. Examine the cells under a microscope for culture density and cell morphology. Carefully transfer the T-25 flask from the incubator to the hood.

4. Remove the culture medium from the flask, and gently wash the cells twice with 2 ml DPBS without calcium and magnesium to remove all traces of serum.

5. Pipet 300 μl of the trypsin–EDTA solution into the flask, and gently rock the flask back and forth several times. Let sit for 1 to 2 min or until 90% of cells begin to disperse (round up), as determined by examining under a microscope.

6. Add 4.7 ml fresh, pre-warmed NIH3T3 cell culture medium to the flask, and mix the cells by gently pipetting up and down.

   Avoid over-pipetting as it could damage the cells.

7. Split the culture 1:6 into new T-25 flasks to maintain the cell culture. Split cells every two or three days.

8. For imaging experiments, split the culture 1:3 and plate into 35-mm glass-bottom dishes. After ~24 hr, cultures should reach 40% to 60% confluence, at which point they should be transfected.

REAGENTS AND SOLUTIONS

All solutions should be prepared under sterile conditions in a tissue culture hood using water with 18.2 MΩ-cm resistivity.

HBSS imaging buffer*

1× Hank's Balanced Salt Solution (Gibco, cat. no. 14065)
20 mM HEPES (Sigma, cat. no. H4034-500G)
2 g/liter D-glucose (Thermo Fisher Scientific, cat. no. A2494001)
Adjust pH to 7.4, and filter sterilize using a 0.22-μm filter
Store a 50-ml aliquot at room temperature near the microscope room and the remainder at 4°C

NIH3T3 cell culture medium

Dulbecco's Modified Eagle's Medium, 1 g/liter glucose (Gibco, cat. no. 11885084)
10% calf serum (ATCC, cat. no. 302030)
1% penicillin-streptomycin (Thermo Fisher Scientific, cat. no. 15140122)
Store culture media at 4°C, and warm to 37°C before use

PDGF stock solution

50 μg/ml PDGF-BB (Sigma-Aldrich, cat. no. P4056-50ug)
4 mM HCl
0.1% (w/v) bovine serum albumin

COMMENTARY

Background Information

Assays to measure the mTORC1 kinase activity

Multiple signal inputs—e.g., growth factors, amino acids, glucose, and changes in energy status—stimulate the activity of mTORC1. Typically, mTORC1 kinase activity is measured *in vitro* by incubating an immunoprecipitated mTORC1 complex with purified protein substrate in the presence of ATP. Phosphorylation of the mTORC1 substrate can then be assessed either by measuring the level of ^{32}P transferred to the substrate (if ATP was radiolabeled; Hara et al., 1997; Wang et al., 2009) or by western blot analysis using antibodies that recognize the phosphorylated substrate (Sancak et al., 2007; Thoreen et al., 2009). The assay relies on robust immunoprecipitation of functional mTORC1 from cells using antibodies against subunits in the complex, most commonly endogenous Raptor (Guertin et al., 2006; Sancak et al., 2007) or exogenous epitope-tagged Raptor (Sancak et al., 2007). This approach is useful in the determination of the functional status of mTORC1, quantification of the kinetics of substrate phosphorylation, and the comparison of *in vitro* activity in mutated complexes. Conventionally these assays are relatively time-consuming, and a loss of activity can occur during the immunoprecipitation process. The interaction between mTOR and Raptor is sensitive to Triton X-100 or NP-40, so the zwitterionic detergent 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) must be used to maintain mTOR-Raptor binding (Kim et al., 2002).

Cellular mTORC1 activity can also be measured by detecting the phosphorylation level of mTORC1 substrates in cell lysates using western blots (Sancak et al., 2007), which accounts for the potential role of endogenous phosphatase activity. Antibodies have been described that exclusively recognize phosphorylated mTORC1 substrates, including T37/T46 of 4EBP1 (Gingras et al., 1999; Thoreen et al., 2009), T389 of S6K (Burnett et al., 1998), and S757 of Ulk1 (Kim et al., 2011).

A complementary FRET-based approach has recently been used to quantify endogenous mTORC1 activity in living cells. In this approach, a phosphorylation-dependent conformational change in the reporter TORCAR results in changes in FRET (Zhou et al., 2015). In particular, TORCAR can be genetically targeted to a specific subcellular location, such as plasma membrane, lysosomes, and nucleus. Subcellular targeted TORCAR allows the detection of location-specific mTORC1 signaling in response to distinct upstream signals—e.g., growth factors and amino acids—which allows a better understanding of the cellular regulation of mTORC1 signaling.

Table 1 Possible Problems and Solutions

Problem	Possible causes	Possible solutions
Dim cells	Poor transfection	Optimize transfection
	Incorrect filter set	Verify microscope filter set
No stable baseline	Changes in cell morphology	Optimize imaging conditions (e.g., temperature, imaging buffer)
	Photobleaching	Reduce exposure time or decrease the illumination using neutral density filters
Smaller/slower responses than expected	Incorrect drug addition	Mix well or use a perfusion system
	Drug concentration too low	Verify amount of drug needed to fully activate kinase
	Low expression of reporter	Optimize transfection
	High basal activity	Optimize the starvation conditions

Critical Parameters

Starvation of cells for imaging

This step focuses on starvation of cells for an optimal TORCAR response. mTORC1 activity can be stimulated by growth factors and amino acids, so cells should be starved for both serum and amino acids to lower the basal phosphorylation of the reporter before stimulation of mTORC1.

Criteria for selecting proper cells

Selecting proper cells is essential for successful imaging experiments. Unhealthy cells are likely to detach from the dish during imaging. The criteria for selecting cells include proper cell morphology, desired expression of reporter, and cellular distribution. First, cell morphology should be inspected to confirm the healthy status of the cells to be imaged. For example, healthy NIH3T3 cells should have a fibroblast-like, elongated shape, rather than being balled-up or rounded. Second, select cells with moderate fluorescence intensities. Intensities that are too high could indicate excessive expression of the probe, which might disrupt endogenous signaling pathways, whereas intensities that are too low may lead to reduced signal-to-noise ratios and make it difficult to detect changes in FRET. Proper cellular distribution is examined by fluorescence intensity. If TORCAR is not targeted to a specific subcellular location, the yellow fluorescence and cyan fluorescence should be uniform and evenly distributed throughout the cells. If TORCAR is targeted to a subcellular location, selected cells should exhibit well-localized fluorescence. For example, co-staining with LysoTracker could be used to verify the targeting of TORCAR to lysosomes.

Control experiments

It is important to confirm that changes in FRET ratio from TORCAR are due to the changes in mTORC1 activity. Specificity can be examined by pretreating cells with mTOR inhibitors—e.g., torin1 and torin2—or the allosteric inhibitor of mTORC1, rapamycin, before stimulating the cells. These treatments should block the FRET response. Addition of the inhibitors following the stimulation, should reverse the TORCAR response. Reversal of the response, however, may not be as robust as the blockade of response by pretreatment, depending on the cellular phosphatase activities. An alternative negative control experiment might be to use a reporter variant containing the T37A/T46A mutations (TORCAR-TA).

Troubleshooting

Table 1 describes some potential problems that may arise during the TORCAR experiment, possible causes for each problem, and strategies for overcoming or avoiding these problems.

Anticipated Results

Treatment with PDGF should produce an increase in CFP fluorescence intensity, and a decrease in FRET.

Time Considerations

The steps in the Basic Protocol can be completed over a period of 3 days. This includes passaging cells into 35-mm imaging dishes (~30 min, day 1), transfecting the cells (30 min to 1 hr, day 2), and acquiring and analyzing the data (1 hr per dish, day 3).

Acknowledgements

This work is supported by National Institutes of Health grants R01 DK073368 and DP1 CA174423 (to J.Z.).

Literature Cited

Aylett, C.H., Sauer, E., Imseng, S., Boehringer, D., Hall, M.N., Ban, N., and Maier, T. 2016. Architecture of human mTOR complex 1. *Science* 351:48-52. doi: 10.1126/science.aaa3870.

Burnett, P.E., Barrow, R.K., Cohen, N.A., Snyder, S.H., and Sabatini, D.M. 1998. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc. Natl. Acad. Sci. U.S.A.* 95:1432-1437. doi: 10.1073/pnas.95.4.1432.

Dibble, C.C. and Cantley, L.C. 2015. Regulation of mTORC1 by PI3K signaling. *Trends Cell Biol*. 25:545-555. doi: 10.1016/j.tcb.2015.06.002.

Dibble, C.C. and Manning, B.D. 2013. Signal integration by mTORC1 coordinates nutrient input with biosynthetic output. *Nat. Cell Biol.* 15:555-564. doi: 10.1038/ncb2763.

Gingras, A.C., Gygi, S.P., Raught, B., Polakiewicz, R.D., Abraham, R.T., Hoekstra, M.F., Aebersold, R., and Sonenberg, N. 1999. Regulation of 4E-BP1 phosphorylation: A novel two-step mechanism. *Genes Dev*. 13:1422-1437. doi: 10.1101/gad.13.11.1422.

Goberdhan, D.C., Wilson, C., and Harris, A.L. 2016. Amino acid sensing by mTORC1: Intracellular transporters mark the spot. *Cell Metab*. 23:580-589. doi: 10.1016/j.cmet.2016.03.013.

Guertin, D.A., Stevens, D.M., Thoreen, C.C., Burds, A.A., Kalaany, N.Y., Moffat, J., Brown, M., Fitzgerald, K.J., and Sabatini, D.M. 2006. Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. *Dev. Cell* 11:859-871. doi: 10.1016/j.devcel.2006.10.007.

Hara, K., Yonezawa, K., Kozlowski, M.T., Sugimoto, T., Andrabi, K., Weng, Q.P., Kasuga, M., Nishimoto, I., and Avruch, J. 1997. Regulation of eIF-4E BP1 phosphorylation by mTOR. *J. Biol. Chem.* 272:26457-26463. doi: 10.1074/jbc.272.42.26457.

Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N., Hidayat, S., Tokunaga, C., Avruch, J., and Yonezawa, K. 2002. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell* 110:177-189. doi: 10.1016/S0092-8674(02)00833-4.

Ilagan, E., Manning, B.D. 2016. Emerging role of mTOR in the response to cancer therapeutics. *Trends Cancer* 2:241-251. doi: 10.1016/j.trecan.2016.03.008.

Kim, J., Kundu, M., Viollet, B., and Guan, K.L. 2011. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat. Cell Biol.* 13:132-141. doi: 10.1038/ncb2152.

Kim, D.H., Sarbassov, D.D., Ali, S.M., King, J.E., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. 2002. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* 110:163-175. doi: 10.1016/S0092-8674(02)00808-5.

Laplante, M. and Sabatini, D.M. 2012. mTOR signaling in growth control and disease. *Cell* 149:274-293. doi: 10.1016/j.cell.2012.03.017.

Sancak, Y., Thoreen, C.C., Peterson, T.R., Lindquist, R.A., Kang, S.A., Spooner, E., Carr, S.A., and Sabatini, D.M. 2007. PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. *Mol. Cell* 25:903-915. doi: 10.1016/j.molcel.2007.03.003.

Thoreen, C.C., Kang, S.A., Chang, J.W., Liu, Q., Zhang, J., Gao, Y., Reichling, L.J., Sim, T., Sabatini, D.M., and Gray, N.S. 2009. An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. *J. Biol. Chem.* 284:8023-8032. doi: 10.1074/jbc.M900301200.

Wang, L., Lawrence, J.C., Jr., Sturgill, T.W., and Harris, T.E. 2009. Mammalian target of rapamycin complex 1 (mTORC1) activity is associated with phosphorylation of raptor by mTOR. *J. Biol. Chem.* 284:14693-14697. doi: 10.1074/jbc.C109.002907.

Zhou, X., Clister, T.L., Lowry, P.R., Seldin, M.M., Wong, G.W., and Zhang, J. 2015. Dynamic visualization of mTORC1 activity in living cells. *Cell Rep*. 10:1767-1777. doi: 10.1016/j.celrep.2015.02.031.