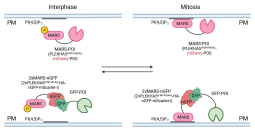
Aug 19, 2024

Applying the Mitosis-enabled Anchor-away/Recruiter System (MARS) for conditional protein recruitment to the plasma membrane during mitosis



DOI
dx.doi.org/10.17504/protocols.io.n2bvjnzypgk5/v1

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OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.n2bvjnzypgk5/v1

Protocol Citation: Xiaofu Cao, Jeremy M Baskin 2024. Applying the Mitosis-enabled Anchor-away/Recruiter System (MARS) for conditional protein recruitment to the plasma membrane during mitosis. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.n2bvjnzypgk5/v1**

Manuscript citation:
Cao X, Huang S, Wagner MM, Cho Y-T, Chiu D-C, Wartchow KM, Lazarian A, McIntire LB, Smolka MB, Baskin JM. “A phosphorylation-controlled switch confers cell cycle-dependent protein relocalization.” Nat Cell Biol (2024).

Protocol status: Working
We use this protocol and it's working

Created: July 19, 2024

Last Modified: August 19, 2024

Protocol Integer ID: 103739

Keywords: mitosis, plasma membrane, conditional protein recruitment, pleckstrin homology domain, phosphorylation

Funders Acknowledgement:
National Institutes of Health
Grant ID: R01GM143367

Abstract

Tools for acute manipulation of protein localization enable elucidation of spatiotemporally defined functions, but their reliance on exogenous triggers can interfere with cell physiology. This limitation is particularly apparent for studying mitosis, whose highly choreographed events are sensitive to perturbations. Here we exploit the serendipitous discovery of a phosphorylation-controlled, cell cycle-dependent localization change of the adaptor protein PLEKHA5 to develop a system for mitosis-specific protein recruitment to the plasma membrane that requires no exogenous stimulus. Mitosis-enabled Anchor-away/Recruiter System (MARS) comprises an engineered, 15-kDa module derived from PLEKHA5 capable of recruiting functional protein cargoes to the plasma membrane during mitosis, either through direct fusion or via GFP–GFP nanobody interaction. This protocol outlines the cloning strategy to generate MARS-POI and GFP-POI fusion constructs, procedures to examine POI recruitment by MARS via transient transfection, and steps to generate cell lines stably expressing 2xMARS-nGFP.

Image Attribution

Image from Figure 5 in Cao X, Huang S, Wagner MM, Cho Y-T, Chiu D-C, Wartchow KM, Lazarian A, McIntire LB, Smolka MB, Baskin JM. “A phosphorylation-controlled switch confers cell cycle-dependent protein relocalization.” Nat Cell Biol (2024).

Materials

Plasmids:

MARS: Addgene 205232
2xMARS: Addgene 205233
MARS-nGFP: Addgene 205234
2xMARS-nGFP: Addgene 205235
pCDNA3-MARS-p85(iSH2): Addgene 205236
pCDNA3-2xMARS-PLD: Addgene 205237
pCDH-2xMARS-nGFP-puro: Addgene 205238
pSBtet-2xMARS-nGFP-puro: Addgene 205239
PAX2 (Lammerding lab, Cornell University): can be replaced by psPAX2, Addgene 12260
VSVG (Lammerding lab, Cornell University): can be replaced by pMD2.G, Addgene 12259
pCMV(CAT)T7-SB100: Addgene 34879

Reagents:

BsrGI: New England Biolabs R3575
XbaI: New England Biolabs R0145
NEBuilder HiFi DNA Assembly Master Mix: New England Biolabs E2621
Rapid DNA Ligation Kit: ThermoFisher K1423
Transfectagro reduced-serum Medium: Corning 40-300-CV
Lipofectamine 2000 Transfection Reagent: Invitrogen 11668019
Polybrene Infection / Transfection Reagent: Sigma-Aldrich TR-1003
Puromycin dihydrochloride >98%: VWR 75844-852

Before start

To use MARS to recruit target proteins to the plasma membrane during mitosis, one can fuse the module to proteins of interest (POI) directly, or alternatively, one can use the MARS-tagged GFP nanobody (nGFP) to recruit GFP-labeled POI (either transiently overexpressed, overexpressed in stable cell lines, or endogenously tagged).

Section I. Cloning of the MARS fusion protein expression plasmid

1 MARS-related plasmids have been deposited to Addgene and their plasmid numbers are listed in the Materials tab. To fuse a single MARS unit to POI, start with pCDNA3-MARS-p85(iSH2) (Addgene 205236). To fuse the tandem MARS module to POI, start with pCDNA3-2xMARS-PLD (Addgene 205237).

2 Digest either plasmid with BsrGI and XbaI at 37 °C to remove p85(iSH2) or PLD. Gel purify the digested vectors.

Note

The BsrGI site is within the mCherry protein at the C-terminus (last three residues). When designing the POI insert sequences, please remember to include the C-terminal mCherry residues into the inserts.

3 Prepare POI insert sequences by PCR or gene block synthesis service, and assemble the inserts into the digested vectors from step 2 using Gibson Assembly or a ligation reaction with T4 DNA ligase.

4 Perform bacterial transformation using the reaction mixture in step 4 into DH5α *E.coli* competent cells, and plate on LB-ampicillin or LB-carbenicillin plates.

5 Pick a few colonies for each construct, grow overnight cultures in LB-ampicillin, and purify the plasmids. Confirm the plasmid sequences by Sanger sequencing or whole-plasmid Oxford Nanopore sequencing.

Note

The steps listed above describe the cloning strategy when one uses the MARS-containing plasmids as the cloning vectors with target POI as inserts. One can also amplify the single or tandem MARS units with mCherry from these two plasmids as inserts, and assemble them into vectors containing target POI.

Section II. Transient transfection of MARS-POI or 2xMARS-nGFP with GFP-POI to examine POI recruitment by live-cell microscopy

6 Seed 200,000 Flp-In HeLa T-Rex cells (or other HeLa cell line) to each 35 mm glass bottom imaging dish in DMEM supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-

streptomycin (P/S). Cells should reach about 30-40% confluency for transfection the next day.

7 Prepare transfection mixture in a polystyrene tube: per 35 mm dish, mix 150 µL Transfectagro (or Opti-MEM) Reduced-serum Medium without FBS and 2 µL Lipofectamine 2000. Incubate at room temperature for 5 min.

8 While the transfection mixture is in incubation, prepare transfection DNA as the following:

a. For MARS-POI transfection, per 35 mm dish, mix 750-1000 ng of MARS-POI plasmid with 150 µL Transfectagro (or Opti-MEM) Reduced-serum Medium without FBS in a polystyrene tube.

b. For 2xMARS-nGFP and GFP-POI co-transfection, per 35 mm dish, mix 800 ng of 2xMARS-nGFP plasmid (Addgene 205235) and 500-1000 ng of GFP-POI plasmid with 150 µL Transfectagro (or Opti-MEM) Reduced-serum Medium without FBS in a polystyrene tube.

9 For each 35 mm dish, add 152 µL of transfection mixture (Transfectagro (or Opti-MEM) Reduced-serum Medium + Lipofectamine 2000) to each polystyrene tube with transfection DNA. Mix thoroughly and incubate at room temperature for 20 min.

10 Change culture media in the 35 mm dishes to Transfectagro (or Opti-MEM) Reduced-serum Medium with 10% FBS (2 mL per dish).

11 Slowly add the final transfection mixture to the dishes: 302 µL per 35 mm dish.

12 6 to 8 hours post-transfection, aspirate the transfection media and exchange to regular growth medium (DMEM with 10% FBS and 1% P/S).

13 24 to 30 hours post transfection, rinse dishes twice with DMEM without Phenol Red or Tyrode's-HEPES (T/H) buffer (135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mg/mL glucose, 5 mg/mL bovine serum albumin, 20 mM HEPES, pH 7.4), and proceed to live-cell confocal microscopy to examine cellular localization of MARS-POI or GFP-POI with 2xMARS-nGFP in interphase cells and mitotic cells.

a. For MARS-POI, use a 561 nm laser to excite the mCherry within the fusion protein and visualize the subcellular localization of MARS-POI. If MARS successfully recruits POI, the fusion protein should localize to the plasma membrane only during mitosis.

b. For GFP-POI and 2xMARS-nGFP imaging, use a 488 nm laser to excite GFP and a 561 nm laser to excite the mScarlet-i in the 2xMARS-nGFP construct. If 2xMARS-nGFP successfully recruits GFP-POI, GFP-POI should co-localize with 2xMARS-nGFP, and both constructs display plasma membrane localization only during mitosis.

Note

If the expression level of GFP-POI is high and substantially exceeds a 1:1 stoichiometry with 2xMARS-nGFP, one may not observe GFP-POI recruitment. In this case, we recommend lowering the expression level of GFP-POI by either transfecting less of the GFP-POI plasmid or use a weaker promoter for GFP-POI expression (e.g., PGK).

14 After confirmation of successful recruitment of target POI by MARS, one can proceed to test the functionality of the fusion enzymes.

Section III. Generating cell lines stably expressing 2xMARS-nGFP constitutively using lentivirus transduction

15 Day 0: Seed HEK 293TN cells in 60 mm dishes one day before transfection to achieve approximately 50% confluency on the day of transfection.

16 Day 1 afternoon: prepare transfection mixture in a polystyrene tube: per 60 mm dish, mix 450 µL Transfectagro (or Opti-MEM) Reduced-serum Medium without FBS and 7.5 µL Lipofectamine 2000. Incubate at room temperature for 5 min.

17 While the transfection mixture is in incubation, prepare packaging plasmids and the MARS lentiviral plasmid pCDH-2xMARS-nGFP-puro (Addgene 205238) as the following:
per 60 mm dish, mix 2800 ng of PAX2 plasmid, 900 ng of VSVG plasmid, and 3750 ng of pCDH-2xMARS-nGFP-puro with 400 µL Transfectagro (or Opti-MEM) Reduced-serum Medium without FBS in a polystyrene tube.

18 For each 60 mm dish, add 457 µL of transfection mixture (Transfectagro (or Opti-MEM) Reduced-serum Medium + Lipofectamine 2000) to each polystyrene tube with lentiviral plasmids. Mix thoroughly and incubate at room temperature for 20 min.

19 Change culture media in the 35 mm dishes to Transfectagro (or Opti-MEM) Reduced-serum Medium with 10% FBS (5 mL per dish).

20 Slowly add the final transfection mixture to the dishes: 860 µL per 60 mm dish.

21 Day 2 morning: 12 to 18 hours post-transfection (overnight), aspirate the transfection media and exchange to 5 mL of DMEM with 10% FBS.

22 Day 2 afternoon to Day 4 morning: Starting from 24 h post-transfection, harvest virus-containing conditioned media (VCM) every 12 h, for a total of four times. After the last collection, filter the VCM through a 0.45 µM filter. VCM can be stored at 4 °C for up to one week or at −80 °C for long-term storage.

23 Day 3: To transduce target cells (Flp-in HeLa) in 6-well plates, seed cells in a 6-well plate one day before transduction to achieve approximately 50% confluency on the next day. Include one "kill control" well where cells are not treated with VCM.

24 Day 4 morning to Day 5 morning: Per well in a 6-well plate: incubate cells with 3 mL of transduction medium (2 mL of VCM + 1 mL of fresh DMEM with 10% FBS) and 8 µg/mL polybrene. For the kill control well, add 3 mL of DMEM with 10% FBS and 8 µg/mL polybrene. Every 12 h, replace old transduction medium with fresh transduction medium and polybrene. Repeat transduction 3 times.

25 Day 5 afternoon: After the third transduction, aspirate transduction medium and leave the cells in regular DMEM with 10% FBS and 1% P/S for 12-24 h.

26 Day 6: Trypsinize the transduced cells and expand from one well of a 6-well plate to a 10-cm dish. Add puromycin to a final concentration of 2 µg/mL for selection. Culture the 2xMARS-nGFP expressing cells and kill control cells in puromycin-containing media until all kill control cells die.

27 Expression level and subcellular localization of 2xMARS-nGFP can be examined by confocal microscopy as described in Section II. If necessary, cells can be sorted by FACS with a 561 nm laser to further enrich the 2xMARS-nGFP-expressing population and/or select the population of cells with the desired expression level of 2xMARS-nGFP based on mScarlet-i fluorescence intensity.

Section IV. Generating cell lines stably expressing 2xMARS-nGFP under doxycycline induction using the Sleeping Beauty transposon system

28 Seed target cells (Flp-in HeLa or similar) in 6-well plates one day before transfection to achieve approximately 70% confluency on the day of transfection. Include one kill control well.

29 Prepare transfection mixture in a polystyrene tube: per well in a 6-well plate, mix 150 µL Transfectagro (or Opti-MEM) Reduced-serum Medium without FBS and 3 µL Lipofectamine 2000. Incubate at room temperature for 5 min.

30 While the transfection mixture is in incubation, prepare transfection DNA:
per 35 mm dish, mix 1900 ng of pSBtet-2xMARS-nGFP-puro (Addgene 205239) and 100 ng of pCMV(CAT)T7-SB100 (Addgene 34879) with 150 µL Transfectagro (or Opti-MEM) Reduced-serum Medium without FBS in a polystyrene tube. For kill control well, only pCMV(CAT)T7-SB100 plasmid is added.

31 For each well of a 6-well plate, add 152 µL of transfection mixture (Transfectagro (or Opti-MEM) Reduced-serum Medium + Lipofectamine 2000) to each polystyrene tube with transfection DNA. Mix thoroughly and incubate at room temperature for 20 min.

32 Change culture media in the 35 mm dishes to Transfectagro (or Opti-MEM) Reduced-serum Medium with 10% FBS (2 mL per well).

33 Slowly add the final transfection mixture to the dishes: 303 µL per well.

34 6 to 8 hours post transfection, aspirate the transfection media and exchange to DMEM with 10% FBS and 1% P/S.

35 24 to 30 hours post transfection, trypsinize transfected cells and expand cells from each well of the 6-well plate to a 10-cm dish. Add puromycin to a final concentration of 2 µg/mL for selection. Culture the cells in puromycin-containing media until all kill control cells die.

36 Expression of 2xMARS-nGFP can be induced by addition of doxycycline at 2 µg/mL for 48 h. Expression level and subcellular localization of 2xMARS-nGFP can be examined by confocal microscopy as described in Section II. If necessary, cells can be sorted by FACS with a 561 nm laser to further enrich the 2xMARS-nGFP-expressing population and/or select the population of cells with the desired expression level of 2xMARS-nGFP based on mScarlet-i fluorescence intensity.

Protocol references

Cao X, Huang S, Wagner MM, Cho Y-T, Chiu D-C, Wartchow KM, Lazarian A, McIntire LB, Smolka MB, Baskin JM. “A phosphorylation-controlled switch confers cell cycle-dependent protein relocalization.” Nat Cell Biol (2024).