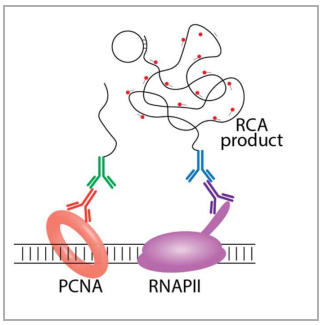

Proximity Ligation Assay to Detect Transcription-Replication Conflicts in Yeast

DEC 06, 2022

WORKS FOR ME 1

COMMENTS 0

DOI
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ABSTRACT

Replication and transcription machineries can come into conflict with each other when spatial and temporal separation of these processes are not possible. We present a protocol to examine transcription-replication conflicts in the budding yeast *Saccharomyces cerevisiae*. We have modified existing proximity ligation assay protocols to detect proximity between RNA polymerase II (transcription machinery) and PCNA (replisome) in yeast. This protocol can be applied to any two proteins or modifications of interest in which you have antibodies suitable for immunofluorescence.

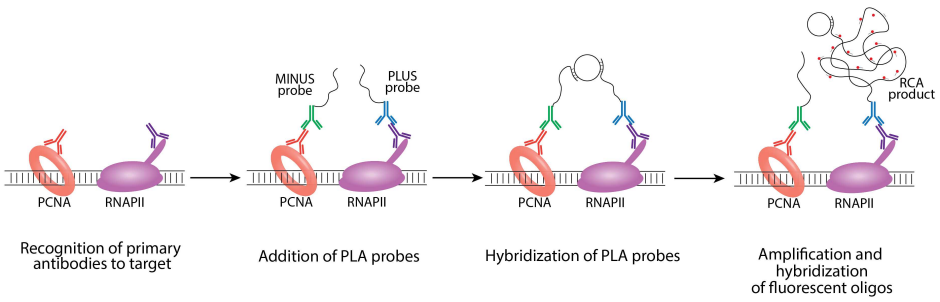

Figure 1. Proximity Ligation Assay Schematic

This proximity ligation assay (PLA) protocol was modified from Alberts et al. *JOVE* 2019 with the following changes:

- Different antibodies targeting RNA polymerase II and PCNA instead of cytosolic chaperone proteins
- Using nocodazole as a negative control (inhibiting replication by arresting cells in M phase)
- Altered wash conditions and slides used
- Added details about imaging and image analysis (using Cell Profiler)

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CITATION

Alberts N, Mathangasinghe Y, Nillegoda NB (2019). In Situ Monitoring of Transiently Formed Molecular Chaperone Assemblies in Bacteria, Yeast, and Human Cells.. Journal of visualized experiments : JoVE.

LINK

https://doi.org/10.3791/60172

Cell Profiler can be used for quantifying foci per nucleus. Pipelines used are included for download and details about image analysis are shown below in the steps of the protocol.

CITATION

Stirling DR, Swain-Bowden MJ, Lucas AM, Carpenter AE, Cimini BA, Goodman A (2021). CellProfiler 4: improvements in speed, utility and usability.. BMC bioinformatics.

LINK

https://doi.org/10.1186/s12859-021-04344-9

DOI

dx.doi.org/10.17504/protocols.io.kxygx93rzg8j/v1

PROTOCOL CITATION

Rebecca E Brown, Catherine H Freudenreich 2022. Proximity Ligation Assay to Detect Transcription-Replication Conflicts in Yeast. **protocols.io** https://dx.doi.org/10.17504/protocols.io.kxygx93rzg8j/v1

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KEYWORDS

Proximity Ligation Assay, Transcription-Replication Conflicts, Saccharomyces cerevisiae

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PROTOCOL INTEGER ID

73132

MATERIALS TEXT

General materials:

Slide preparation:

- 0.1% Poly-L-lysine: Sigma Aldrich, Cat# P8920-100ML
- Polytetrafluoroethylene (PTFE) Printed Slide, 30 well, 2mm: Electron Microscopy Sciences, Cat# 63434-02
- Rectangular cover glass (1.5mm thickness, 22x50): Millipore Sigma (Corning), Cat# CLS2980225-100EA

Chemicals and Enzymes:

- Nocodazole: Selleckchem, Cat# R17934
- Dimethyl Sulfoxide: Fisher Scientific, Cat# BP231-100
- Zymolyase 100T: amsbio, Cat# 120493-1
- Paraformaldehyde 96%, extra pure: Acros Organics, Cat# 30525-89-4
- Sodium phosphate Dibasic Anhydrous: Fisher Scientific, Cat# S374-1
- Sodium phosphate monobasic: Sigma Aldrich, Cat# S9638
- D-sorbitol: Acros Organics, Cat# 50-70-4
- Potassium phosphate monobasic: Sigma Aldrich, Cat# P9791
- 2-Mercaptoethanol: Sigma Aldrich, Cat# M6250-100ML
- TritonX-100: Fisher Scientific, Cat# BP151-500

Antibodies:

- Anti-PCNA Antibody [5E6/2]: abcam, Cat# ab70472
- RNA Polymerase II/POLR2A [p Ser2] Antibody: Novus Biologicals, Cat# NB100-1805

Duolink® Proximity Ligation Assay Reagents:

Any Duolink® reagents that are compatible with primary antibodies chosen can be used.

Starter kit:

- Duolink® In Situ Red Starter Kit Mouse/Rabbit: Millipore Sigma, Cat# DUO92101

Individual reagents:

- Duolink® In Situ Detection Reagents Red: Millipore Sigma, Cat# DUO92008
- Duolink® In Situ PLA® Probe Anti-Rabbit PLUS: Millipore Sigma, Cat# DUO92002
- Duolink® In Situ PLA® Probe Anti-Mouse MINUS: Millipore Sigma, Cat# DUO92004
- Duolink® *In Situ* Mounting Medium with DAPI: Millipore Sigma, Cat# DUO82040
- Duolink® In Situ Wash Buffers, Fluorescence: Millipore Sigma, Cat# DUO82049

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Instruments:

- Benchtop centrifuge
- Refrigerator/incubator (temperature range of 4-37 °C)
- Roller drum/shaking incubator (temperature range of 30-37 °C)
- Vacuum aspirator
- Widefield Fluorescence Microscope (we used Leica Dmi8 Thunder and DeltaVision Ultra microscopes)

Software:

- Cell Profiler to quantify PLA foci
- ImageJ or other software to view images
- Prism or other software for making figures and analysis of data

SAFETY WARNINGS

Follow safety guidelines in accordance with university/organization guidelines. Refer to safety warnings of individual reagents provided by the manufacturers.

BEFORE STARTING

1. Make all solutions before starting the protocol:

Nocodazole:

- Make 1.5 mg/mL nocodazole solution in DMSO. Dilute to 15 µg/mL concentration in yeast rich liquid media.

4% Paraformaldehyde (PFA):

- Dissolve 0.8 g PFA in pre-warmed 17 mL 0.1 M Na_2HPO_4.
- Add 3 mL of 0.1 M NaH_2PO_4.
- Final pH at 7.6.

Wash buffer:

- 1.2 M Sorbitol in 100 mM KPO_4 pH 6.5

Zymolyase solution:

- 0.5 mg/mL Zymolyase 100 T, 1.2 M Sorbitol, 100 mM KPO_4 pH 6.5, 20 mM 2-Mercaptoethanol.
- Make fresh for each experiment.

Permeabilizing solution:

- 1% TritonX-100 in 100 mM KPO_4 pH 6.5.

Wash buffer A:

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- Make according to Duolink® manufacturer guidelines.

Wash buffer B:

- Make according to Duolink® manufacturer guidelines.

2. Prepare humidity chamber (see step 18).

2h

Preparation of Poly-L-Lysine Coated Slides

1 Prepare poly-L-lysine coated polytetrafluoroethylene (PTFE) slide to ensure that yeast cells adhere to slide:

NOTE: Slides should be prepared either day of or day before proximity ligation assay protocol is started.

NOTE: Be sure not to touch the wells of the slide for all remaining steps of this protocol. Make sure to label the slides with pencil only.

2 Gently scrub PTFE slide using toothbrush with soft bristles (i.e. baby toothbrush).

15m

3 Rinse slide with deionized water and let slide dry completely in hood for 00:15:00 .

10m

4 Place 5 µL of 0.1% poly-L-lysine onto each well and let sit for 00:10:00 at Room temperature .

1h

5 Rinse slide with deionized water and let slide dry completely in hood (for at least 01:00:00 at Room temperature , overnight is acceptable).

1d

Cell Growth and Collection

6 **NOTE:** For all centrifugation steps, make sure to spin at low speeds (< 3000 rpm is recommended).

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7 Grow cells overnight at 30 °C to saturation (use roller drum or shaking incubator) and dilute back to an OD_{600} ~0.2. 16h

NOTE: Cells can be grown in media of choice. For this protocol, yeast synthetic complete dropout media was used for all experiments except for those treating cells with nocodazole, in which cells were grown in yeast rich media instead.

8 Grow cells to an OD_{600} ~1.5. 6h

NOTE: Growth time will vary per strain (typically ~4-10 hrs after diluting back to OD_{600} ~0.2).

8.1 For experiments treating cells with nocodazole, grow cells to OD_{600} ~1.0. Add nocodazole ([M] 15 µg/mL final concentration in cultures) and grow cells for 01:00:00 before collection. 1h

NOTE: An important negative control to include is a sample that does not express one of the targets (in this case PCNA and RNA polymerase II) to ensure that primary antibodies are specific. Since PCNA and RNAPII are essential proteins, replication or transcription must be inhibited as a negative control and should result in reduced PLA foci. Here we chose to inhibit replication by treating cells with nocodazole to arrest cells in M phase.

9 Collect cells by centrifugation at 3000 rpm, Room temperature for 00:05:00. Collect 1 mL of cells per condition (i.e. for each suspension of prepared spheroplasts in sorbitol - see step 15). 5m

NOTE: Volume and OD_{600} of cells collected may need to be altered to optimize the density of cells upon the slide while imaging.

Cell Fixation 40m

10 Fix cells with [M] 4 % (m/v) paraformaldehyde (PFA) for 00:15:00 at room temperature. Use 100 µL of [M] 4 % (m/v) PFA per 1 mL of cells collected. Collect cells by centrifugation at 3000 rpm, Room temperature for 00:05:00 after incubation in PFA. 20m

11 Wash cells 3x in with wash buffer ([M] 1.2 molar (M) sorbitol in [M] 100 millimolar (mM) KPO_4 pH 6.5). Use 100 µL of wash buffer per 1 mL of cells collected. Pellet cells between washes by centrifugation at 3000 rpm, Room temperature for 00:05:00 . 20m

Cell Wall Digestion to Prepare Spheroplasts 45m

12 Cell wall is degraded through enzymatic digestion by Zymolyase: 20m

Digest cell walls with Zymolyase solution ([M] 0.5 mg/mL Zymolyase 100 T, [M] 1.2 molar (M) sorbitol, [M] 100 millimolar (mM) KPO_4 pH 6.5, [M] 20 millimolar (mM) 2-Mercaptoethanol) at 30 °C for

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00:20:00 .

NOTE: Treat spheroplasts carefully to prevent disruption of nuclear structure.

5m

13 Spin down cells at 2000 rpm, Room temperature for 00:05:00 to remove Zymolyase solution.

20m

14 Wash cells 3x in with wash buffer ([M] 1.2 molar (M) sorbitol in [M] 100 millimolar (mM) KPO_4 pH 6.5). Use 100 µL of wash buffer per 1 mL of cells collected. Pellet cells between washes by centrifugation at 2000 rpm, Room temperature for 00:05:00 .

35m

Attaching Spheroplasts to Slide

35m

15 Resuspend spheroplasts in 30 µL - 100 µL of 1.2M sorbitol. Attach spheroplasts to poly-L-lysine coated slides by pipetting 5 µL into each well. Let spheroplasts sit for 00:30:00 at Room temperature before aspirating liquid and proceeding with next steps.

NOTE: Altering volume of sorbitol to suspend spheroplasts may need to be altered to optimize the density of cells upon the slide while imaging.

40m

Cell Permeabilization

20m

16 Wash cells 3x in with wash buffer ([M] 1.2 molar (M) sorbitol in [M] 100 millimolar (mM) KPO_4 pH 6.5). Use 5 µL of wash buffer per well. Let wash buffer sit on well for 00:05:00 . Aspirate liquid using a pipette tip connected to a vacuum source after each wash.

NOTE: Make sure not to touch wells when aspirating solution. Touch tip to the edge of the well to aspirate the solution.

20m

17 Wash attached spheroplasts 3x with permeabilizing solution ([M] 1 % (v/v) TritonX-100 in [M] 100 millimolar (mM) KPO_4 pH 6.5). Use 5 µL of permeabilizing solution per well. Let permeabilizing solution sit on well for 00:05:00 . Aspirate liquid using a vacuum source after each wash.

1h 5m

Proximity Ligation Assay (PLA) - Blocking

18 The steps in the PLA sections below follow the Duolink® PLA Fluorescence Protocol (Duolink PLA Fluorescence Protocol) using reagents from the kit provided by Millipore Sigma (see materials).

NOTE: Before proceeding with steps, prepare a humidity chamber and equilibrate to 37 °C .

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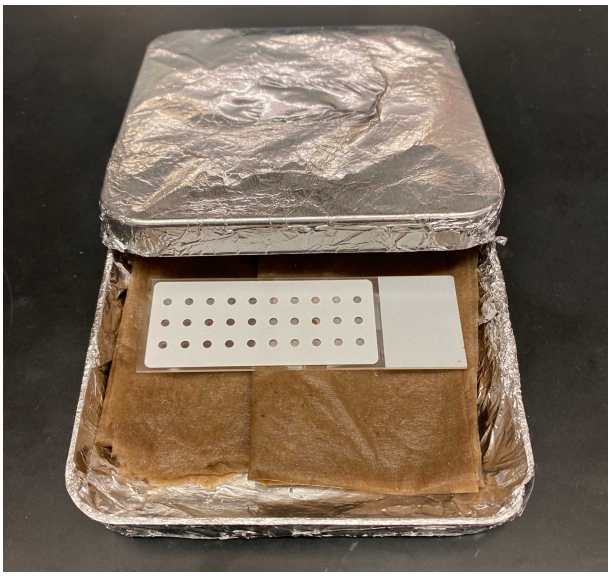

Figure 2. Humidity chamber for proximity ligation assay. Coat petri dish with aluminum foil to create a dark chamber. Wet paper towels and place on bottom of petri dish (should re-wet paper towels between incubations to ensure chamber remains humid). The poly-L-lysine coated PTFE slide can sit on top of paper towels.

19 Vortex the Duolink® Blocking Solution.

20 Add 5 µL of Blocking Solution to each sample well (be sure to cover entire sample with Blocking Solution).

21 Incubate slide in heated humidity chamber for 01:00:00 at 37 °C. 1h

PLA - Primary Antibody Incubation 1d

22 Vortex Duolink® Antibody Diluent.

23 Dilute your primary antibodies to suitable concentration in Duolink® Antibody Diluent. The following antibodies/dilutions were used: 5m

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- 1:400 dilution of anti-PCNA [5E6/2] (abcam ab70472)
- 1:400 dilution of anti-RNAPII [p Ser2] (Novus Biologicals NB100-1805)

NOTE: Antibody dilutions may need to be optimized if using different antibodies or for different strains/growth conditions.

24 Aspirate Duolink® Blocking Solution from slide wells.

NOTE: Make sure not to touch wells when aspirating solution. Touch tip to the edge of the well to aspirate the solution.

5m

25 Add diluted primary antibody solution to each well as stated below:

- Experimental condition: create mix of the two primary antibody dilutions (2.5 µL of each antibody dilution per well). Add 5 µL of mixed primary antibodies to each well, making sure to cover the entire sample.
- Single antibody controls: Add 5 µL of individual primary antibody to each well, making sure to cover the entire sample.

NOTE: An important control to include with each PLA experiment is single antibody controls. Adding only one antibody to the reaction should eliminate or at least drastically decrease the amount of PLA foci detected.

12h

26 Incubate slides in a humidity chamber at 4 °C overnight.

NOTE: Optimal incubation temperature/time for primary antibodies chosen will need to be determined.

1h 30m

PLA - PLA Probe Incubation

30m

27 **NOTE:** Heat up humidity chamber to 37 °C before proceeding with these steps.

While chamber is heating up: Vortex PLUS and MINUS PLA probes and Duolink® Antibody Diluent.

5m

28 Make PLA probe solution: Dilute the PLUS/MINUS PLA probes 1:5 in Duolink® Antibody Diluent.

- For each 5 µL reaction: add 1 µL of each probe and 3 µL of Duolink® Antibody Diluent.

29 Aspirate primary antibody solution from slide wells.

NOTE: Make sure not to touch wells when aspirating solution. Touch tip to the edge of the well to aspirate the solution.

10m

30 Wash slide wells 2x for 00:05:00 in 1x Wash Buffer A at Room temperature. Let wash buffer sit on slide and aspirate solution between each wash.

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31 Aspirate excess Wash Buffer A from slide wells.

32 Apply 5 µL of PLA probe solution to each slide well, making sure to cover the entire sample. 5m

33 Incubate slides in pre-heated humidity chamber for 01:00:00 at 37 °C. 1h

PLA - Ligation 1h

34 **NOTE:** Wait to add Ligase to solution until immediately prior to addition to sample. Make sure ligation buffer is completely thawed/mixed prior to use. Ligation buffer can be aliquoted to prevent multiple freeze/thaw cycles. 5m

Make 1X ligation buffer solution: Dilute the 5X Duolink® Ligation buffer 1:5 in molecular biology grade water.

- For each 5 µL reaction: add 1 µL of 5X Duolink® Ligation buffer and 4 µL of molecular biology grade water.

35 Aspirate PLA probe solution from each slide well.

NOTE: Make sure not to touch wells when aspirating solution. Touch tip to the edge of the well to aspirate the solution.

36 Wash slide wells 2x for 00:05:00 in 1x Wash Buffer A at Room temperature. Let wash buffer sit on slide and aspirate solution between each wash. 10m

37 Make Ligase solution: During the wash steps retrieve Ligase from freezer using freezer block. Add Ligase to 1x ligation buffer at a 1:40 dilution. 5m

- For each 5 µL reaction: add 0.125 µL of Ligase and 4.875 µL of 1x ligation buffer.

38 Aspirate excess Wash Buffer A from slide wells.

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39 Apply 5 µL of Ligase solution to each slide well, making sure to cover the entire sample. 5m

40 Incubate slides in pre-heated humidity chamber for 00:30:00 at 37 °C. 30m

PLA - Amplification 2h

41 **NOTE:** Wait to add Polymerase until immediately prior to addition to sample. Amplification buffer is light-sensitive (protect all solutions containing buffer from light). 5m

Make 1X amplification buffer solution: Dilute the 5X Duolink® Amplification buffer 1:5 in molecular biology grade water.

- For each 5 µL reaction: add 1 µL of 5X Duolink® Amplification buffer and 4 µL of molecular biology grade water.

42 Aspirate Ligase solution from each slide well.

NOTE: Make sure not to touch wells when aspirating solution. Touch tip to the edge of the well to aspirate the solution.

43 Wash slide wells 2x for 00:02:00 in 1x Wash Buffer A at Room temperature. Let wash buffer sit on slide and aspirate solution between each wash. 5m

44 Make Amplification solution: During the wash steps retrieve Polymerase from freezer using freezer block. Add Polymerase to 1x amplification buffer at a 1:80 dilution. 5m

- For each 5 µL reaction: add 0.0625 µL of Ligase and 4.9375 µL of 1x amplification buffer.

45 Aspirate excess Wash Buffer A from slide wells.

46 Apply 5 µL of Amplification solution to each slide well, making sure to cover the entire sample. 5m

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47 Incubate slides in pre-heated humidity chamber for 01:40:00 at 37 °C. 1h 40m

PLA - Final Slide Preparation 45m

48 **NOTE:** Light sensitive reagents - keep slides protected from light at all times.

Aspirate Amplification solution from slide wells.

NOTE: Make sure not to touch wells when aspirating solution. Touch tip to the edge of the well to aspirate the solution.

49 Wash slide wells 2x for 00:10:00 in 1x Wash Buffer B at Room temperature. Let wash buffer sit on slide and aspirate solution between each wash. 20m

50 Wash slides once in 0.01x Wash Buffer B for 00:01:00. 1m

51 Aspirate excess Wash Buffer B from slide wells.

52 Mount the slides with a coverslip using a minimal volume of Duolink® PLA Mounting Medium with DAPI. Make sure to leave space on slide around coverslip to allow for sealing with nail polish. Seal edges of coverslip to slide with clear nail polish. 5m

53 Wait at least 00:15:00 and up to 2 days before imaging samples using a fluorescence or confocal microscope. For storage of 1-2 days, keep slide in the dark at 4 °C. Slide can be stored longer term at -20 °C. 15m

PLA - Imaging 2d

54 Image samples using an oiled 100x objective. See below for details imaging on a Leica Dmi8 Thunder or DeltaVision Ultra widefield fluorescence microscope with Duolink® In Situ Red Detection Reagents:

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54.1 Using Leica Dmi8 Thunder:
For imaging:

- Use 575nm excitation for PLA foci
- Use 395nm excitation for DAPI

For deconvolution:

- Small Volume Computational Clearing (Under Thunder/Lightning in Process tab)
- Adaptive strategy
- Refractive Index/Mounting Medium - 1.461/custom

54.2 Using DeltaVision Ultra:
For imaging:

- Use Red channel for PLA foci
- Use Blue channel for DAPI
- Used 1.5180 Laser Liquid Oil for use with objective requiring oil

For deconvolution:

- Add deconvolution to processing

Image Analysis

55 Quantification of foci per nucleus can be done by manual counting, using Cell Profiler, or other available software. Below is analysis done with Cell Profiler:

56 Three dimensional images need to be split into separate channels, positions, and timepoints using the following code:

bfconvert imagename.file_ending imagename_C%c_P%s_T%t.tif

57 Use Cell Profiler to create maximum intensity projections of images. Pipeline used is linked here and can be downloaded/modified for future use.

max_project.cppipe

See directions and example screenshots below:

57.1 Use Images module to compile list of files to be analyzed:

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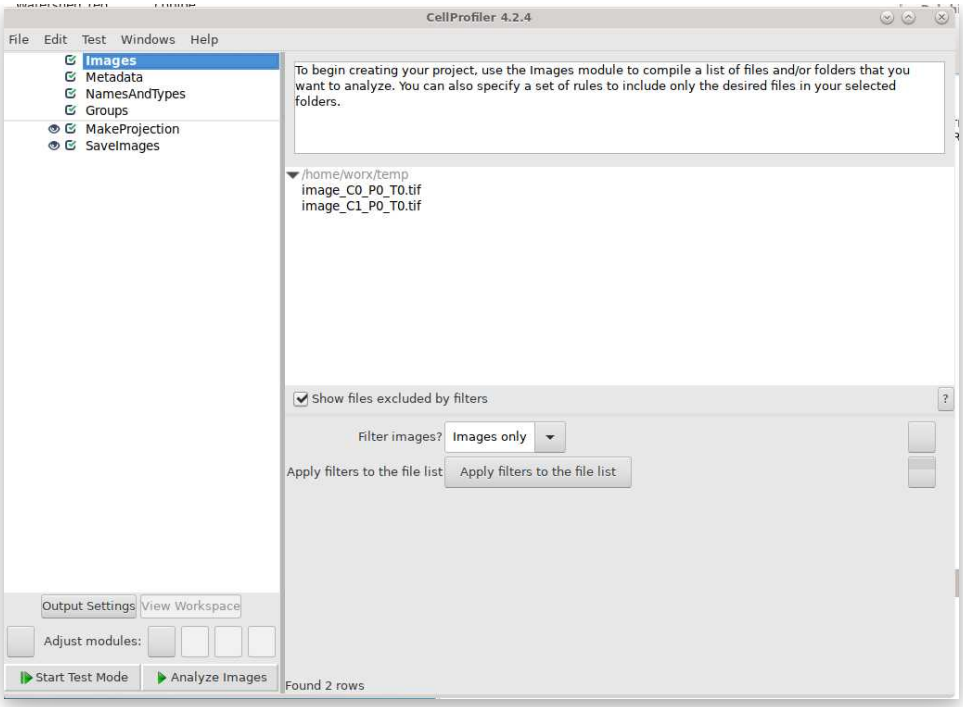

Figure 3. Example of image upload using Cell Profiler.

57.2 Use MetaData module extract data from file names:

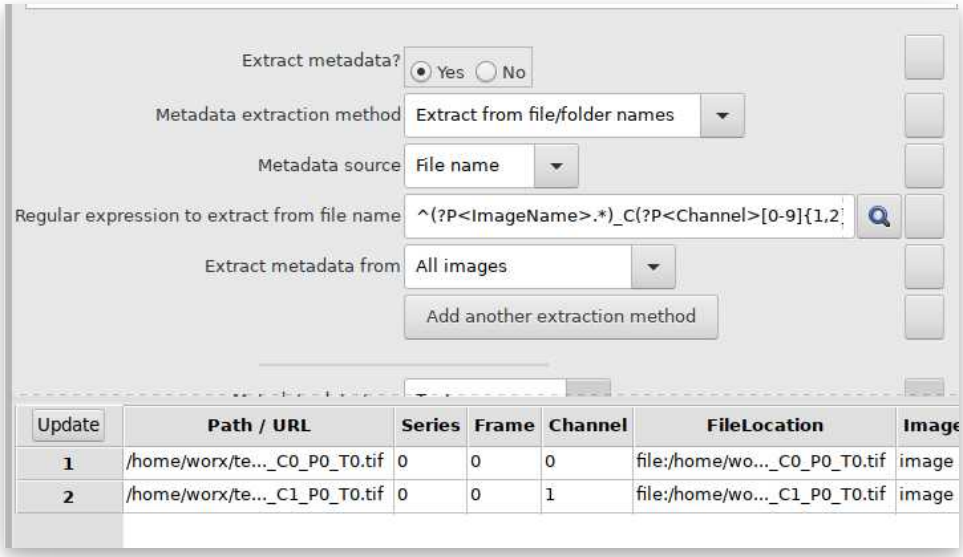

Figure 4. Example of extracting metadata from file names using Cell Profiler.

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Figure 5. Example of editing regular expression to extract image name, channel, position, and timepoint from image files using Cell Profiler.

57.3 Use NamesAndTypes module to assign names to image files:

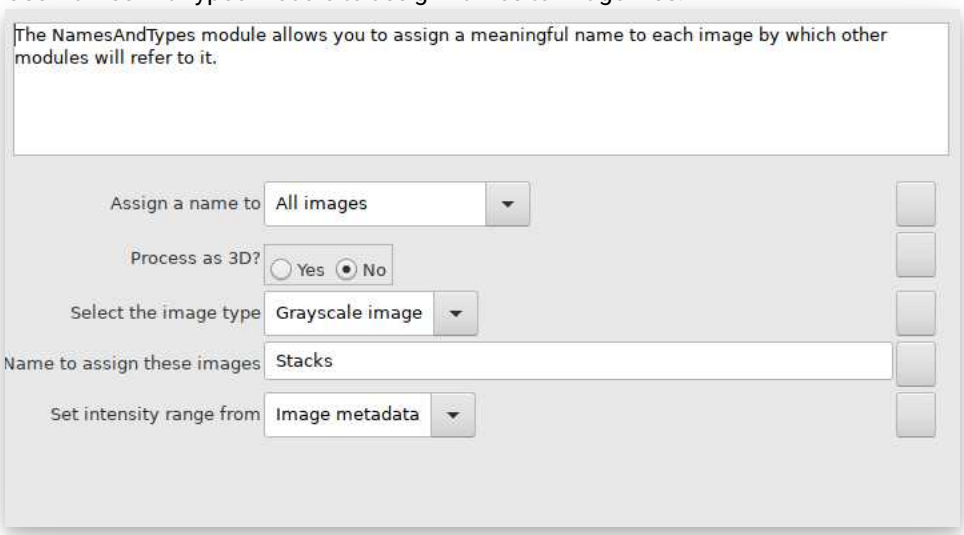

Figure 6. Example of assigning names to files using Cell Profiler. Name assigned is how images will be referred to in later steps.

57.4 Use Groups module to group files by name of file and channel used:

Citation: Rebecca E Brown, Catherine H Freudenreich Proximity Ligation Assay to Detect Transcription-Replication Conflicts in Yeast https://dx.doi.org/10.17504/protocols.io.kxygx93rzg8j/v1

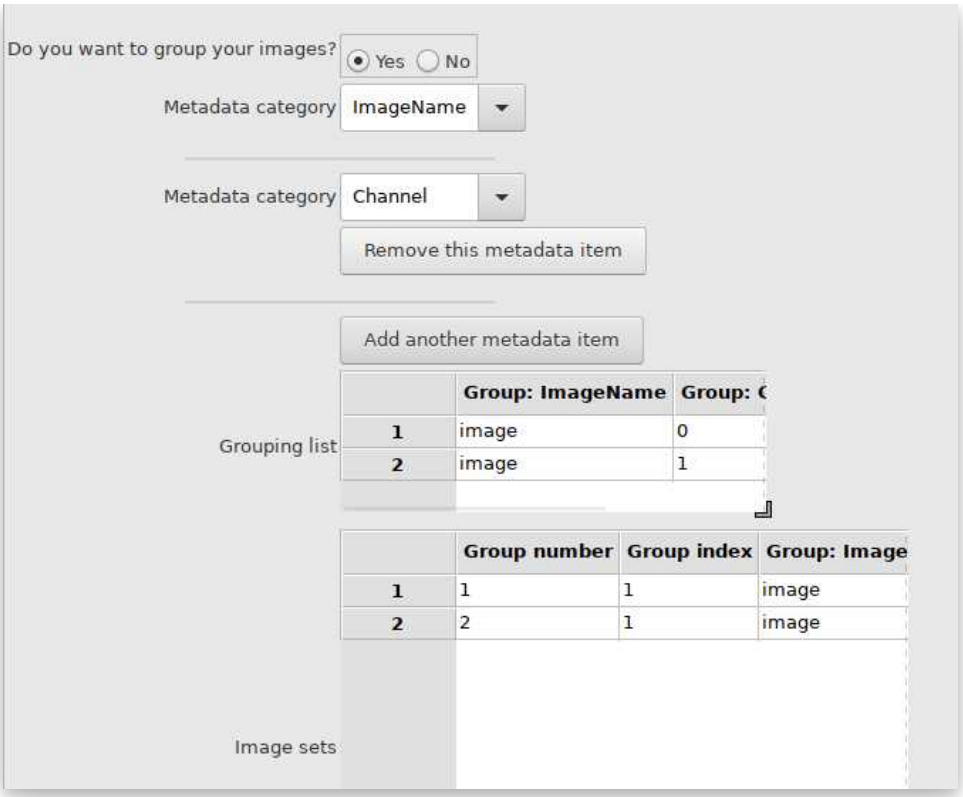

Figure 7. Example of grouping files using Cell Profiler.

57.5 Use MakeProjection module to create maximum intensity projection images:

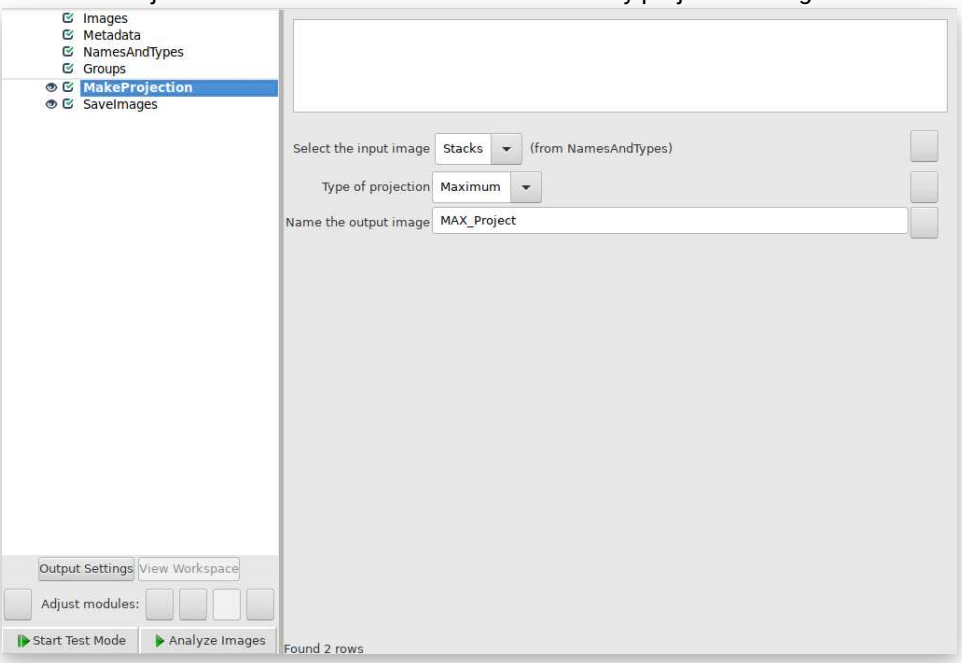

Figure 8. Example of making maximum intensity projection images using Cell Profiler.

57.6 Use SaveImages module to save maximum intensity projection images:

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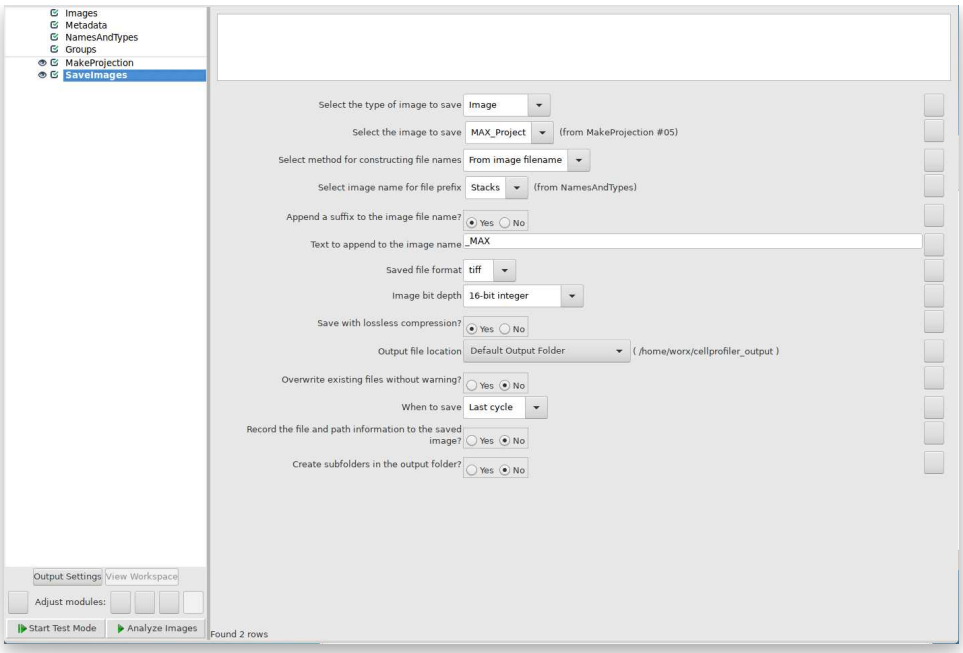

Figure 9. Example of saving maximum intensity projection images using Cell Profiler. Image bit depth should be compatible with image files saved from microscope used.

58 Use Cell Profiler to quantify foci per nucleus. Pipeline used is linked here and can be downloaded/modified for future use.

PLA_Analysis_221129.cppipe

See directions and example screenshots below:

58.1 Use MetaData module extract data from file names:

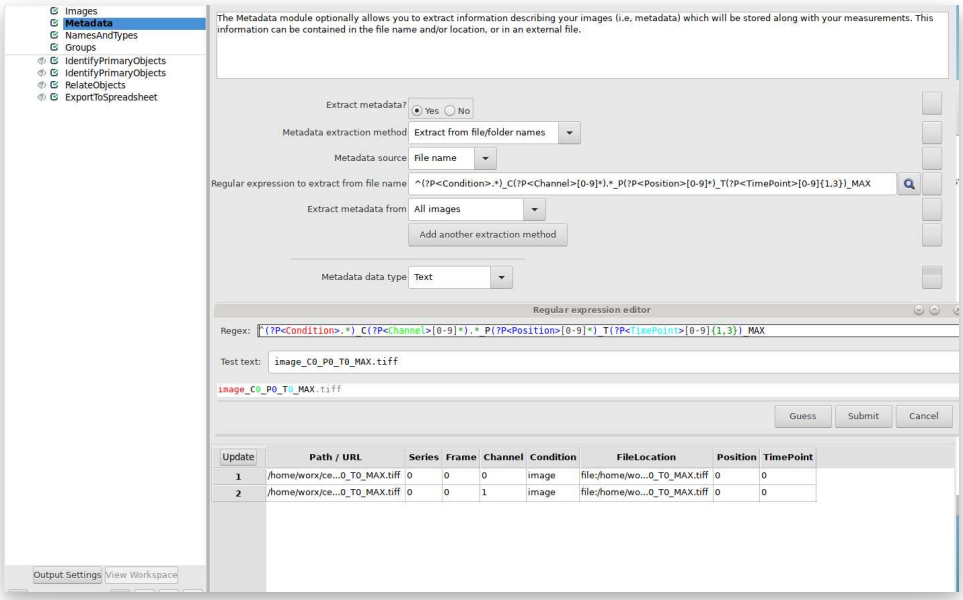

Figure 10. Example of extracting metadata from file names using Cell Profiler.

Citation: Rebecca E Brown, Catherine H Freudenreich Proximity Ligation Assay to Detect Transcription-Replication Conflicts in Yeast https://dx.doi.org/10.17504/protocols.io.kxygx93rzg8j/v1

58.2 Use NamesAndTypes module to assign names to image files:

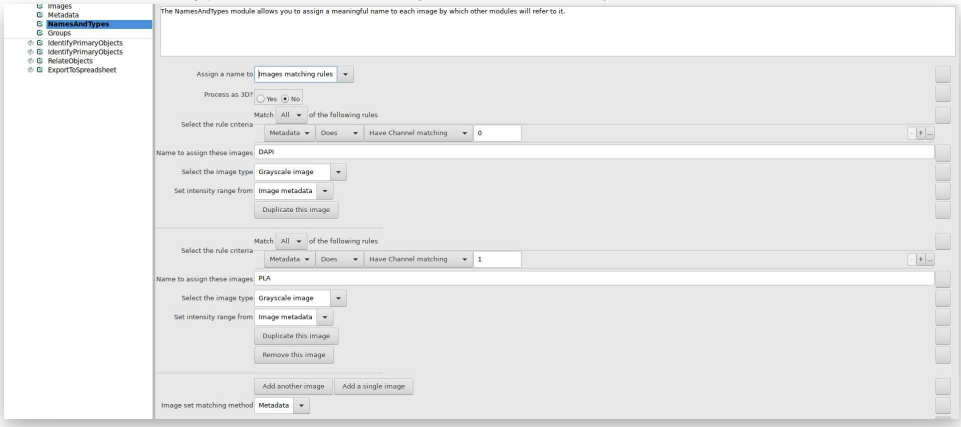

Figure 11. Page 1 - Example of assigning names to files using Cell Profiler. This assigns DAPI and PLA foci channels names to images for later analysis. Name assigned is how images will be referred to in later steps.

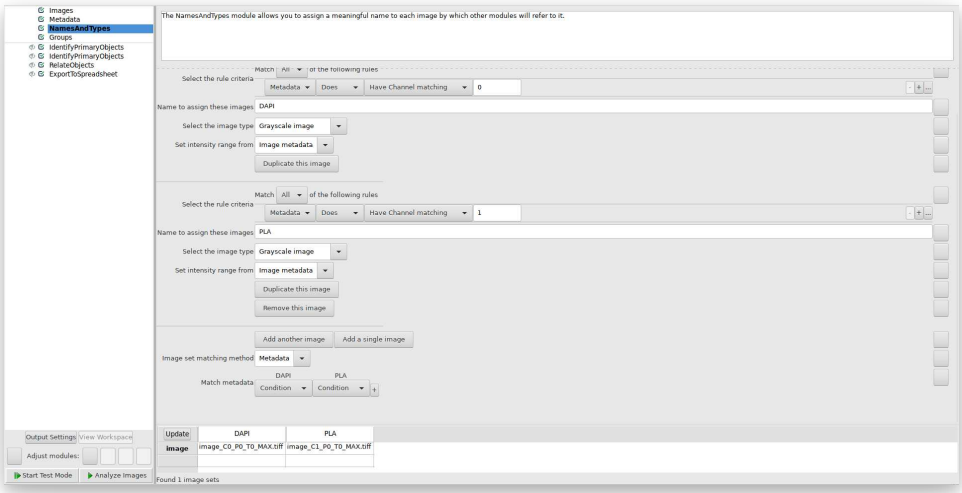

Figure 12. Page 2 - Example of assigning names to files using Cell Profiler. This assigns DAPI and PLA foci channels names to images for later analysis. Name assigned is how images will be referred to in later steps.

58.3 Use IdentifyPrimaryObjects module to identify nuclei:

Citation: Rebecca E Brown, Catherine H Freudenreich Proximity Ligation Assay to Detect Transcription-Replication Conflicts in Yeast https://dx.doi.org/10.17504/protocols.io.kxygx93rzg8j/v1

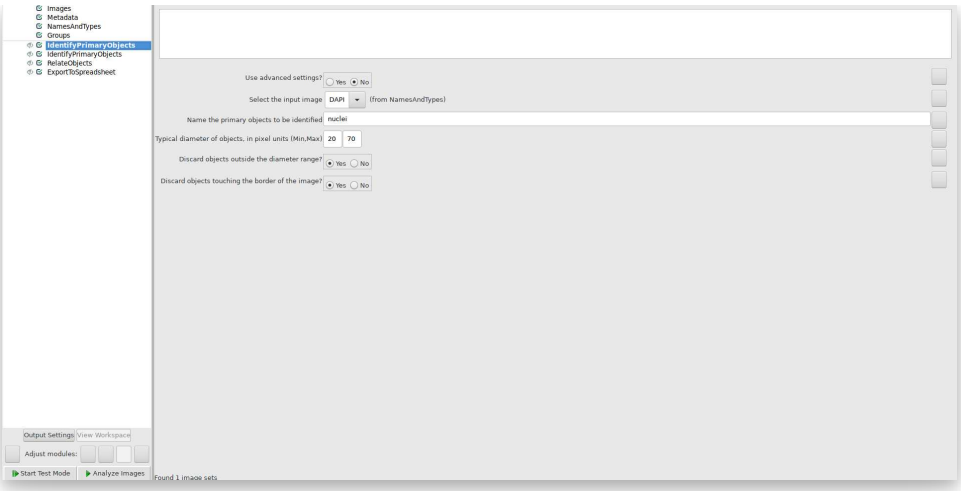

Figure 13. Example of identifying nuclei using Cell Profiler. Diameter range of nuclei will have to be modified based on images used.

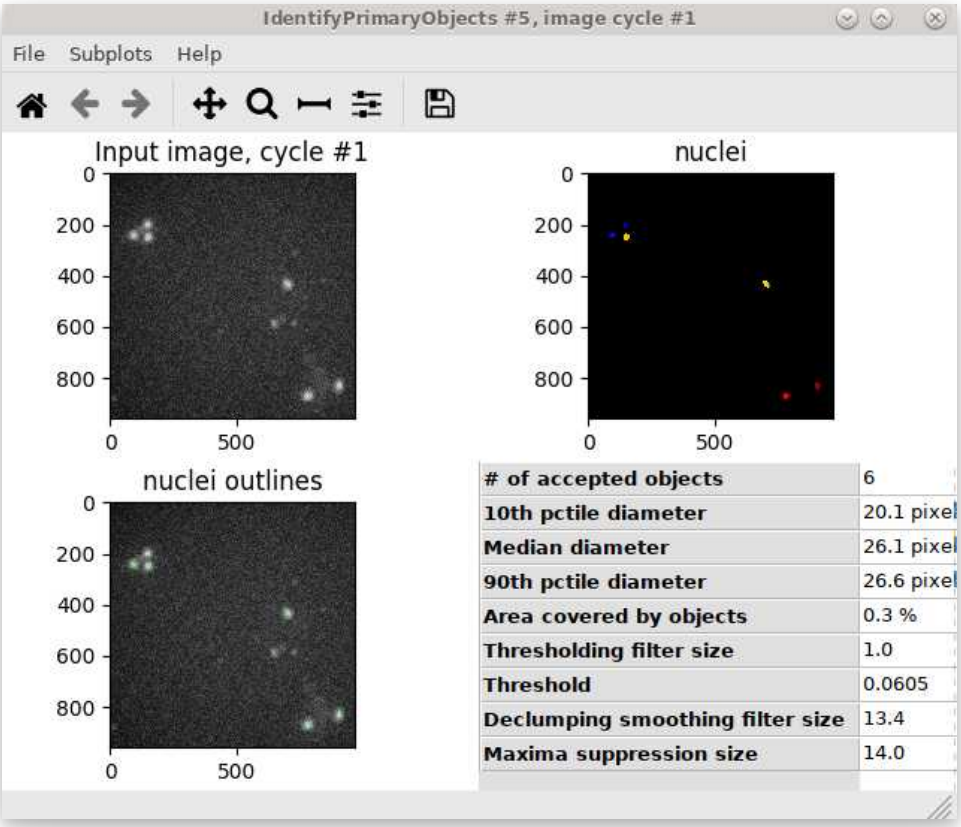

Figure 14. Example image of nuclei being identified as objects using Cell Profiler. Threshold will need to be modified to ensure identification of correct objects from image.

58.4 Use IdentifyPrimaryObjects module to identify foci:

Citation: Rebecca E Brown, Catherine H Freudenreich Proximity Ligation Assay to Detect Transcription-Replication Conflicts in Yeast https://dx.doi.org/10.17504/protocols.io.kxygx93rzg8j/v1

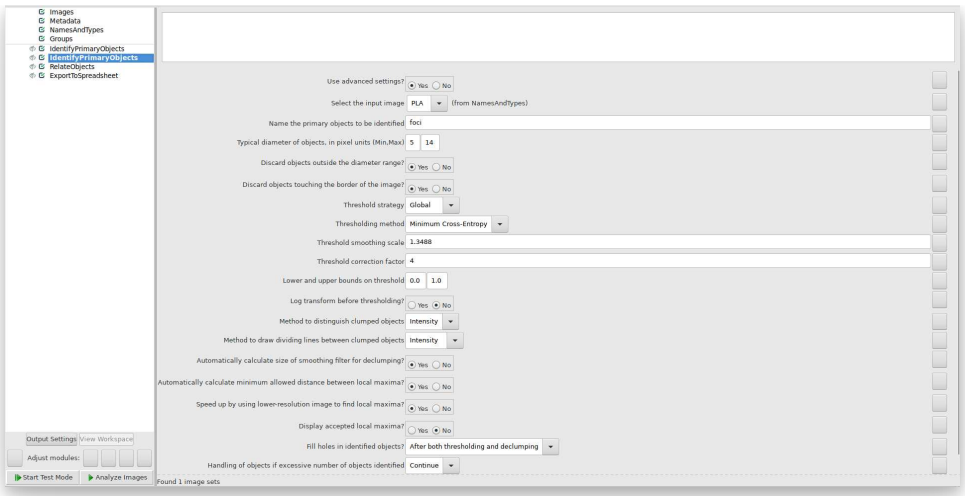

Figure 15. Example of identifying PLA foci using Cell Profiler. Diameter range of foci will have to be modified based on images used.

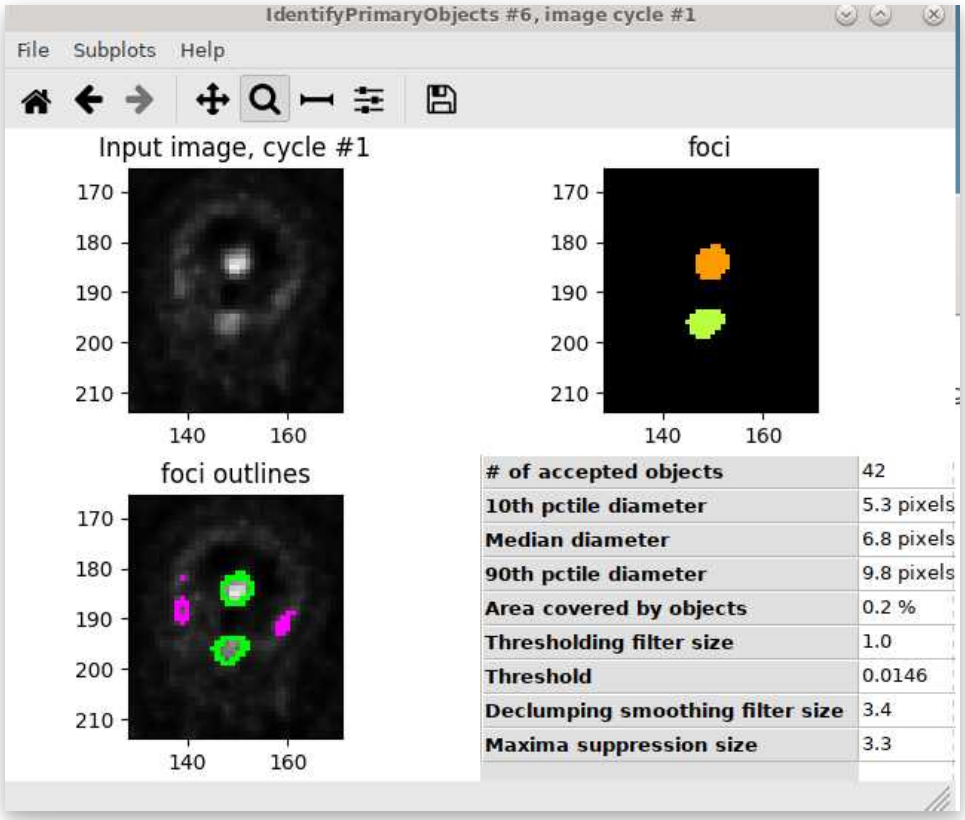

Figure 16. Example image of PLA foci being identified as objects using Cell Profiler. Threshold will need to be modified to ensure identification of correct objects from image.

58.5 Use RelateObjects module to group together nuclei and PLA foci (this will allow for identification of only foci within nuclei):

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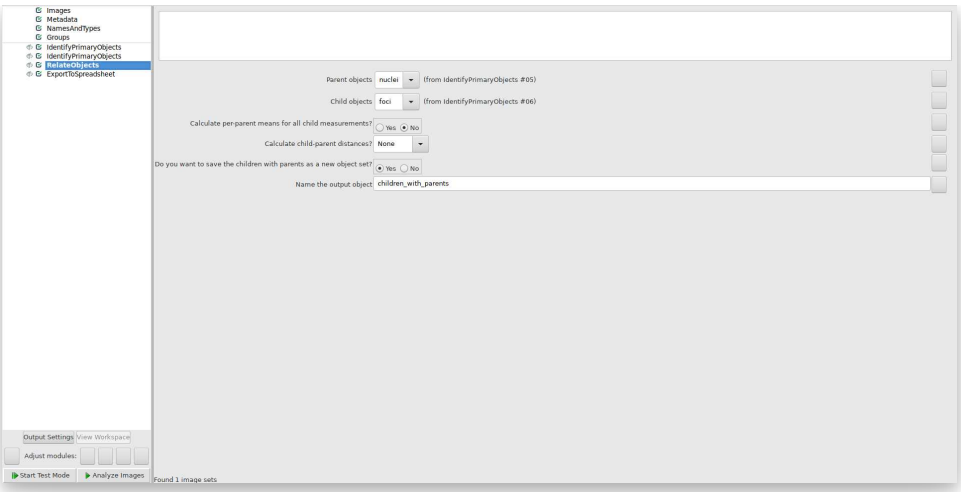

Figure 17. Example of relating foci and nuclei using Cell Profiler.

58.6 Use ExportToSpreadsheet module to export quantification of foci per nucleus:

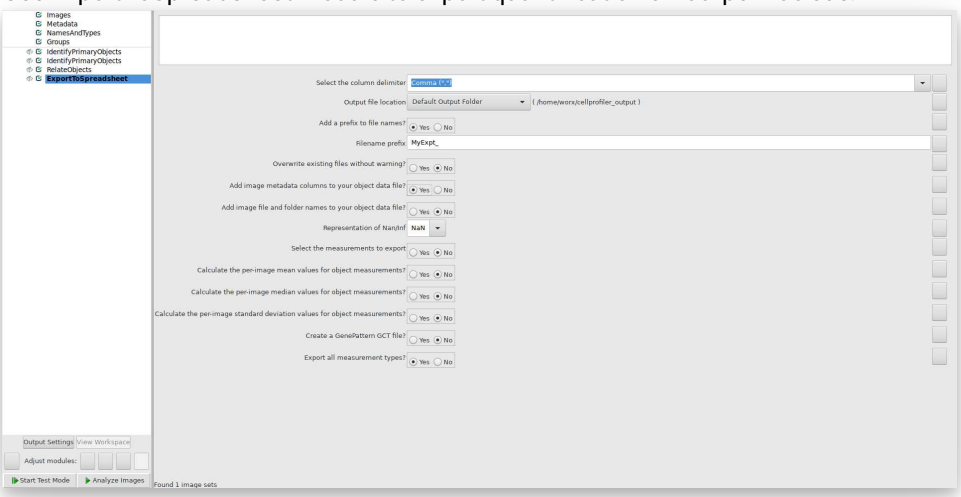

Figure 18. Example of exporting data to spreadsheet using Cell Profiler.

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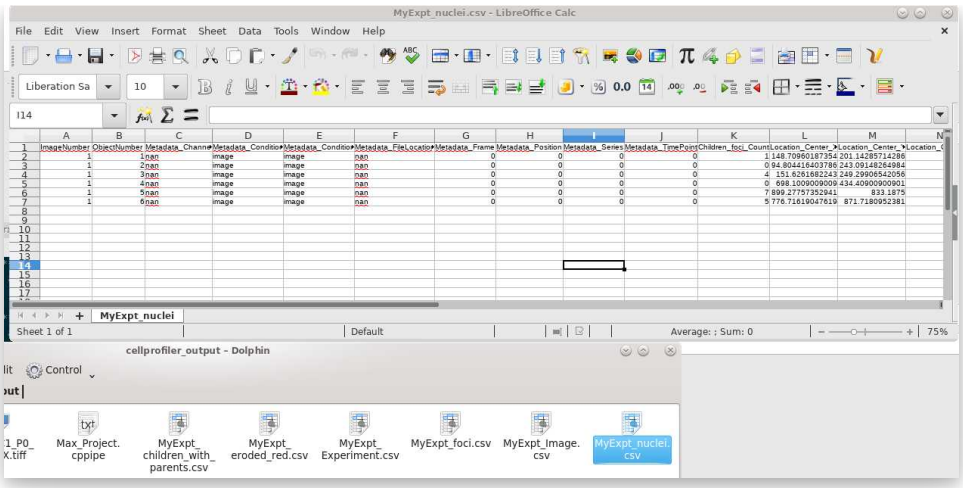

Figure 19. Example of data exported from Cell Profiler. Foci within each nucleus is output in "Children_foci_Count" in column K. The file highlighted in CellProfiler_output window is file that contains desired data.

Example of Data

59 Data shown is from Brown et al. *PLOS Biology* 2022.

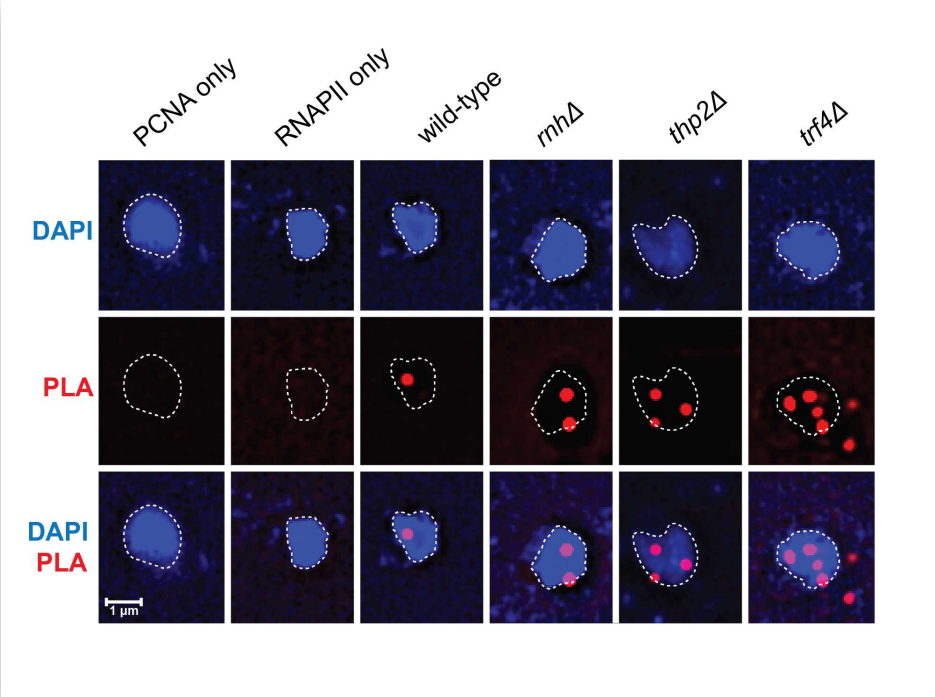

Figure 20. Example of PLA microscopy images.

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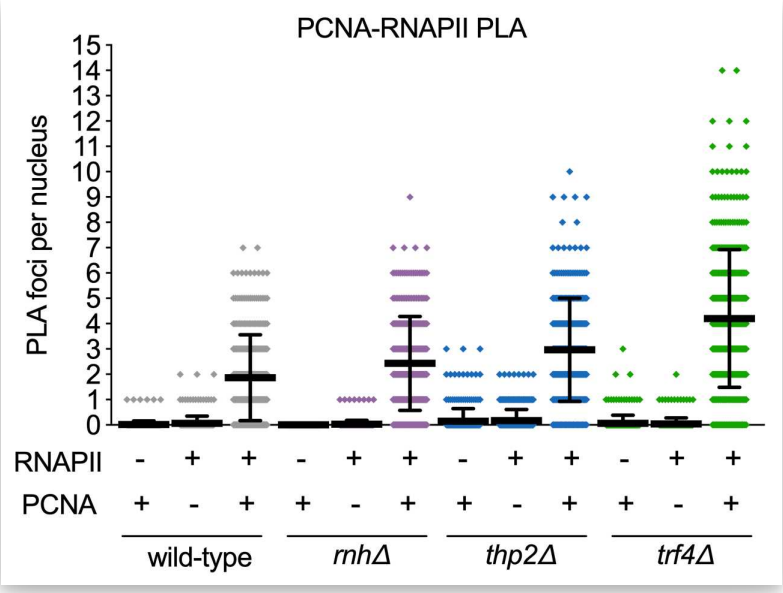

Figure 21. Example quantification of PLA foci in indicated strains (experimental/double antibody condition and single antibody controls shown). Graph shown was made with GraphPad Prism software using the scatter dot plot graph style. Error bars show the mean +/- the standard deviation.

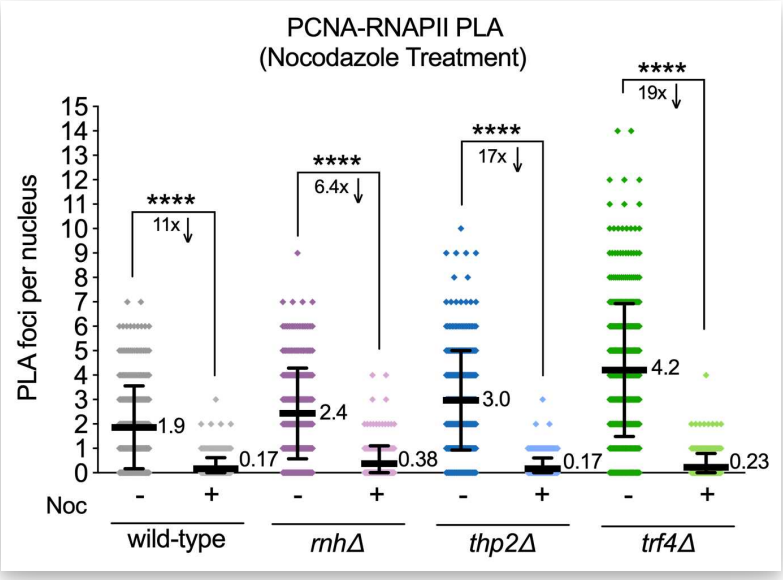

Figure 22. Example quantification of PLA foci in indicated strains (treatment with nocodazole arresting cells in M phase as a way to inhibit replication as a negative control). Statistical analysis was performed using a nonparametric Mann-Whitney test.

Citation: Rebecca E Brown, Catherine H Freudenreich Proximity Ligation Assay to Detect Transcription-Replication Conflicts in Yeast https://dx.doi.org/10.17504/protocols.io.kxygx93rzg8j/v1