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TITLE:

Visualization and Quantification of TGFβ/BMP/SMAD Signaling under Different Fluid Shear Stress Conditions using Proximity-Ligation-Assay

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KEYWORDS:

fluid shear stress, proximity ligation assay, endothelial cells, SMADs, BMP, TGFβ, atherosclerosis, flow

SUMMARY:

Here, we establish a protocol to simultaneously visualize and analyze multiple SMAD complexes using proximity ligation assay (PLA) in endothelial cells exposed to pathological and physiological fluid shear stress conditions.

ABSTRACT:

Transforming Growth Factor β (TGFβ)/Bone Morphogenetic Protein (BMP) signaling is tightly regulated and balanced during the development and homeostasis of the vasculature system Therefore, deregulation in this signaling pathway results in severe vascular pathologies, such as pulmonary artery hypertension, hereditary hemorrhagic telangiectasia, and atherosclerosis. Endothelial cells (ECs), as the innermost layer of blood vessels, are constantly exposed to fluid shear stress (SS). Abnormal patterns of fluid SS have been shown to enhance TGFβ/BMP signaling, which, together with other stimuli, induce atherogenesis. In relation to this, atheroprone, low laminar SS was found to enhance TGFβ/BMP signaling while atheroprotective, high laminar SS, diminishes this signaling. To efficiently analyze the activation of these pathways, we designed a workflow to investigate the formation of transcription factor complexes under low laminar SS and high laminar SS conditions using a commercially available pneumatic pump system and proximity ligation assay (PLA).

Active TGFβ/BMP-signaling requires the formation of trimeric SMAD complexes consisting of two regulatory SMADs (R-SMAD); SMAD2/3 and SMAD1/5/8 for TGFβ and BMP signaling, respectively) with a common mediator SMAD (co-SMAD; SMAD4). Using PLA targeting different

subunits of the trimeric SMAD-complex, i.e., either R-SMAD/co-SMAD or R-SMAD/R-SMAD, the formation of active SMAD transcription factor complexes can be measured quantitatively and spatially using fluorescence microscopy.

The usage of flow slides with 6 small parallel channels, that can be connected in series, allows for the investigation of the transcription factor complex formation and inclusion of necessary controls.

The workflow explained here can be easily adapted for studies targeting the proximity of SMADs to other transcription factors or to transcription factor complexes other than SMADs, in different fluid SS conditions. The workflow presented here shows a quick and effective way to study the fluid SS induced TGFβ/BMP signaling in ECs, both quantitatively and spatially.

INTRODUCTION:

Proteins of the transforming growth factors beta (TGFβ) superfamily are pleiotropic cytokines with a variety of members, including TGFβs, bone morphogenetic proteins (BMPs), and Activins[1,2]. Ligand binding induces the formation of receptor oligomers leading to the phosphorylation and, thereby, activation of cytosolic regulatory SMAD (R-SMAD). Depending on the sub-family of ligands, different R-SMADs are activated[1,2]. While TGFβs and Activins mainly induce phosphorylation of SMAD2/3, BMPs induce SMAD1/5/8 phosphorylation. However, there are accumulating evidences that BMPs and TGFβs/Activins also activate R-SMADs of the respective other sub-family, in a process termed as 'lateral signaling'[3-8] and that there are mixed SMAD complexes consisting of both, SMAD1/5 and SMAD2/3, members[3,9]. Two activated R-SMADs subsequently form trimeric complexes with the common mediator SMAD4. These transcription factor complexes are then able to translocate into the nucleus and regulate the transcription of target genes. SMADs can interact with a variety of different transcriptional co-activators and co-repressors, leading to the diversification of the possibilities to regulate target genes[10]. Deregulation of SMAD signaling has severe implications in a variety of diseases. In line with this, unbalanced TGFβ/BMP signaling may lead to severe vascular pathologies, such as pulmonary artery hypertension, hereditary hemorrhagic telangiectasia, or atherosclerosis[3,11-14].

Endothelial cells (ECs) form the innermost layer of blood vessels and are, therefore, exposed to shear stress (SS), a frictional force exerted by the viscous flow of the blood. Interestingly, ECs residing at the parts of the vasculature, which are exposed to high levels of uniform, laminar SS, are kept in a homeostatic and quiescent state. In contrast, ECs that experience low, non-uniform SS, e.g., at bifurcations or the aortic arch, are proliferative and activate inflammatory pathways[15]. In turn, sites of dysfunctional ECs are prone to develop atherosclerosis. Interestingly, ECs in these atheroprone areas display aberrantly high levels of activated SMAD2/3 and SMAD1/5[16-18]. In this context, enhanced TGFβ/BMP signaling was found to be an early event in the development of atherosclerotic lesions[19] and interference with BMP signaling was found to markedly reduce vascular inflammation, atheroma formation, and associated calcification[20].

Proximity Ligation Assay (PLA) is a biochemical technique to study protein-protein interactions *in situ*[21,22]. It relies on the specificity of antibodies of different species that can bind target proteins

of interest, allowing highly specific detection of endogenous protein interactions at a single-cell level. Here, primary antibodies have to bind to their target epitope at a distance of less than 40 nm to allow for the detection[23]. Therefore, PLA is greatly beneficial over traditional co-immunoprecipitation approaches, where several million cells are needed to detect endogenous protein interactions. In PLA, species-specific secondary antibodies, covalently linked to DNA fragments (termed Plus and Minus probes), bind the primary antibodies and if the proteins of interest interact, Plus and Minus probes come in close proximity. The DNA gets ligated in the following step and the rolling circle amplification of the circular DNA is made possible. During amplification, fluorescently labeled complementary oligonucleotides bind to the synthesized DNA, allowing these protein interactions to be visualized by conventional fluorescence microscopy.

The protocol described here enables scientists to quantitatively compare the number of active SMAD transcription complexes at atheroprotective and atheroprone SS conditions *in vitro* using PLA. SS is generated via a programmable pneumatic pump system that is able to generate laminar unidirectional flow of defined levels and allows stepwise increases of flow rates. This method allows for the detection of interactions between SMAD1/5 or SMAD2/3 with SMAD4, as well as mixed-R-SMAD complexes. It can easily be expanded to analyze interactions of SMADs with transcriptional co-regulators or to the transcription factor complexes other than SMADs. **Figure 1** shows the major steps of the protocol presented below.

[Place **Figure 1** here]

PROTOCOL:

A graphical overview of the major steps in this protocol is shown in **Figure 1**.

1. Cell culture and fluid shear stress exposure

NOTE: Human umbilical vein ECs (HUVECs) were used as an example to study SS induced interaction of SMADs. The protocol described below can be applied to every SS responsive cell type.

1.1 Coat 6-channel slide with 0.1% porcine skin gelatin in PBS for 30 min at 37 °C.

1.2 Seed HUVECs in pre-coated 6-channel slides at a density of 2.5 x 10^6 cells per mL in 30 µL of M199 full medium.

NOTE: For further information on how to seed cells in the flow slide, see reference[24].

1.3 Let cells adhere for 1 h at 37 °C in a humidified incubator.

1.4 Add 60 µL of pre-warmed M199 full medium to each of the reservoirs.

1.5 Culture for 2 days, with a gentle medium exchange once a day, at 37 °C in a humidified incubator.

1.5.1 Aspirate the reservoirs completely, add 120 µL of pre-warmed M199 full medium in one of the reservoirs, and aspirate from the other side.

1.5.2 Add 60 µL of pre-warmed M199 full medium to both reservoirs.

1.6 Assemble and start the flow set-up as detailed in the reference[25].

1.6.1 Mount tubing on fluidic units. Here, silicone tubing with a diameter of 0.8 mm and 1.6 mm are used to apply shear stress of 1 dyn/cm^2 and 30 dyn/cm^2, respectively.

NOTE: The material and tubing length should remain constant, as changes could influence the resulting shear stress. In general, other combinations of pump systems and tubing can be used, as long as the resulting shear stress is known, and the pump creates a steady laminar flow.

1.6.2 Fill the reservoirs with an appropriate amount of pre-warmed M199 full medium (minimum 10 mL).

1.6.3 Connect fluidic units with the tubing to the pump system and perform a pre-run without cells to equilibrate the medium and to remove any remaining air[25].

1.6.4 Serially connect the single channels on the 6-channel slide to one another by using serial connection tubing. The first and the last channel on the slide will be connected to the tubing assembled in 1.6.1 (see **Figure 1A** for a scheme). Be careful not to introduce any air into the system as this could severely harm the cells. Further information on the serial connection can be found in reference[26].

1.6.5 For the exposure of cells to high levels of shear stress (>20 dyn/cm^2), use a ramp phase, i.e., increase the shear stress stepwise with adaptation phases. Steps can be set in increments of 5 dyn/cm^2 per 30 min.

2. Fixation

2.1 Detach slides from the pumps after the fluid SS exposure. Use clamps on the tubing when detaching, to avoid the medium spill in the incubator.

2.2 Immediately transfer flow slides on ice, while the remaining tubing is detached sequentially. When removing the tubing from reservoirs, the reservoir on the other side should be kept closed with a finger to avoid trapping air bubbles in the channel. This might interfere with fixation steps.

2.3 Keeping the cells on ice, aspirate the medium carefully from the reservoirs but not from the channel where the cells reside. Subsequently, wash samples with cold sterile PBS (4 °C) with three

times the channel volume (90 µL). Add PBS in one reservoir and aspirate carefully from the other reservoir. Repeat this step in each of the 6 channels per slide.

NOTE: For all washing and incubation steps the respective solution is added in one of the reservoirs which leads to an exchange of solutions in the channel. To allow for complete substitution of solutions in the channel, the excess solution is then aspirated from the other reservoir. Solution on the top of the cells in the channel is not removed. Cells should not dry at any time. Therefore, it is important to wash carefully without any air bubble insertion into the slides.

2.4 Fix the cells by adding 90 µL of buffered 4% PFA solution in the same reservoir where the PBS was added beforehand and similarly aspirate the liquid from the other reservoir. Repeat this step in each channel in each slide. After the addition of PFA solution, transfer the samples from ice to room temperature (RT) and incubate for 20 min.

CAUTION: PFA is toxic and should be handled carefully. Use gloves and work under a fume hood.

2.5 Wash cells 3x with PBS (RT) by adding it in one reservoir and aspirating carefully from the other reservoir. Empty just the reservoirs, ensuring not to dry out the channel. Repeat this step for each of the 6 channels per slide.

2.6 Quench the PFA-fixation by adding 90 µL of ambient 50 mM ammonium chloride in PBS in one of the reservoirs. Aspirate excess PFA solution from the other reservoir. Repeat for each channel in the slide. Incubate the samples for 10 min at RT.

2.7 Wash as described in step 2.5.

NOTE: At this point, the samples may be stored at 4 °C overnight, or the protocol can be immediately continued with blocking and primary antibody incubation (see step 3).

3. Blocking and primary antibody incubation

3.1 To permeabilize the cells, add 90 µL of 0.3% Triton-X-100 in PBS in an emptied reservoir, and aspirate from the other reservoir for each channel. Incubate for 10 min at RT.

3.2 Wash as described in step 2.5.

3.3 Add 90 µL of sterile PLA blocking solution in one reservoir of a channel and aspirate from the other side. Repeat this step for each channel. Block for 1 h at 37 °C in a humidified chamber.

3.3.1 To make a humidified chamber, use a 10 cm dish with wet tissue sealed with wax film and place the dish in the incubator. Alternatively, other humidity chamber formats can be used that supply a humid atmosphere.

NOTE: Alternatively, self-made blocking solution can be used (e.g., 3% (w/v) BSA in PBS, sterile filtered).

3.4 Prepare primary antibodies (1:100) in PLA antibody diluent. Prepare 30 µL of the solution per channel. Add both primary antibodies simultaneously and vortex.

NOTE: Alternatively, self-made antibody diluent can be used (e.g., 1% (w/v) BSA in PBS). Antibodies used here are combinations of SMAD1-SMAD2/3, SMAD2/3-SMAD4 and phospho-SMAD1/5-SMAD4. Detailed information can be found in the **Table of Materials**.

3.5 Before the application of primary antibodies, aspirate the blocking solution from the reservoirs and, also, carefully from the channel. Pipette 30 µL of the primary antibody solution immediately into the empty channel by tilting the channel while adding the solution.

NOTE: Perform the removal of the blocking solution and addition of the antibody solution channel-by-channel to ensure cells do not dry out in between.

3.6 Incubate samples with the primary antibodies overnight in humidified chambers at 4 °C.

NOTE: The incubation can also be performed for 1 h at room temperature, if interested in continuing with the following steps on the same day.

4. PLA probe incubation

NOTE: For all steps in section 4.1-7.3, the washing buffers A and B are stored at 4 °C and need to be warmed to RT prior to the use.

4.1 Dilute PLA-probes (+)-mouse and (-)-rabbit to 1:5 in PLA antibody diluent (or 1% BSA) solution. Prepare 30 µL per channel.

4.2 Wash samples 2x for 5 min using 90 µL of the wash buffer A at RT by adding it in one of the reservoirs and aspirating carefully from the other reservoir. Repeat this step for each of the 6 channels per slide.

4.3 Aspirate the wash buffer A carefully and add 30 µL of PLA probe solution (prepared in step 4.1), similar to the addition of primary antibodies in step 3.5.

4.4 Incubate samples for 1 h at 37 °C in a humidified chamber.

5. Ligation

5.1 Wash samples 2x for 5 min using 90 µL of the wash buffer A at RT, as described in 4.2.

5.2 Prepare a 1:5 dilution of the ligation buffer in deionized water. Use this buffer to dilute the ligase enzyme to 1:40 (on ice). Use 30 µL per channel.

5.3 Aspirate the wash buffer A completely and add the ligation solution as described in 3.5.

5.4 Incubate samples for 30 min at 37 °C in a humidified chamber.

6. Amplification

6.1 Wash samples 2x for 2 min using 90 µL of wash buffer A at RT, as described in 4.2.

6.2 Prepare the amplification buffer by diluting it 1:5 in deionized water and use it to dilute the polymerase enzyme to 1:80 (on ice). Protect from light. Prepare 30 µL per channel.

6.3 Aspirate the wash buffer A completely and immediately add the prepared amplification solution into the empty channel, as described in 3.5. Incubate samples for 100 min at 37 °C in a humidified chamber.

7. Mounting

7.1 Wash samples 2x for 10 min using 90 µL of Wash Buffer B at RT as described in 4.2. Add DAPI (1:500) from 1 mg/mL stock solution (in deionized water) in the first wash to stain nuclei. Do not dry the channel.

7.2 Dilute the wash buffer B in deionized water (1:10) and wash 1x with 90 µL of 0.1x buffer B solution as described in 4.2.

7.3 Aspirate the wash buffer B completely and immediately add 2-3 drops of the liquid mounting medium into one reservoir. Distribute it in the channel by tilting the slide. Store samples at 4 °C in a humidified environment until imaging.

8. Image acquisition

8.1 Acquire images using a fluorescence microscope. Ensure that the respective filters fitting the fluorescent PLA probes are available.

NOTE: It is beneficial to make use of a confocal microscope, if possible, as obtained PLA spots are more defined. This also supports further image processing and data analysis.

9. Image analysis and quantification using ImageJ/FIJI

9.1 Process exported images (.tiff) with an image processing program, such as ImageJ[27].

NOTE: All scripts used within this study and that are necessary for the automatic counting of cellular, nuclear, and all PLA events (per cell) can be found in a GitHub repository: https://github.com/Habacef/Proximity-Ligation-Assay-analysis. Perform statistical analysis using any suitable program or tool.

REPRESENTATIVE RESULTS:

We have previously used PLA to detect interactions of different SMAD proteins[3] and analyzed shear stress induced changes in SMAD phosphorylation[28].

Here, both methods were combined with the protocol described above. HUVECs were subjected to shear stress of 1 dyn/cm^2 and 30 dyn/cm^2 and analyzed for interactions of SMAD transcription factors. We show that, when compared to the high shear stress (30 dyn/cm^2), the low shear stress (1 dyn/cm^2) leads to a significant increase in SMAD1-SMAD2/3 interactions, the so called mixed-SMAD complexes in both, the cytosol, and nuclei of analyzed cells (**Figure 2A**, lower panel). PLA events are visible as distinct spots in both samples, and one can distinguish between cytosolic and nuclear events with reference to DAPI staining (**Figure 2A**, upper panel). In contrast, antibody controls, where only one of both primary antibodies but still both PLA probes were incubated, showed negligible numbers of PLA signals (**Figure 2B**). Thus, it can be concluded that the experiment was successful.

[Place **Figure 2** here]

To show how different concentrations of antibodies change PLA results, the same experiment was performed with a 1:50 instead of a 1:100 dilution of antibodies. Under these conditions, twice the amount of antibody result in more than four-fold higher PLA signals per cell (compare **Figure 2A** and **Figure 2C**). The difference in signals between 1 dyn/cm^2 and 30 dyn/cm^2 decreases when a higher antibody concentration is used and statistical significance is lost for the total and cytosolic PLA events (**Figure 2A**, lower panel; **Figure 2C**, lower panel). This might be due to the signal coalescence and problems of distinguishing PLA events. If such accumulation of signals occurs, antibody concentrations should be decreased.

Furthermore, we showed that self-made buffers for blocking and antibody dilution can be used as an alternative for commercial buffers included in *in situ* PLA kits. PLA events per cell were compared for SMAD1-SMAD2/3 (**Figure 3A** versus **Figure 3D**), SMAD2/3-SMAD4 (**Figure 3B** versus **Figure 3E**) and pSMAD1/5-SMAD4 (**Figure 3C** versus **Figure 3F**) complexes under 1 dyn/cm^2 and 30 dyn/cm^2 using either commercial solutions (**Figure 3A-C**) or self-made BSA/PBS based solutions (**Figure 3D-F**). Quantifications for both commercial and self-made diluent/ blocking solutions show the same trend of PLA signals in cytosolic and nuclear areas. However, the total number of PLA events per cell is higher when using commercial solutions (**Figure 3A-C**, lower panels versus **Figure 3D-F**, lower panels).

[Place **Figure 3** here]

We also included an example for a failed PLA experiment. Here, a combination of SMAD2/3-SMAD4 antibodies was used where the SMAD4 antibody was not suited for performing immunofluorescence experiments. When compared to single antibody controls, no increase in spots in either the 1 dyn/cm^2 or 30 dyn/cm^2 samples was observed (**Figure 4A** versus **Figure 4B**). As the formation of SMAD2/3-SMAD4 complexes is induced by shear stress (see **Figure 3B,E**), it can be concluded that this PLA experiment was unsuccessful. This highlights the importance of choosing the correct antibody combinations to detect PLA events, as orientation and distance of bound antibodies might be crucial for successful annealing of the oligonucleotide probes.

[Place **Figure 4** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic representation of the protocol described. (**A**) Cells seeded in 6-channel slides are exposed to shear stress with a pneumatic pump system. (**B**) Fixed cells are used for PLA experiment or for control conditions. (**C**) Images of PLA experiments are acquired with a fluorescence microscope and are analyzed using ImageJ analysis software.

Figure 2: SMAD2/3-SMAD1 PLA comparing different antibody concentrations. (**A**) Confocal fluorescence images and quantification of SMAD2/3-SMAD1-PLA in HUVECs exposed to 24 h of indicated SS levels. Antibodies dilution ratio: 1:100. (**B**) Confocal images of single antibody controls for PLA in **A**. (**C**) Confocal fluorescence images and quantification of SMAD2/3-SMAD1-PLA in HUVECs exposed to 24 h of indicated SS levels. Antibodies dilution ratio: 1:50. Scale bars for **A**-**C**: 20 µm and 10 µm in zoom in. Dyne equals dyn/cm^2. Antibodies used were rabbit anti-SMAD2/3 and mouse anti-SMAD1 (see **Table of Materials**). For each condition 5 random areas along the center of the flow channel were used for image acquisition. N=1 biological replicate.

Figure 3: PLA experiment comparing commercial and self-made antibody buffers. Confocal images (upper part of each panel) and quantification (lower part of each panel) of different SMAD-SMAD PLAs in HUVECs exposed to 24 hours of indicated shear stress levels. (**A-C**) Commercial buffers (see **Table of Materials**). (**D-F**) Self-made buffers (3% BSA in PBS for blocking, 1 % BSA in PBS for antibody dilution). All primary antibodies were diluted 1:100. Scale bar, 20 µM (10 µM for zoom in). Statistical significance was calculated with two-sided t-Test. ns – non-significant, *P<0.05, **P<0.01, ***P<0.001. Values are depicted as mean + SEM. Dyne equals dyn/cm^2. Antibodies used were rabbit anti-SMAD2/3, mouse anti-SMAD1, rabbit anti-phospho SMAD1/5 and mouse anti-SMAD4 (see **Table of Materials**). For each condition 5 random areas along the center of the flow channel were used for image acquisition. N=1 biological replicate.

Figure 4: Example for failed PLA experiment. Confocal images, scale bar: 20 µm and 10 µm in zoom in. (**A**) SMAD2/3-SMAD4 PLA. (**B**) Single antibody controls. Antibodies used were mouse anti-SMAD2/3 and rabbit anti-SMAD4 (see **Table of Materials**). Dyne equals dyn/cm^2. For each condition 5 random areas along the center of the flow channel were used for image acquisition. N=1 biological replicate.

DISCUSSION:

The PLA based protocol described here offers an efficient way to determine close proximity of two proteins (e.g., their direct interaction) in EC exposed to shear stress with quantitative and spatial resolution. By using flow slides with multiple parallel channels, several different protein interactions can be examined at the same time in cells under identical mechanical conditions. In contrast, custom-build flow chamber systems often make use of a single channel that is built around a glass coverslip, which would allow only a single PLA experiment without the necessary controls per slide and pump. Although this protocol focuses on the detection of SMAD interactions, it can be adapted to detect any other protein interactions. However, analysis of results must be done carefully as two proteins may also reside in close proximity without forming complexes. If definite statements on interactions of proteins are desired, PLA experiments should be complemented with additional methods such as co-immunoprecipitations. Additionally, PLA cannot be used to detect the protein-protein interaction in live cells since samples need to be fixed for subsequent antibody binding and DNA amplification steps.

For successful detection of protein interactions by PLA, the most critical step is to choose a combination of primary antibodies that fulfil several criteria: (1) The antibodies detecting the individual protein-partners must be generated in different species (e.g., mouse or rabbit) as the secondary antibodies are species-specific; ideally, antibodies were already successfully tested by conventional immunofluorescence microscopy; (2) the distance between epitope bound antibodies should be <40 nm[23]; (3) as the affinities for antibody-epitope binding might differ, the final concentration of used antibodies has to be adjusted for each experimental setting, as shown here (**Figure 2A**, lower panel versus **Figure 2C**, lower panel). Therefore, if very few but specific PLA events are detected, it may be worth increasing the amount of antibody used. However, this must be carefully titrated to avoid oversaturation and unspecific binding events. Also, antibody concentration used in control samples must match the concentration used in the PLA samples.

As for any other biochemical assay, suitable controls are indispensable in PLA. Unspecific binding of the used antibodies might, for example, lead to PLA signals originating from just one primary antibody. Therefore, essential antibody controls for PLA experiments should include addition of only one of the two primary antibodies but both PLA Probes (Plus and Minus). Furthermore, controls with no antibodies added can be used to determine unspecific binding of the PLA probes to the sample. In general, those technical controls should yield no to a very few PLA signals. If several signals are observed, concentration of the primary antibody used, and its specificity should be reconsidered. In addition, it is useful to include a positive biological control, if possible. In the protocol described above, this could be stimulation with a BMP ligand that is known to induce phosphorylation of SMADs and, therefore, trimeric complex formation with SMAD4.

For PLA experiments, it is normally recommended to use cells that are 50-70% confluent, since this simplifies the penetration of reagents. However, when performing experiments with ECs, we would strictly argue against this, except if semi-confluency is part of the experimental set-up. *In vivo* ECs form monolayers with tight cell-to-cell junctions, which are essential for EC homeostasis and mechano-transduction[29]. Therefore, experiments on non-confluent ECs could give rise to false results. Furthermore, non-confluent cells are more prone to detach from flow channel slides

if high levels of shear stress are used during the experiment. We advise to seed cells (2-2.5 x 10^6 cells/ mL, see protocol step 1.2) two to three days in advance of experimental start as EC monolayers need time to form and develop mature junctions. Therefore, we would not recommend seeding a higher number of cells (>2.5 x 10^6 cells/mL) in flow channels just one day before the experiment to achieve a confluent monolayer.

Although various liquid mounting media containing DAPI exist it is worth to include a separate DAPI staining step and mount the cells with liquid mounting medium without DAPI, at least if polymer-based flow slides are used. This prevents heavy background signals during image acquisition. Images should be acquired from positions in the channel center rather than the edges since shear stress is inhomogeneous at the channel walls. We recommend to take at least 5-10 images per biological replicate and condition of random areas along the center region. 3 or more biological replicates are normally used to gain statistical relevance. For image analysis we advise to use the ImageJ/FIJI macro function. In the protocol above, we mentioned an ImageJ macro that is suitable to count cytosolic and nuclear PLA events based on DAPI staining. Users should be aware that parameters like particle size need to be adjusted depending on nuclear size or bigger/smaller PLA dots. The macro saves the threshold PLA images and masks that should be compared to raw images to evaluate specificity of PLA signal detection.

In conclusion, the method presented here allows rapid and efficient spatial and quantitative analysis of transcription factor complex formation at atheroprotective and atheroprone SS conditions. It will allow scientists to further decipher the impact of SMAD complex formation in atherogenesis and vascular disease in general. It can, furthermore, be adapted to investigate the consequences of proximity of different proteins in those vascular pathologies.

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DISCLOSURES:

The authors declare no conflict of interest.

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Figure 1

Click here to access/download;Figure;Fig1.png

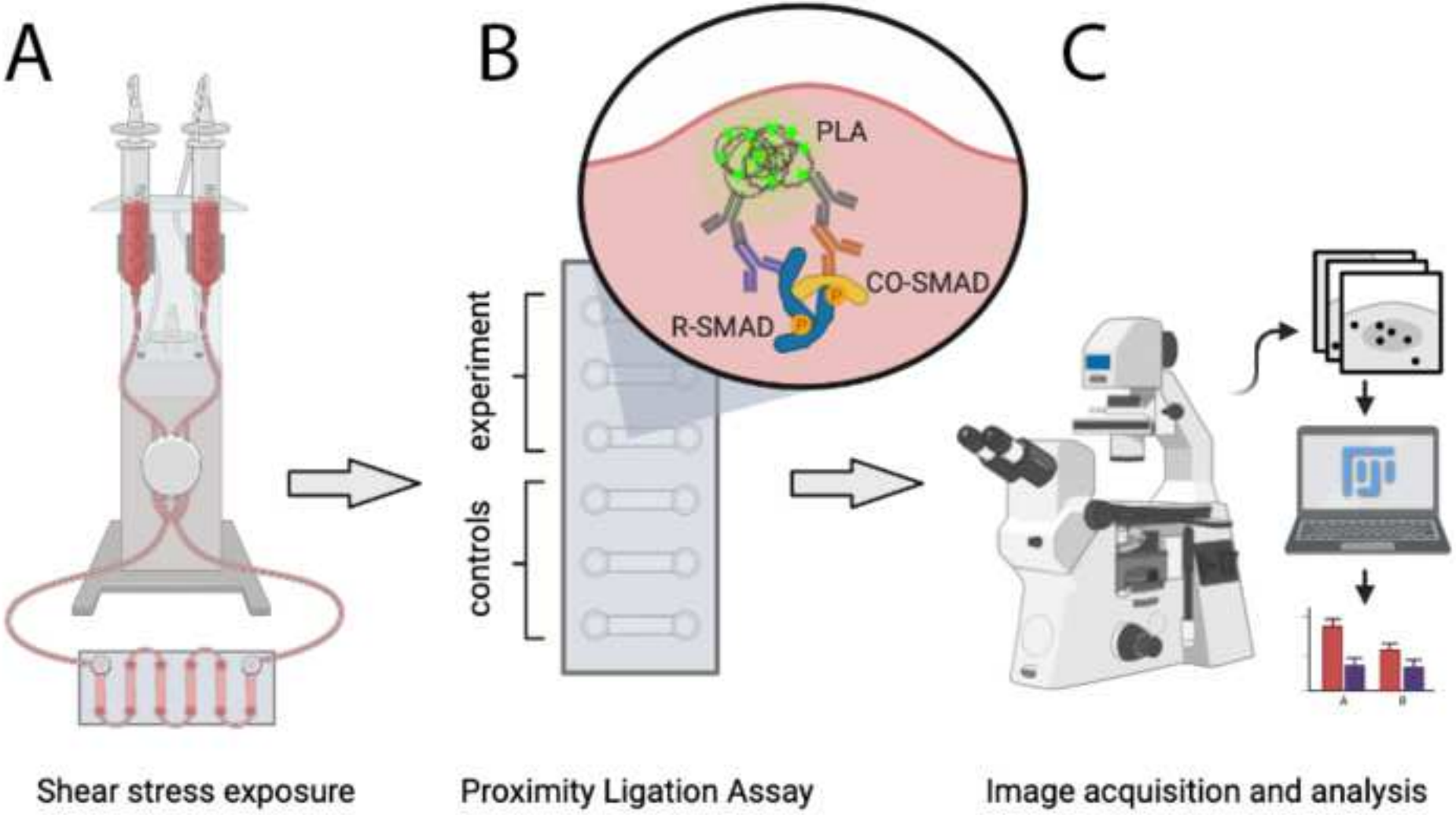


Figure 2

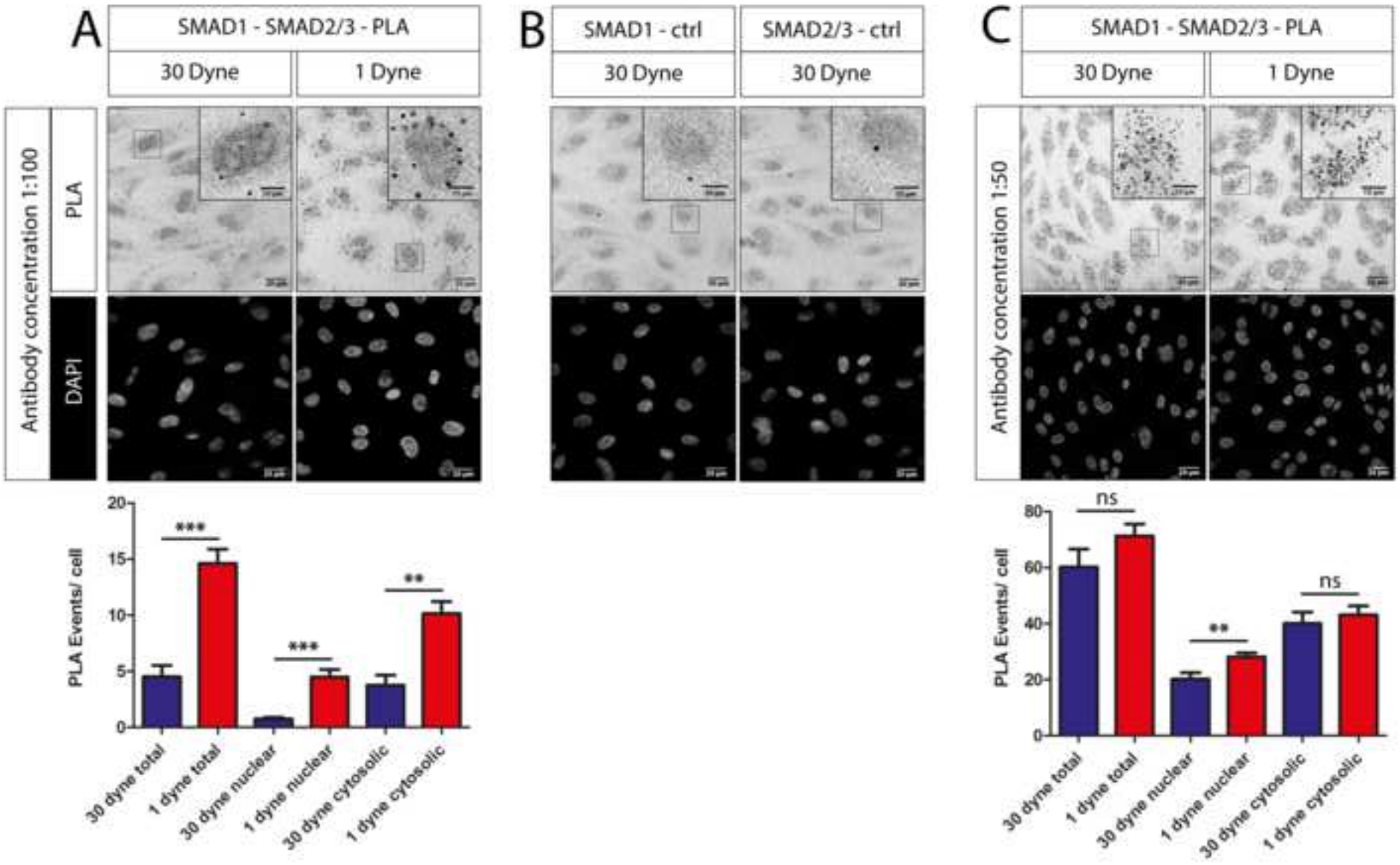


Figure 3

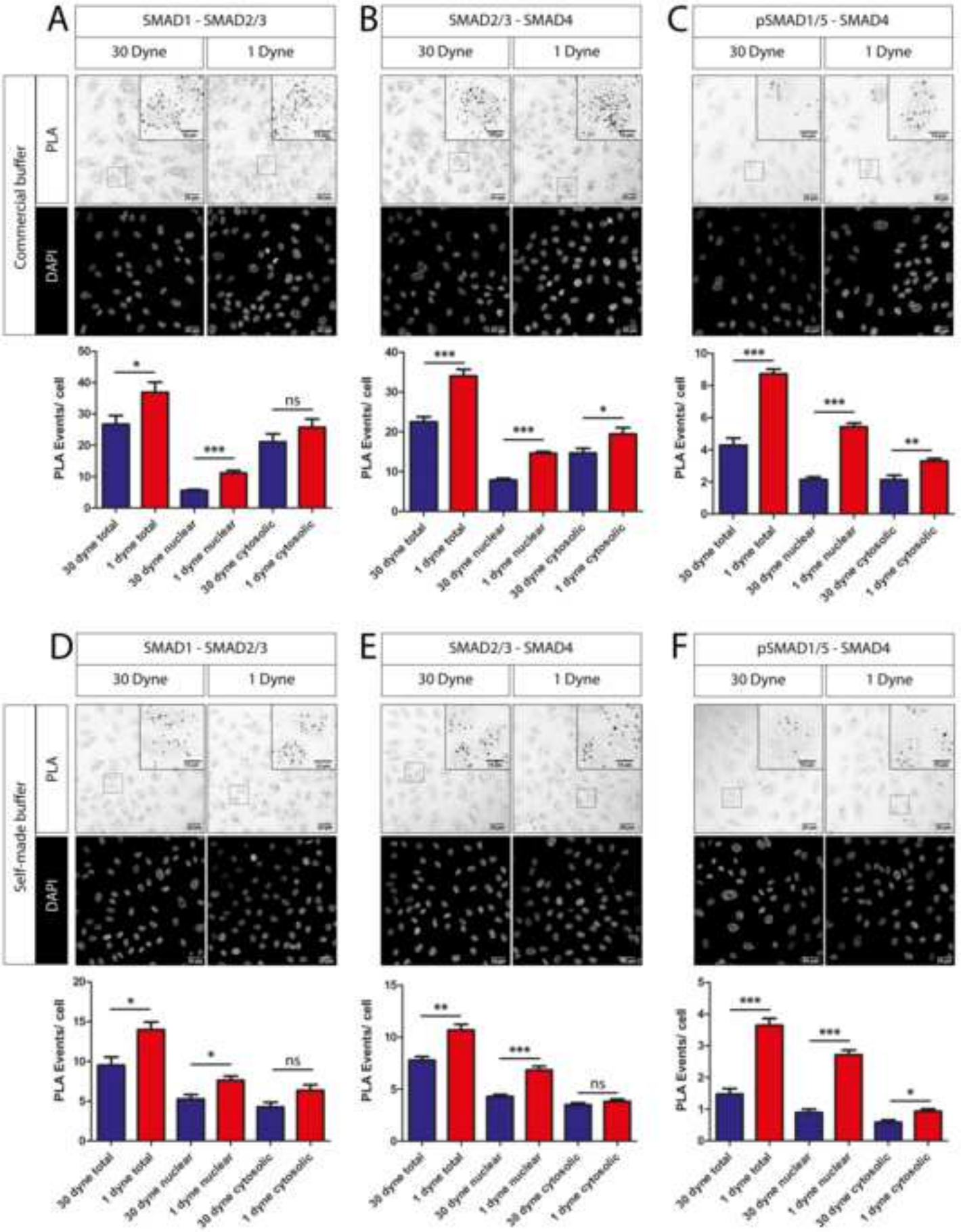
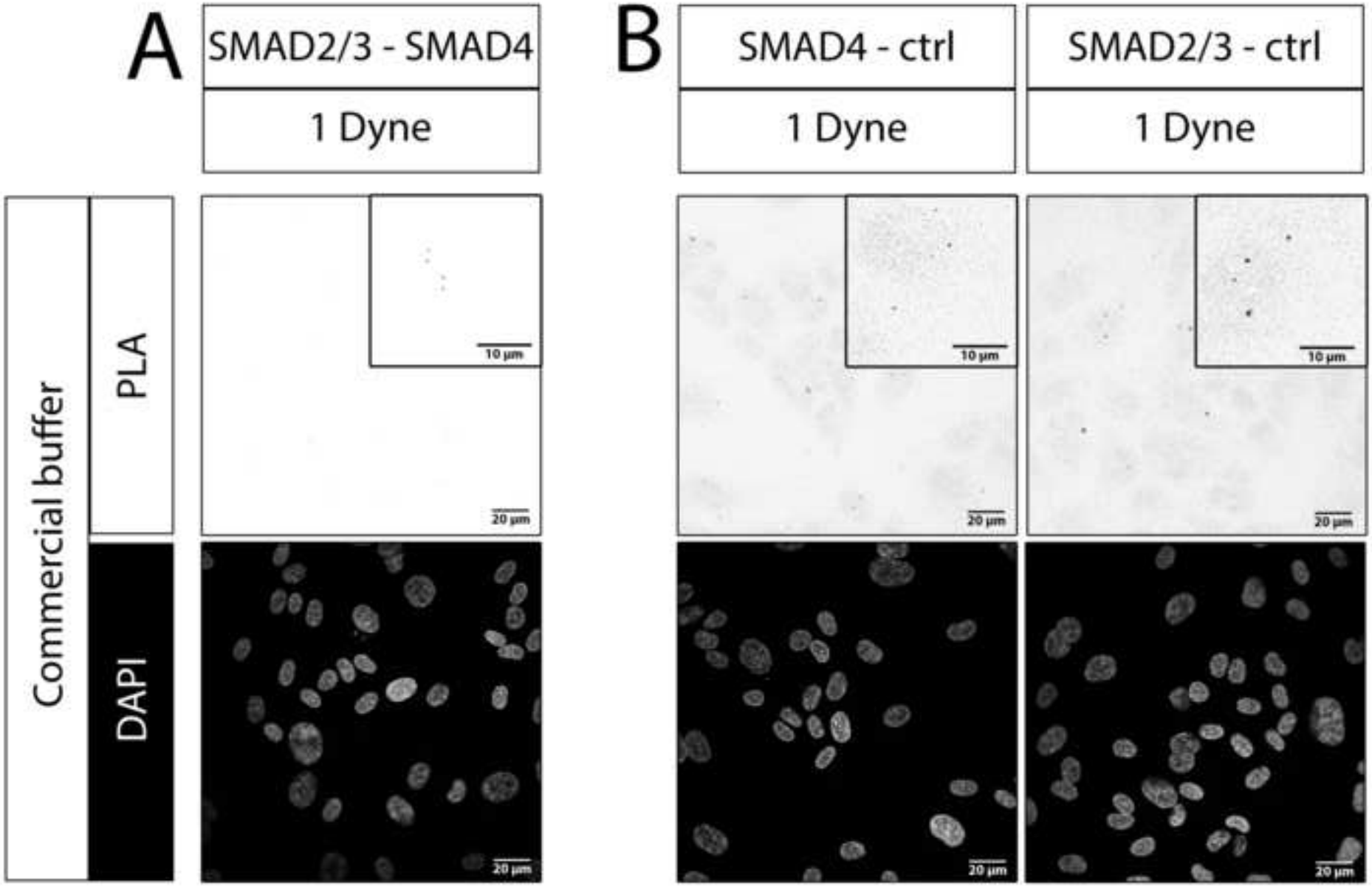


Figure 4



Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
µ-Slide VI 0.4	ibidi	80606	6-channel slide
Ammonium Chloride	Carl Roth	K298.1	Quenching
Bovine Serum Albumin	Carl Roth	8076.4	Blocking
DAPI	Sigma Aldrich/ Merck	D9542	Stain DNA/Nuclei
DPBS	PAN Biotech	P04-53500	PBS
Duolink In Situ Detection Reagents Green	Sigma Aldrich/ Merck	DUO92014	PLA kit containing Ligase, ligation buffer, polyn
Duolink In Situ PLA Probe Anti-Mouse MINUS	Sigma Aldrich/ Merck	DUO92004	MINUS probe
Duolink In Situ PLA Probe Anti-Rabbit PLUS	Sigma Aldrich/ Merck	DUO92002	PLUS probe
Duolink In Situ Wash Buffers, Fluorescence	Sigma Aldrich/ Merck	DUO82049	PLA wash buffers A and B
Endothelial Cell Growth Supplement	Corning		supplement for medium (ECGS)
Fetal calf Serum			supplement for medium
FIJI			Image Analysis software
Formaldehyde solution 4% buffered	KLINIPATH/VWR	VWRK4186.BO1	PFA
Full medium			M199 basal medium +20 % FCS +1 % P/S + 2 nI
Gelatin from porcine skin, Type A	Sigma Aldrich	G2500	Use 0.1% in PBS for coating of flow channels
GraphPad Prism v.7	GarphPad		Statistical Program used for the Plots and stati
Heparin sodium salt from porcine intestinal mucosa	Sigma Aldrich	H4784-250MG	supplement for medium (Hep)
HUVECs			
ibidi Mounting Medium	ibidi	50001	Liquid mounting medium
ibidi Pump System	ibidi	10902	pneumatic pump
Leica TCS SP8	Leica		confocal microscope
L-Glutamin 200mM	PAN Biotech	P04-80100	supplement for medium (L-Glu)
Medium 199	Sigma Aldrich	M2154	Base medium
mouse anti- SMAD1 Antibody	Abcam	ab53745	Suited for PLA

mouse anti- SMAD2/3 Antibody	BD Bioscience		610843	Not suited for PLA in combination with CST 95
mousee anti- SMAD4 Antibody	Sanata Cruz Biotechnology	sc-7966		Suited for PLA
Penicillin 10.000U/ml /Streptomycin 10mg/ml	PAN Biotech	P06-07100		supplement for medium (P/S)
Perfusion Set WHITE	ibidi		10963	Tubings used for 1 dyn/cm2
Perfusion Set YELLOW and GREEN	ibidi		10964	Tubings used for 30 dyn/cm^2
rabbit anti- phospho SMAD1/5 Antibody	Cell Signaling Technologies		9516	Suited for PLA
rabbit anti- SMAD2/3 XP Antibody	Cell Signaling Technologies		8685	Suited for PLA
rabbit anti- SMAD4 Antibody	Cell Signaling Technologies		9515	Not suited for PLA in combination with BD 610
Serial Connector for µ-Slides	ibidi		10830	serial connection tubes
Triton X-100	Carl Roth		6683.1	Permeabilization

nerase and amplification buffer (with green labeled oligonucleotides)

M L-Glu + 25 µg/mL Hep + 50 µg/mL ECGS

stical calculations

15

)843

Dear Editor,

thank you and the Reviewers for the valuable comments on our manuscript. We are submitting here a revision, where all points raised by the editor, reviewer 1 and reviewer 2 were considered. We have included new experiments and 2 new figures. We apologize for the delay, in part caused by the pandemic. Enclosed in this letter we are addressing all changes made directly following the points listed.

With this revised manuscript we are confident, that our improved manuscript can now be accepted at JoVE and are looking forward to the next steps.

With kind regards on behalf of all authors,

Petra Knaus

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We carefully did proofread the manuscript and also checked again the use of abbreviations.

2. Please provide an **email address** for each author.

Email addresses for each author are provided now in italics below the affiliations.

3. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the statement(s), but before punctuation.

We changed the citations to numbered superscripts before punctuation.

4. JoVE cannot publisappropriate h manuscripts containing **commercial language**. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript (abstract, main text, figure legends; the only place brand names and sources can appear is in the Table of Materials; use generic terms instead in the manuscript. Enter the **generic description** used in the text in the comments column of the Table of Materials.
For example: µ-Slides VI, ibidi GmbH; Duolink ll Blocking Solution; Duolink Antibody Diluent; ibidi mounting medium; GraphPad Prism; Duolink in-Situ PLA kits etc

We screened the manuscript and removed all commercial language from the text and protocol.

5. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "**we", "you", "our" etc**.).

We revised the text and avoided the use of personal pronouns whenever suitable.

6. Please ensure that all text in the protocol section is written in the **imperative tense** as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

All steps in the protocol are now written in imperative tense.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We added further references on how to assemble the fluid shear stress set up and to seed cells in flow channels.

8. After **including a one line space between each protocol step**, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.
We highlighted 3 pages of the protocol with a yellow background.

9. Lines 48-50 + 402-403: If possible, please rephrase the application and the recommendation to use ibidi mounting medium instead of Duolink In-situ mounting medium in terms of why this should be done (presence or absence of components? Any other property of the media?) rather than make it sound like an endorsement of ibidi GmBH.

We removed commercial language in former lines 48-50.

In former lines 402-403 we exchanged "ibidi mounting medium" to now "liquid mounting medium". We already stated in the first version of the manuscript that (Duolink) Mounting medium with DAPI leads to strong background signals in flow channels, a remark we wanted to highlight.

10. Please add **limitations of the PLA to the discussion**.

We added limitations of PLA in the discussion: PLA is not suited to make definite statements on protein interaction and should be complemented by co-immunoprecipitations or other techniques if this is desired. Additionally, PLA is not suited for usage in living cells.

We already mentioned several limitations regarding use of antibodies in the first version of the manuscript.

11. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

A scale bar and its definition (also for zoom-ins) was already present in the first version of the manuscript.

12. Please provide issue numbers in parentheses after the volume numbers in the reference list. [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (italics). Volume (bold) (Issue), FirstPage–LastPage (YEAR).]

We changed the citations accordingly.

13. Please sort the Materials Table alphabetically by the name of the material.

The materials and methods table is now sorted alphabetically.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The PLA based protocol described here offers an efficient way to determine close proximity of two proteins in shear stress exposed endothelial cells. The method allows quantitative and spatial resolution of the interaction. Importantly, this approach uses flow slides with multiple parallel channels, which allows for several interactions to be examined at once in one experiment. This compares favorably with the more common, custom-build flow chamber systems, which often makes use of a single channel and, thus, only allows a single PLA experiment. The necessary controls can therefore not be run in parallel. Although the protocol described here focused on the detection of SMAD transcription factors, it can be adapted to detect any other protein interactions.

Major Concerns:

The only major concern is the lack of information on detection, analysis, and reproducibility. For example, it is not clear what area is detected. Is it a single image from the microscope, the whole chamber, a few selected cells? Similarly, for analysis, what area was chosen? The most promising cells, the whole chamber? Finally, statistical analysis is performed but it is not clear on what. Are these biological replicates? Did the authors take pictures in different parts of the chamber? More information about these aspects would be highly welcome as it would help users of the approach generate more reproducible and higher quality data.

We agree with Reviewer 1 that the information on image acquisition was incomplete. We added the following sentence to the figure legends: "*For each condition 5 random areas along the center of the flow channel were used for image acquisition. N=1 biological replicate".*

We furthermore adapted the discussion and included the following sentence in new lines 392-394

We recommend to take at least 5-10 images per biological replicate and condition of random areas along the center region. 3 or more biological replicates are normally used to gain statistical relevance.

Minor Concerns:
In 1.6.1. the authors state that, in order to save money, the tubing can be assembled in the lab. However, the material and tubing length should remain constant, as changes could influence the resulting shear stress. This may be emphasized more strongly (e.g. as note) as it seems to be an important aspect.

We thank Reviewer 1s for this advice and changed the respective part accordingly.

Reviewer #2:
Manuscript Summary:
This manuscript by Mendez, Obendorf and Knaus is interesting, well written overall, and scientifically sound. The team has previously published research papers on both techniques of focus - PLA and shear stress experiments. The protocol is detailed, clear and technically sound; researchers in the filed will be able to reproduce it without additional information. It will definitely be useful for both developmental biology and disease studies on the genetic regulation of endothelial cells. While the protocol is presented clearly, several points should be improved in terms of clarity and accuracy within the rest of the manuscript. I stress especially on a point from Results which I included under "Major concerns" just because in my opinion this is the point that requires most attention.

Major Concerns:
- Fig. 3: I do not understand why Fig. 3 represents results obtained using 1:50 antibody concentration, when this is clearly suboptimal than 1:100. The authors themselves stress that the higher concentration is problematic (265-268). Under this concentration, the total significance of the difference of PLA events under high and low stress is lost (Fig. 3A). Even so, the authors go on to compare commercial and self-made buffers with 1:50, and most of the results are not significant. Furthermore, the authors claim that "Quantifications for both commercial and self-made diluent/ blocking solutions show the same trend of PLA signals in cytosolic and nuclear areas". This, however, is only evident from pSMAD1/5-SMAD4. For SMAD1-SMAD2/3, the small but significant nuclear difference is lost with self-made buffers; for SMAD2/3-SMAD4, the results with both buffers are very different. I am therefore not sure that Fig. 3 as it is can be used to prove that both buffers can be used the same way.
- In respect to the point above, I strongly recommend that the authors repeat the Fig. 3 experiment with antibody concentration of 1:100. Then, for better clarity, the comparison of the 1:100 and 1:50 antibody could become part of Fig. 2 (the present Fig. 3A becomes Fig. 2C) and all buffer comparisons are in Fig. 3 (another image from the same experiment can be used for Fig. 3A, instead of referring to Fig. 2A). Then in Fig. 2, instead of "commercial buffers", the side panels of A and C can read "1:100" and "1:50", respectively.

We highly appreciate this comment. We agree that the buffer comparison should be done at a concentration of 1:100 and therefore repeated the experiment as proposed by Reviewer 2. We have created a **new Figure 2** which now shows a comparison of 1:50 and 1:100 dilutions of SMAD1-SMAD2/3 with commercial buffers, as proposed by Reviewer 2. This will help the

reader to more easily understand, why appropriate antibody dilutions are crucial for successful PLA experiments.

New Figure 3 now shows a comparison of commercial and self-made buffers at 1:100 antibody dilution. We rewrote the respective result section accordingly. Now the results clearly show that the self-made buffer samples follow the very same trend as the commercial ones, while PLA events are still lower, as already stated before (see new Figure 3).

Minor Concerns:
- Readability: There are many acronyms in the manuscript. While this is unavoidable in the field, I suggest that the authors improve readability by abbreviating only where necessary. For example: "fluid shear stress" is mentioned only few times and doesn't need to be abbreviated; "shear stress" is mentioned more often, but many times not abbreviated anyway; "low laminar shear stress" and "high laminar shear stress" are mentioned only in the abstract and definitely don't need acronyms. In addition, "which" is improperly used instead of "that" throughout the text.

We eliminated unnecessary acronyms as mentioned here and corrected the use of 'which' and 'that'. Thank you for pointing out.

- Introduction: The first paragraph on TGFβ/BMP signalling is a bit too long for a methods paper and will benefit from being made more concise (especially the first part).

We agree and removed the section of receptor complex formation from the BMP/TGFβ introduction as this is not of interest for this paper.

On Line 95-96, write "species-specific secondary antibodies" instead of "specific secondary antibodies" - this way the statement from few lines above, that primary antibodies are of different species, will make sense.

We changed the respective part according to the reviewer's suggestion (new line 96).

- Line 83: What do you mean by "activated"?

We see that the term of Endothelial Activation might not be trivial and exchanged it for *"proliferative and activate inflammatory pathways"* (new line 83).

- Line 104: What do you mean by "consecutive protocol"? I think "consecutive" is not needed here.

We changed the respective part according to the reviewer's suggestion.

- Introduction: While there is introduction of PLA, nothing at all is mentioned on the shear stress fluid system. The reader then jumps straight into the protocol without knowing what that system is and how does it work. Figure 1 will be here but this is not enough - for

example, controls are shown but we only get to know what the controls are at the bottom of page 5. Please, insert a sentence or two in the last part of the intro to explain the shear stress system so the reader gets the full picture.

We agree with Reviewer 2 that there was a lack of introduction into the used shear stress system and have added a sentence to the last part of the introduction.

- Line 143: "with a finger" is deleted but I think it is useful to keep it.

Since this line might be useful for protocol users we reinserted it into the revised manuscript.

- Fig. 2 and Fig. 3: It is not clear which cell is magnified in the small rectangle: is it one of the cells visible in the larger area? If yes - which cell? Please clarify, either in the text or on the image. Be consistent which you write first, Smad1 or Smad2/3 - Figures 2A and 3A don't match. The images show 30 Dyne first and the graphs under them - 1 Dyne first. Please, be consistent for better clarity. I suggest re-doing the graphs to match the images, as 30 Dyne is the "normal" condition. Also, you want to emphasize the increase of SMADs under low shear stress (not the decrease under high stress - which wouldn't make sense but it looks like this from the graphs).

We appreciate the comment made by reviewer 2. We added a frame around the spot, which was used for the zoom-ins.

We completely agree with the second part of this comment and changed the order of SMAD2/3-SMAD1 to SMAD1-SMAD2/3 in Figure 2A. We also agree that the graphs should match the order of the images and redid all corresponding graphs so that 30 dyne is now always mentioned first.

- Line 272: "Fig. 2A vs. 2D" should read "Fig. 3A vs. 3D".

Corrected

- Line 285: Write "As the formation of SMAD2/3-SMAD4 complexes is induced by shear stress" instead of "As the induction of SMAD2/3-SMAD4 complex formation is induced by shear stress".

We changed the respective part according to the reviewer’s suggestion (new line 285-286).

- Lines 339-342: The authors address a general PLA limitation that detection of two proteins does not necessarily mean they interact directly. Is this mentioned because it is considered as a relevant to this study limitation? If yes, could the authors comment further on it?

We do not see this point as a limitation on this particular study but rather as a limitation of PLA in general. We added the advice to compensate for that by using co-immunoprecipitation or another suitable technique (new lines 348-350).

- Line 346: It is stated that antibodies must be of different species but not why. The authors could add "…because the secondary antibodies are species-specific" to the end of the sentence.

We changed the respective part according to the reviewer's suggestion (new line 355).

- The Discussion will benefit from a concluding paragraph. It can be a short summary on the general impact on the protocol for disease research.

We now end the manuscript with a concluding paragraph following the reviewer's suggestion.

- The Discussion is interesting but a little bit lengthy and could be made a bit more concise.

We removed the paragraph on air bubbles (former lines 391-397) from the manuscript as this was already sufficiently mentioned in the protocol. We furthermore shortened several aspects in the discussion to make it more concise and easier to read.