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Supporting Information for

Nanoscale regulation of ROS signaling at the plasma membrane tunes the plant response to osmotic stress

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Tables 1 and 2

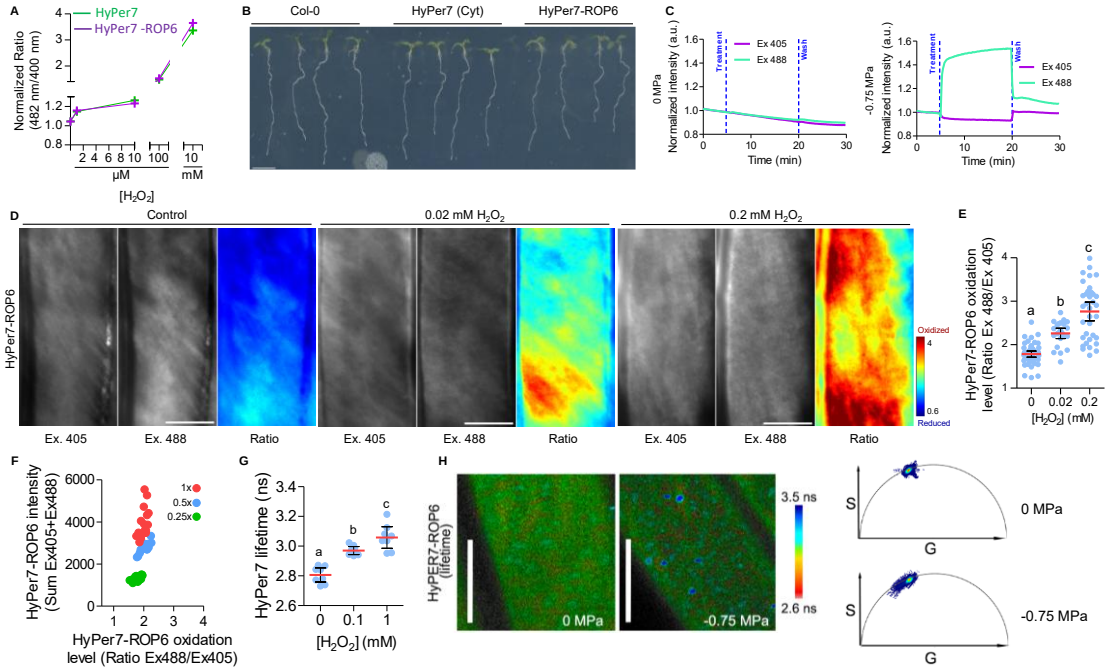

Fig. S1: ROP6 fusion does not affect HyPer7 sensitivity to H_2O_2 nor plant development

A, HyPer7 (green) and HyPer7-ROP6 (purple) ratio in control condition after progressive oxidation with 1, 10, 100 µM and 10 mM exogenous H2O2. B, 5 days old Col-0 and transgenic lines expressing HyPer7 and HyPer7-ROP6, grown on MS/2 vertical petri dishes. C, Typical time course showing normalized intensity of 488 and 405 individual channels in 5 days old plantlets expressing HyPer7-ROP6 upon control condition (0 MPa) or upon osmotic stimulation (-0.75 MPa). D, Intensity-based (Ex405 and Ex488) and ratio-based (Ratio) TIRF images of HyPer7-ROP6-expressing root epidermal cells after application of increasing concentration of exogenous H2O2 for 15 min and its respective quantification in E. F, Ratiometric (488/405) and total intensity (405+488) quantification of in vitro HyPer7-ROP6 at various concentrations (1x, 0.5x, and 0.25x). G, HyPer7-ROP6 lifetime quantification of HyPer7-ROP6 expressing root epidermal cells after application of different H2O2 concentrations. H, Confocal micrographs showing HyPer7-ROP6 lifetime in control condition (0 MPa) or upon osmotic stimulation (-0.75 MPa) and their corresponding phasor plots.

Mean with error bars correspond to the 95% confident interval. For E and G, letters show significant differences according to a one-way ANOVA and Tukey's multiple comparisons test at p<0.05. In E, n cells > 24 from >9 seedlings from 3 independent replicates. In G, n cells >9 from >6 seedlings from 2 independent replicates. For D and H, scale bar is 10 µm.

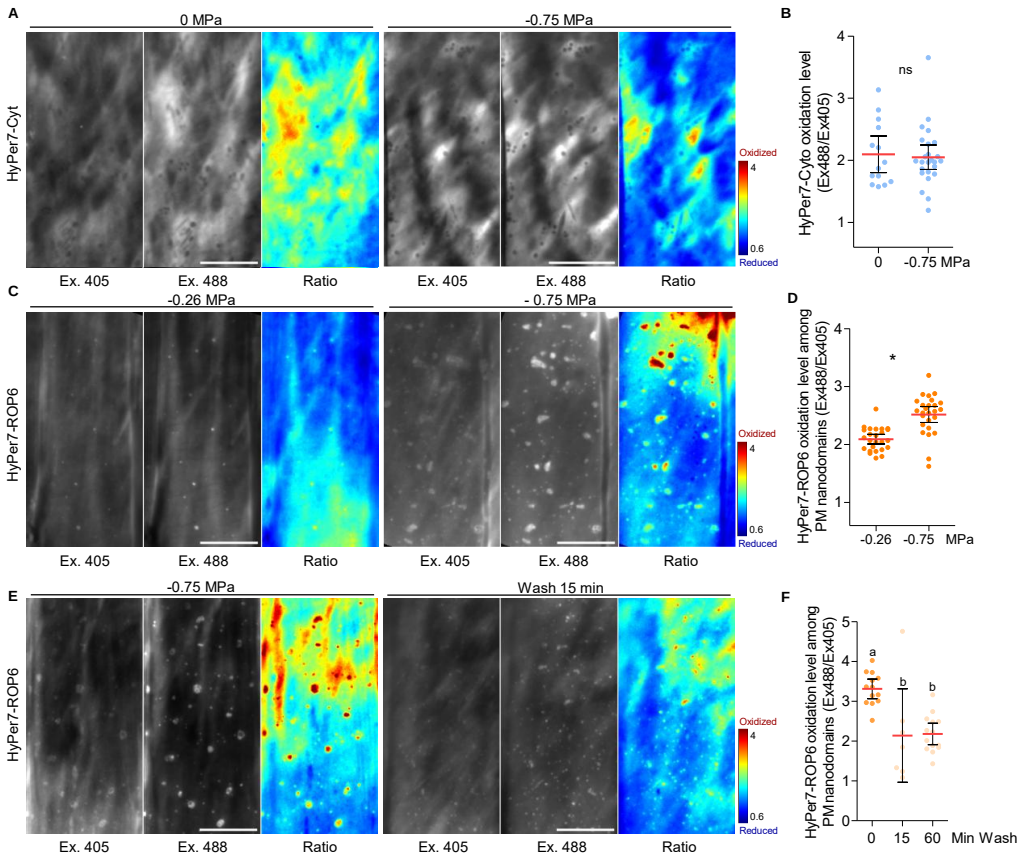

Fig. S2: HyPer7-ROP6 localized oxidation is dependent on the strength of the osmotic signal and is not observed with the soluble HyPer7

A, Intensity-based (Ex405 and Ex488) or ratio-based (Ratio) TIRF images of HyPer7 expressing root epidermal cells in control condition (0 MPa) or after a 15 min osmotic stimulation (-0.75 MPa) and its respective quantification in B. C, Intensity-based (Ex405 and Ex488) and ratio-based (Ratio) TIRF images of HyPer7-ROP6 expressing root epidermal cells after a 15 min mild (-0.26 MPa) or stronger (-0.75 MPa) osmotic stimulation. D, Quantification of mean ratio values within HyPer7-ROP6 nanodomains after 15 min mild (-0.26 MPa) or stronger (-0.75 MPa) osmotic stimulation. E, Intensity-based (Ex405 and Ex488) and ratio-based (Ratio) TIRF images of HyPer7-ROP6 expressing root epidermal cells after 15 min osmotic stimulation (-0.75 MPa) and followed by a 15min wash with control buffer. F, Quantification of mean ratio values within HyPer7-ROP6 nanodomains after 15 min osmotic stimulation (-0.75 MPa) and followed by a 15min or 1h wash with control buffer.

Mean with error bars correspond to the 95% confident interval. For B t-test, p-value=0,7692, n.s. means no significant differences n cells >14 from >3 seedlings from 3 independent replicates. For D t-test, p-value<0,0001 n cells >25 from >3 seedlings from 3 independent replicates. For F, letters show significant differences according to a one-way ANOVA and Tukey's multiple comparisons test at p<0.05. n cells > 7 from >3 seedlings from 2 independent replicates. Scale bar is 10 µm.

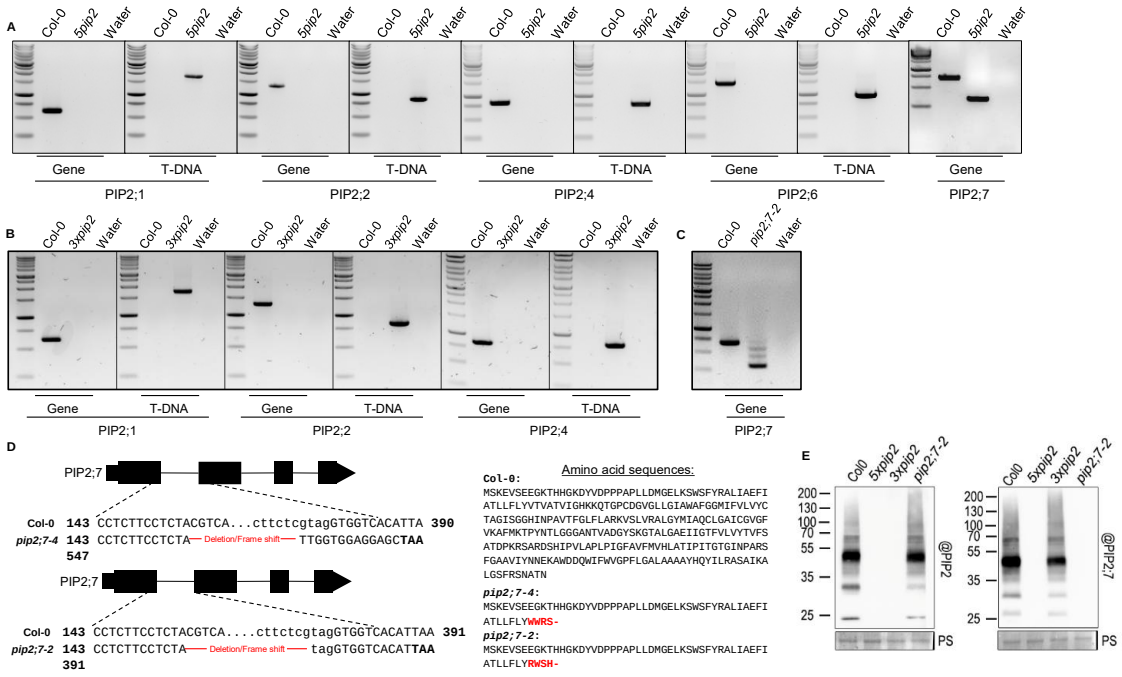

Fig. S3: Genetic characterization of PIP2 mutants

A, PCR electrophoresis for wild-type genes (PIP2;1, PIP2;2, PIP2;4, PIP2;6, PIP2;7) and corresponding T-DNA insertions or CRISPR mutations in Col-0 and the quintuple PIP2 mutant (5xpip2). Same is shown for the triple PIP2 mutant (3xpip2) in B, and for the simple CRISPR mutant pip2;7-2 in C. D, PIP2;7 gene model showing regions in which CRISPR -driven deletions in I coding sequence have occurred for pip2;7-4 (allele in 5xpip2 background) and pip2;7-2 (allele in Col-0). The corresponding amino acid sequences are shown aside. E, Western blot from root protein extract from Col-0, pip2;7-2, 3xpip2 or 5xpip2 and revealed with antibody against PIP2 that recognizes PIP2;1, PIP2;2 and PIP2;3 or against PIP2;7. PS, Ponceau red.

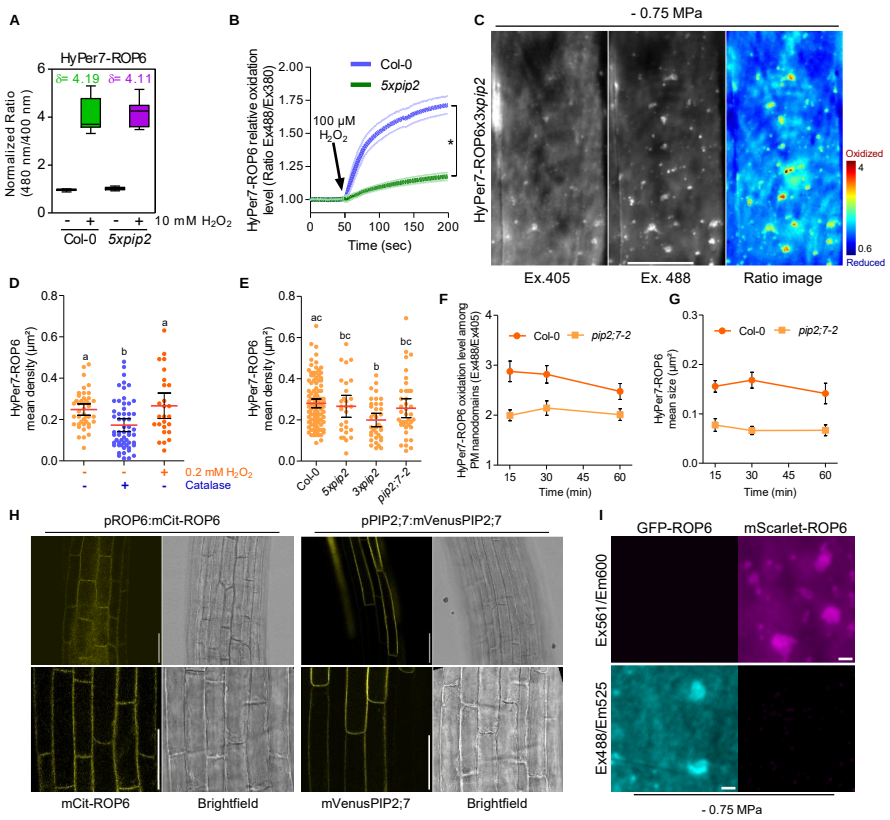

Fig. S4: Characterization of H_2O_2 diffusion, osmotically-induced ROS nano-environments and cellular growth in PIP2 mutant background.

A, Relative oxidation dynamics of HyPer7-ROP6 in Col-0 and 5xpip2 backgrounds in response to application of 10 mM exogenous H2O2. B, Time course ratiometric imaging of 5 days old plantlets expressing HyPer7-ROP6 in Col-0 or 5xpip2 background perfused with 100 µM exogenous H2O2. C, Intensity-based (Ex405 and Ex488) and ratio-based (Ratio) TIRF images of HyPer7-ROP6 expressing root epidermal cells in 3xpip2 background after osmotic stimulation (15min, -0.75 MPa). D, Quantification of HyPer7-ROP6 nanodomains density after osmotic stimulation (15min, -0.75 MPa) cotreated with catalase or exogenous H2O2 (0.2 mM). E, Quantification of HyPer7-ROP6 plasma membrane nanodomains density after osmotic stimulation (15min, -0.75 MPa) in Col-0, 5xpip2, 3xpip2, and pip2;7-2 backgrounds. Quantification of mean ratio values within HyPer7-ROP6 nanodomains, F and size, G after 15 min, 30 min or 60 min osmotic stimulation (-0.75 MPa). H, pROP6:mCit-ROP6 or pPIP2;7:mVenus-PIP2;7 confocal images in root epidermal cells. I, TIRF images of root cells expressing either p35S:GFP-ROP6 or pPIN2:mScarlet-ROP6 after 15 min osmotic stimulation (-0.75 MPa). Images were taken with similar settings than in figure 4 F and G.

*Mean with error bars correspond to the 95% confident interval. For B, *is t-test, p-value=0,0002 at t=200 sec, n > 16 seedlings from 2 independent replicates. For D and E, letters show significant differences according to a one-way ANOVA and Tukey's multiple comparisons test at p<0.05. For D , n cells > 25 from n > 15 seedlings from 3 independent replicates. For E, n cells > 28 from n > 15 seedlings from 3 independent replicates. For C, scale bar is 10 µm. For H, scale bar is 50 µm. For I, scale bar is 1 µm.*

Table S1: Primer table

HyPer7
Cloning primers for domestication

Domestication of HyPer7 in position B3-B4

Reaction 1

Oligo forward	GCGCCGTCTCGCTCGAATGCACCTGGCTAATGAGGA
Oligo reverse	GCGCCGTCTCGTAAGACCCTGAATGCGCTGT

Reaction 2

Oligo forward	GCGCCGTCTCGCTTACCAACACCCTTCAAGG
Oligo reverse	GCGCCGTCTCGCTCACGAACCACCGGTATCGCAGATGAAGC

Domestication of ROP6 in position B5

Oligo forward	GCGCCGTCTCGCTCGTTCGATGAGTGCTTCAAGGTTTATCAAG
Oligo reverse	GCGCCGTCTCGCTCAAAGCTCAGAGTATAGAACAACCTTTCTG

Domestication of p35S (already available)

GB0030 https://goldenbraidpro.com/feature/GB0030/

Domestication of Tnos (already available)

GB0037 https://goldenbraidpro.com/feature/GB0037/

pPIN2:mScarlet3-ROP6

primer name	sequence	purpose
pGG_BK651-F	taagcGAAGACtgCTGCaggcatgcgacgtcgagc	amplification of binary vector backbone from pDGE651 with BbsI restriction sites
pGG_BK651-R	tgacccGAAGACgcGATTgtaacgatgacagagcgttgc	
pGG_ora-651-F	gcgtccgGAAGACgcAATCatactgagaccagtggtatgggg	amplification of orange selection from pAGM 4673 (GoldenGate) with BbsI
pGG_ora-651-R2	gcgtccgGAAGACaaGCAGaccttgagacccacttgagtgg	restriction sites,
pPIN2-SiCEgateA-F	ctgGCTCTTCaCCTagtttcaatagtttcatcctgtttta	amplification of PIN2 promoter with SapI restriction sites for modified GreenGate cloning
pPIN2-SiCEgateA-R	tgaGCTCTTCtGTTtcctttgatttacttttttccggcg	
mScarlet3-SiCEgateB-F	ctgGCTCTTCaACAatatggatagcaccgaggcagtga	amplification of mScarlet3 with SapI restriction sites for modified GreenGa
mScarlet3-SiCEgateB-R	tgaGCTCTTCaGCCggagccaccggagccgccggtggag	
ROP6genomic_SiCEgateC-F	ctgGCTCTTCgGCTccatgagtgcttcaaggtttatcaag	amplification of genomic ROP6 with SapI restriction sites for modified GreenGate cloning
ROP6genomic_SiCEgateC-R	tgaGCTCTTCcTGAtcagagtatagaacaacctttctg	
ROP6-3'UTR_SiCEgateD-F	ctgGCTCTTCtCAGttcaacaagaagaaaggaatcaac	amplification of the 3'-UTR of ROP6 with SapI restriction sites for modified
ROP6-3'UTR_SiCEgateD-R	tgaGCTCTTCgCAGcttaagacaattggtgtgaatcta	
HSPt-pICH41276-F	tgagGAAGACaagcttggatatgaagatgaagatgaaata	amplification of HSP terminator with BbsI restriction site for assembly
HSPt-R	tgagGAAGACaacttatctttaatcatattcc	
HSPt-rbcS E9-F	tgagGAAGACaataaggcttagagctttcgttcgtatca	amplification of rbcS E9 terminator with BbsI restriction site for assembly
rbcS E9t-pICH41276-R	tgagGAAGACaaagcgttgtcaatcaattggcaagtc	together
HSP-rb_SiCEgateE-F	tctcGGTCTCtctgcGGatatgaagatgaagatgaaatatttgg	amplification of double terminator HSP-rbcS E9 with BsaI restriction sites for
HSP-rb_SiCEgateE-R	tctcGGTCTCttagtttgtcaatcaattggcaagtc	

Genotyping primers

pip2;1

Gene

Name	Seq
pip2;1 5'S	CTAACCACTCCAACAGAGAAG
Pip2;1_spec Rev	CCGATTAAATAGTAATGTCAACCAAG

TDNA

Name	Seq
RB T-DNA	GGCTCCTTCAACGTTGCGGTT
Pip2;1_spec Rev	CCGATTAAATAGTAATGTCAACCAAG

pip2;2

Gene

Name	Seq
Pip2;2_spec Fw	CAGTCTTCACAAACACAACAT
Pip2;2_spec Rev	ACAACCATTTTGTAATTGTTGTTATT

TDNA

Name	Seq
Wisc_p745	AAAGTCCGCAATGTGTTATTAAGTTGTC
Pip2;2_spec Rev	ACAACCATTTTGTAATTGTTGTTATT

pip2;4

Gene

Name	Seq
PIP2;4_SM-f	CAAAGCATTCCAAAGTTCTTA
PIP2;4SAIL_1251_G12-r	AAAGCAACATTAATTAAAACCACA

TDNA

Name	Seq
dSPM31	TGAACCGACACTTTTTGAACCGACACTTTTAACATAAG
PIP2;4SAIL_1251_G12-r	AAAGCAACATTAATTAAAACCACA

pip2;6

Gene

Name	Seq
pip2 ;6 int1 s (2;6fa)	CTATCTCATCTGGATCAGCTGG
PIP2;6 ex4 antisens	ATGGAGCTCATGAACGTGGCTCCTGAC

TDNA

Name	Seq
Wisc_p745	AAAGTCCGCAATGTGTTATTAAGTTGTC
PIP2;6 ex4 antisens	ATGGAGCTCATGAACGTGGCTCCTGAC

pip2;7 (Gene and CrispR mutant)

Gene

Name	Seq
Fwd	AAACCCACCATGGAAAAGACTA
Rv	gacttacGGGGATGTGAGAGTC

size Wt 653 bp, deletion 224 bp = fragment of 429 bp

PCR Program:

95°C	2 min	
95°C	1 min	33 cycles
58°C	1 min	
72°C	2 min	
72°C	5 min	
20°C	inf	

CRISPR generation of *pip2;7* mutation:

pip2;7-2 guide RNA target regions:

CTACGTCACCGTCGCTACTG
tgttctcgtcttctcgtagG
TAAGGTCTCTTTGGTGCGAG
ACTCCTTACAACACTCTTGG

pip2;7-2 guide RNA target regions:

CTACGTCACCGTCGCTACTG
ACTCCTTACAACACTCTTGG

Table S2: ImageJ macros used for image processing

Processing of ratiometric TIRF images:

```
// Do not forget to open flat-field 405 and 488.tif images for
illumination correction, If not, the macro will crash at the beginning.
// "BatchProcessFolders" – batch processing of TIFF files and
subfolders.
requires("1.33s");
inputDir = getDirectory("Choose your input folder");
outputDir = getDirectory("Choose your output folder");
if(!File.exists(outputDir)) File.makeDirectory(outputDir);

count = 0;
countFiles(inputDir);
n = 0;
processFiles(inputDir);

function countFiles(inputDir) {
    list = getFileList(inputDir);
    for (i=0; i<list.length; i++) {
        if (endsWith(list[i], "/"))
            countFiles(""+inputDir+list[i]);
        else
            count++;
    }
}

function processFiles(inputDir) {
    list = getFileList(inputDir);
    for (i=0; i<list.length; i++) {
        if (endsWith(list[i], "/"))
            processFiles(""+inputDir+list[i]);
        else {
            showProgress(n++, count);
            path = inputDir + list[i];
            processFile(path);
        }
    }
}

function processFile(path) {
    if (endsWith(path, ".tif")) {
        open(path);
        originalTitle = getTitle(); // store file name
        run("Deinterleave", "how=2");

// Runs Z-projection, illumination correction, and creation of ratio
images.
        // ----- Channel 1 (405 nm) -----
        selectWindow(originalTitle + " #1");
        run("Z Project...", "projection=[Average Intensity]");
        run("32-bit");
        imageCalculator("Divide create 32-bit", "AVG_" + originalTitle
+ " #1", "405.tif");

        // ----- Channel 2 (488 nm) -----
```

```
        selectWindow(originalTitle + " #2");
        run("Z Project...", "projection=[Average Intensity]");
        run("32-bit");
        imageCalculator("Divide create 32-bit", "AVG_" + originalTitle
+ " #2", "488.tif");

        // ----- Ratio (488 / 405) -----
        imageCalculator("Divide create 32-bit",
                        "Result of AVG_" + originalTitle + " #2",
                        "Result of AVG_" + originalTitle + " #1");
        selectWindow("Result of Result of AVG_" + originalTitle + "
#2");
        run("Jet");
        run("Brightness/Contrast...");
        setMinAndMax(0.6, 4.0);
        run("Jet");
        rename("Ratio_" + originalTitle);
        saveAs("tiff", outputDir + originalTitle + "_Ratio");
        saveAs("jpeg", inputDir + "Ratio_" + originalTitle);

        // ----- Save 488 nm projection -----
        selectWindow("Result of AVG_" + originalTitle + " #2");
        saveAs("tiff", outputDir + originalTitle + "_Zpro_AVG_488");
        selectWindow(originalTitle + "_Zpro_AVG_488.tif");
        run("Green");
        rename("488_" + originalTitle);
        saveAs("jpeg", inputDir + "Zpro_AVG_488_" + originalTitle);

        // ----- Save 405 nm projection -----
        selectWindow("Result of AVG_" + originalTitle + " #1");
        saveAs("tiff", outputDir + originalTitle + "_Zpro_AVG_405");
        run("Magenta");
        rename("405_" + originalTitle);
        saveAs("jpeg", inputDir + "ZproAVG_405_" + originalTitle);

        // ----- Cleanup -----
        close("AVG_" + originalTitle + " #2");
        close("AVG_" + originalTitle + " #1");
        close(originalTitle + " #2");
        close(originalTitle + " #1");
    }
}

// Close any remaining open images
while (nImages > 0) {
    selectImage(nImages);
    close();
}
```

Determination of the distribution of the ratio values for pixels among HyPer7-ROP6 nanodomains:

```
//This macro is applied on the segmented image prior being applied on
the ratio image.
setAutoThreshold("Default");
// Automatically determines a threshold level using ImageJ's default
algorithm
```

```
setThreshold(2, 255);
// Explicitly applies a lower threshold of 2 and an upper limit of 255
// to remove background pixels below intensity 2.

setOption("BlackBackground", false);
// Ensures that the background will be white and objects black after
conversion to mask.

run("Convert to Mask");
// Converts the thresholded image into a binary mask (foreground vs.
background).

run("Invert");
// Inverts the binary image so that regions of interest (ROIs) appear
white on a black background.

run("Analyze Particles...", "display add");
// Detects and outlines all particle regions in the binary mask,
// adds them as ROIs to the ROI Manager, and displays their outlines.

close();
// Closes the original image window (ROIs remain in memory)to the apply
the following code on the ratio image.

roiManager("Select All");
// Selects all ROIs currently stored in the ROI Manager.

roiManager("Combine");
// Merges all selected ROIs into a single combined ROI.

run("Histogram", "bins=41 x_min=0.80 x_max=5 y_max=Auto");
// Generates an intensity histogram within the combined ROI
// using 41 bins over the range 0.8–5 (range of the ratio values
observed with HyPer7-ROP6 upon osmotic stimulation).
```

Image processing for ratiometric microscopy:

```
path = File.directory;
// Defines the current working directory as the save path.

run("Split Channels");
// Splits the multi-channel image into two grayscale stacks (C1 and
C2).

// ----- Channel 1: 405 nm -----
selectImage("C1-image_Pos0.tif");
run("Enhance Contrast", "saturated=0.35");
// Slightly enhances contrast by saturating 0.35% of pixels at both
ends of the intensity range.

waitForUser("Draw ROI for BG substraction");
```

```
// Pauses execution to allow manual selection of a background ROI for
background subtraction.

run("BG Subtraction from ROI");
// Subtracts the mean intensity of the selected ROI from the entire
image stack.
wait(50);
// Short pause to prevent macro timing conflicts.
run("32-bit");
// Converts the image to 32-bit precision for quantitative analysis.
setThreshold(300, 1e30);
// Sets a lower threshold of 300 to remove low-intensity background
pixels.
run("NaN Background", "stack");
// Converts all pixels below threshold to NaN values
run("Fire");
// Applies the “Fire” LUT for visualization.
title = "405.tif";
saveAs("TIFF", path + title);
// Saves the processed 405 nm image as a TIFF.
run("Select All");
run("Plot Z-axis Profile");
// Generates an intensity profile along the Z-axis (depth) of the image
stack.

selectImage("405.tif-0-0");
saveAs("TIFF", path + "Plot 405");
// Saves the Z-profile plot as a TIFF file.

// ----- Channel 2: 488 nm -----
selectImage("C2-image_Pos0.tif");
run("Enhance Contrast", "saturated=0.35");
waitForUser("Draw ROI for BG substraction");
run("BG Subtraction from ROI");
wait(50);
run("32-bit");
setThreshold(200, 1e30);
run("NaN Background", "stack");
run("Fire");
title = "488.tif";
saveAs("TIFF", path + title);
run("Select All");
run("Plot Z-axis Profile");
selectImage("488.tif-0-0");
saveAs("TIFF", path + "Plot488");

// ----- Ratio Calculation (488 / 405) -----
imageCalculator("Divide create 32-bit stack", "488.tif", "405.tif");
// Creates a new 32-bit ratio image by dividing 488 nm stack by 405 nm
stack.
wait(50);
// Short delay to ensure processing stability.
selectWindow("Result of 488.tif");
run("Fire");
// Applies “Fire” lookup table to ratio image.
```

```
saveAs("TIFF", path + "Ratio 488 on 405");
// Saves the ratio stack as a TIFF.

run("Select All");
run("Plot Z-axis Profile");
// Generates a Z-axis profile of the ratio image.

selectImage("Ratio 488 on 405.tif-0-0");
saveAs("TIFF", path + "Plot Ratio 488 on 405");
// Saves the ratio profile as a TIFF file.
```