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Valia Khodr, Laura Clauzier, Paul Machillot, Adrià Sales, Elisa Migliorini, et al.. Development of an automated high-content immunofluorescence assay of pSmads quantification: Proof-of-concept with drugs inhibiting the BMP/TGF- $\beta$  pathways. *Biotechnology Journal*, 2024, 19 (9), 10.1002/biot.202400007 . hal-04717984

**HAL Id: hal-04717984**

**<https://hal.science/hal-04717984v1>**

Submitted on 2 Oct 2024

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## RESEARCH ARTICLE

# Development of an automated high-content immunofluorescence assay of pSmads quantification: Proof-of-concept with drugs inhibiting the BMP/TGF- $\beta$ pathways

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## Abstract

**Introduction:** Bone morphogenetic proteins (BMPs) and transforming growth factors (TGF- $\beta$ ) are members of the TGF- $\beta$  superfamily, known for their roles in several physiological and pathological processes. These factors are known to bind in vivo to BMP and TGF- $\beta$  receptors, respectively, which induces the phosphorylation of Smad (pSmad) transcription factors. This pathway is generally studied with Western blot and luciferase bioluminescence assay, which presents some limitations.

**Purpose:** In this work, we developed and optimized a high-throughput assay to study pSmad pathways using immunofluorescence (IF) as an alternative to Western blot. We aimed to overcome the technical challenges usually faced in the classical IF assay in image acquisition, analysis, and quantification.

**Methods:** We used C2C12 cells as a cellular model. The cells were stimulated with BMP-2 and TGF- $\beta$ 1 that were delivered either in solution (soluble) or via a biomaterial presenting the growth factor (GF), that is in a “matrix-bound” manner. Image acquisition parameters, analysis methods, and quantification of pSmads using IF were optimized for cells cultured on two types of supports: on bare glass and on a biomimetic coating made by self-assembly of the biopolymers hyaluronic acid and poly(L-lysine), which was crosslinked and then loaded with the GFs.

**Results:** We performed high-content kinetic studies of pSmad expression for cells cultured in 96-well microplates in response to soluble and matrix-bound BMP-2 and TGF- $\beta$ 1. The detection limit of the IF-based assay was found to be similar to

**Abbreviations:** ALP, alkaline phosphatase; bBMP-2, matrix-bound BMP-2 loaded in the biomimetic films; BMP, bone morphogenetic protein; BMPR, bone morphogenetic protein receptors; bTGF- $\beta$ 1, matrix-bound TGF- $\beta$ 1 loaded in the biomimetic films; DAPI, 4',6-diamidino-2-phenylindole; DMEM, 1:1 Dulbecco's Modified Eagle Medium; DMSO, dimethyl sulfoxide; EC50, half-maximal effective concentration; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HA, hyaluronic acid; HEK293, human embryonic kidney cells; IC50, half-maximal inhibitory concentration; IF, immunofluorescence; PEI, polyethylenimine; PFA, paraformaldehyde; PLL, poly(L-lysine) hydrobromide; pSmad, phosphorylated Smad; sBMP-2, soluble BMP-2; sTGF- $\beta$ 1, soluble TGF- $\beta$ 1; Sulfo-NHS, N-hydroxysulfosuccinimide sodium salt; TCPS, tissue culture polystyrene; TGF- $\beta$ , transforming growth factor; TGF- $\beta$ R, transforming growth factor receptor.

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Western blot. Additionally, we provide a proof-of-concept for drug testing using inhibitors of BMP and TGF- $\beta$  receptors, under conditions where specific signaling pathways are engaged via the ligand/receptor interactions. Altogether, our findings offer perspectives for future mechanistic studies on cell signaling and for studies at the single cell level using imaging methods.

**KEYWORDS**

biomaterials, BMP, BMPR, drugs, high-content screening, immunofluorescence

## 1 | INTRODUCTION

The transforming growth factor (TGF- $\beta$ ) superfamily is a large group of structurally related growth factors (GFs) grouped into different families, notably the bone morphogenetic protein (BMP) and TGF- $\beta$  families. These GFs have been reported to have various physiological roles in development and organ regeneration<sup>[1]</sup>. They have been described to signal *in vivo*, by binding to a ternary complex of type-I and type-II receptors. Specifically, either the type-I BMP receptors (BMPR) ALK1, ALK2, ALK3, and ALK6, or the TGF- $\beta$  receptor (TGF- $\beta$ R) named also ALK5 forms this complex with the type-II receptors (BMPR-II, ACTR-IIA, ACTR-IIB, or TGF- $\beta$ R-II), which then activate a cascade of signaling pathways such as the canonical Smad pathway and other Smad-independent pathways. In the Smad pathway, the ternary complex of GF and receptors activates Smad 1/5/9 in response to BMP-2, and the Smad 2/3 in response to TGF- $\beta$ , by undergoing a carboxy-terminal phosphorylation from the type-I receptor kinase.<sup>[2,3]</sup> The phosphorylated Smads (pSmads) translocate and accumulate in the nucleus<sup>[4]</sup> and induce the transcription of several regulatory genes.<sup>[5]</sup>

The fact that the pSmad signaling is activated by BMP that has a major osteogenic role *in vivo* and *in vitro*, led to consider Smad as an essential marker of early bone regeneration.<sup>[6]</sup> Consequently, this pathway was extensively studied by the standard molecular techniques such as Western blot, gene reporter assays using a specific promoter (such as bioluminescence luciferase), and immunofluorescence (IF). The Western blot technique has the advantage of having a high sensitivity of the picogram order, and being selective in binding specific target antibody in the presence of heterogeneous mixture of several proteins.<sup>[7]</sup> Similarly the gene reporter technique based on bioluminescence luciferase is characterized with a high sensitivity of the order of 10 fg.<sup>[8]</sup> However, these techniques have limitations in terms of requiring optimization steps, variation of signal quantification, in addition to the limitation of the number of samples in the case of Western blot. Most importantly, these techniques measure the signal average across a population of cells.<sup>[7,9]</sup> On the other hand, the IF technique is generally used for visualization purposes, since the quantification in experiments with several conditions is usually a tedious and lengthy task. However, this technique has the advantage of being compartment-specific and quantitative at a single-cell level. This characteristic could be particularly interesting in detecting phenotypic changes that affect subpopulation of cells.<sup>[10,11]</sup>

The BMP and TGF- $\beta$  Smad signaling is extensively studied, due to its association with several diseases such as fibrodysplasia ossificans progressive, Barrett esophagus, leukemia, and fibrosis.<sup>[12]</sup> Thus, pharmaceutical companies have been focusing on the discovery of new target molecules that can modulate their signaling, in an effort to develop new therapies. The modulation of BMP and TGF- $\beta$  signaling can be done at different steps of their signaling, but receptor kinase inhibitors have been mainly developed.<sup>[13]</sup> Thus, our aim was first to develop a high-content screening assay to quantify pSmad intensity on the standard glass condition. Our second aim was to apply this assay to thin biomimetic coatings made of poly(L-lysine)/hyaluronic acid (PLL/HA) previously developed in the team,<sup>[14,15]</sup> and to optimize it to overcome the difficulties of doing cellular studies on biomaterials.<sup>[16]</sup> Indeed, performing drug screening on biomaterials is not common, yet it has recently been applied to develop a human breast cancer model and hence investigate the effect of extracellular matrix in inducing drug resistance.<sup>[17]</sup> In that context, drug tests on biomaterials could reveal notable processes that could be masked on glass surfaces. Here, using C2C12 skeletal myoblasts as a reference model for BMP-responsive cells,<sup>[18]</sup> we develop a new pSmad assay based on IF. We optimize it for cells cultured on glass and on biomaterials. Using the new pSmad assay, we present a proof-of-concept on two types of receptor-kinase inhibitors that impact pSmad signaling: LDN-193189, an inhibitor of BMP type 1 receptors (ALK1, ALK2, ALK3, and ALK6), and two inhibitors of the TGF- $\beta$  type I receptor ALK5: galunisertib and vactosertib.

## 2 | MATERIALS AND METHODS

### 2.1 | Materials and reagents

Polyethylenimine (PEI) and poly(L-lysine) hydrobromide (PLL) were purchased from Sigma-Aldrich (St Quentin Fallavier, France), and sodium hyaluronate (HA) from Lifecore medical (USA). Paraformaldehyde (PFA), methanol, and acetone were purchased from Sigma-Aldrich (USA). The rinsing solutions 0.15 M NaCl pH 6.4 and 5.5, as well as 1 mM HCL pH 3.7 and 0.15 M NaCl 20 mM HEPES pH 7.4 were all prepared in house. BMP-2 were bought from Bioventus (France) and TGF- $\beta$ 1 from PeproTech (France). LDN-193189, galunisertib, and vactosertib were obtained from Selleckchem

(USA). The glass and tissue culture polystyrene (TCPS) 96-well cell culture microplates, and CellView (10-well cell culture plates, ref: 543979) were purchased from Greiner bio-one (Germany). The 4',6-diamidino-2-phenylindole (DAPI) was acquired from Invitrogen, Thermofisher Scientific, France. Trihydrate trichlorhydrate de 2'-[4-éthoxyphényl]-5-[4-méthyl-1-pipérazinyl]-2,5'-bi-1H-benzimidazole (Hoechst 33342), anti-pSmad 1/5/9 (ref: 13820S), and anti-pSmad 2 (ref: 18338S) antibodies were obtained from Cell Signaling (USA). The secondary antibody conjugated to Alexa555 or Alexa488 was purchased from Invitrogen, Thermofisher Scientific (France).

## 2.2 | Film preparation

The films were prepared as previously described using a modified robot-liquid handling robot (TECAN Freedom EVO 100, Tecan France, France) in 96-well cell culture microplates with a layer-by-layer deposition of PLL and HA polyelectrolytes.<sup>[14,15]</sup> They were then crosslinked with 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC) at a concentration of 70 mg mL<sup>-1</sup> and *N*-hydrosulfosuccinimide sodium salt (Sulfo-NHS) at a concentration of 11 mg mL<sup>-1</sup>. The films were rinsed with a solution of HEPES NaCl at pH 7, as previously described.<sup>[19]</sup> The bound BMP-2, hereby called matrix-bound BMP-2 loaded in the biomimetic films (bBMP-2), and bound TGF- $\beta$ , called matrix-bound TGF- $\beta$ 1 loaded in the biomimetic films (bTGF- $\beta$ 1), were then loaded in a solution of HCl at a concentration of 20 and 0.75  $\mu$ g mL<sup>-1</sup> respectively, for 2 h at 37°C, followed by six washes with an HEPES NaCl pH 7 solution. Before usage, the films were sterilized by UV for 30 min.

## 2.3 | Cell culture

C2C12 skeletal myoblasts cells, obtained from the American Type Culture Collection, ATCC, were cultured in 1:1 Dulbecco's Modified Eagle Medium (DMEM):Ham's F12 medium (Gibco, Life Technologies, France) supplemented with 10% fetal bovine serum in an incubator at 37°C and 5% CO<sub>2</sub>. The cells were cultured up to passage 12. They were then seeded at a density of 10,000 cells cm<sup>-2</sup> in each well.

## 2.4 | Dose-response and kinetic studies

The dose-response assays consisted of using a range of concentrations from 0.001 pg mL<sup>-1</sup> to 100 ng mL<sup>-1</sup> for soluble BMP-2 (sBMP-2), and 0.001 to 1000 ng mL<sup>-1</sup> for soluble TGF- $\beta$ 1 (sTGF- $\beta$ 1), with a volume of 50 and 200  $\mu$ L per well, respectively. In both cases, the cells were exposed to the ligands for 1 h. After quantification of pSmad values, the half-maximal effective concentration (EC50) values were obtained by performing a non-linear dose-response fit on Origin. The kinetic studies consisted on using 400 ng mL<sup>-1</sup> of BMP-2 and TGF- $\beta$ 1 for the

soluble conditions. For matrix-bound conditions, the BMP/TGF- $\beta$  were loaded in the biomaterials at 20  $\mu$ g mL<sup>-1</sup> for BMP-2 and 0.75  $\mu$ g mL<sup>-1</sup> for TGF- $\beta$ 1.

## 2.5 | Kinetics and drug assay

These assays consisted on seeding the cells on glass or on the biomimetic films. For cells on glass, sBMP-2 or sTGF- $\beta$ 1 were at a concentration of 400 and 10 ng mL<sup>-1</sup>, respectively. For cells on biomimetic films, the bBMP-2 and bTGF- $\beta$ 1 were prepared using BMP-2 concentration of 20  $\mu$ g mL<sup>-1</sup> and TGF- $\beta$  concentration 0.75  $\mu$ g mL<sup>-1</sup>. In the kinetic assay, the cells were fixed at different time intervals ranging from 15 min until 3 days. We first exposed the cells to the BMP/TGF ligand for 15 min. Then the drug was added for 30 min, at a concentration range of 0.1 pM–0.1  $\mu$ M for LDN-193189 and 10 pM–10  $\mu$ M for galunisertib and vactosertib. The half-maximal inhibitory concentration (IC50) values were obtained by performing a non-linear dose-response fit using Origin software with the equation  $y = A1 + \frac{A2-A1}{1+10^{(Logx0-x)p}}$ , where A1 being the highest value, A2 the lowest, and *p* is the hill slope.

## 2.6 | Immunofluorescence assay

The cells were fixed with 4% PFA, and stained with DAPI or Hoechst 33342 for the nuclear staining. The pSmad was stained by using a 1/800 dilution of anti-pSmad antibody. The secondary antibody conjugated to either Alexa Fluor 488 or Alexa Fluor 555 was diluted at 1:500 in BSA 3% w/v in PBS.

## 2.7 | Image acquisition

The images were acquired using In Cell GE INCA 2500 imaging system (General Electrics Healthcare, France) using a 20X objective. Similar exposure times were applied for both pSmad1/5/9 and pSmad2. Sixteen to 21 images distributed over each well were acquired and at least 500 cells were analyzed per well. For image analysis, the InCarta software (General Electrics Healthcare, USA) was used to automatically segment nuclei and cells, and to quantify the nuclear pSmad signal intensity.

## 2.8 | Data analysis

The raw intensity data were normalized by the maximum signal measured, and transformed into a percentage of Smad signaling where the background signal corresponds to 0% response. Reported pSmad intensity values are obtained by averaging the values obtained from at least two biological replicates and three technical replicates for each experiment. The reported errors are presented as the standard deviation.

### 3 | RESULTS

#### 3.1 | Immunofluorescence pSmad assay and image analysis

In this study, we developed and optimized a high-content screening IF assay to quantify the translocation of Smad to the nucleus. We first developed it on glass as reference cell culture substrate, then on the biomimetic film coating that enable to present the GFs in a matrix-bound manner. We started by optimizing the experimental conditions, notably cell culture, image acquisition, and subsequent image analysis (Figure 1) to be compatible with both our biomimetic films and the standard glass condition used for imaging.<sup>[20]</sup> For simplicity, sBMP-2 and sTGF- $\beta$ 1 designations will be used to indicate the soluble proteins BMP and TGF- $\beta$  respectively used on glass surfaces, while bBMP-2 and bTGF- $\beta$ 1 will be used to designate the matrix-bound proteins loaded in the biomimetic films. For image acquisition and analysis, we used an automated imaging system (In Cell analyzer) adapted for high-content screening, as described in Section 2. To quantify pSmad 1/5/9 in the nucleus, the raw image of the nuclei stained with DAPI was used to set a threshold in order to remove the background fluorescence and to select solely the nuclei. This analysis generates a “mask image” of the nuclei, which can be subtracted afterwards from the image of pSmad staining. As a result, a new image is created that shows solely pSmad staining inside the nucleus (Figure 1A). The nuclear intensity was then quantified using either InCarta, which is the commercialized program of In Cell analyzer, or can be alternatively analyzed using the widely used image analysis software ImageJ.

In a kinetic IF assay, the cell fixation step is essential since cells need to be fixed at different time points throughout the experiment. Hence, we first optimized the experimental conditions using sBMP-2 and compared three different methods of cell fixation: 4% PFA that is a commonly used fixation protocol, 95% methanol, and 40 mM citrate/60% acetone (Figure 1B). IF of pSmad 1/5/9 showed that pSmad signal was the highest for fixation with 4% PFA and 95% methanol, in comparison to the citrate/acetone mixture. Next, we looked at several other parameters related to (i) the possibility to do a kinetic experiment on the same plate, (ii) to quantify cell adhesion via the cell number, obtained by labeling nuclei, and cell spreading area obtained by actin staining of cell cytoskeleton, (iii) the need for a permeabilization step, and (iv) the possibility to perform an alkaline phosphatase (ALP) staining, since it is considered an early marker of differentiation in bone cells.<sup>[18]</sup> The results are summarized in the table of Figure 1C. Overall, a toxicity was found when using 4% PFA for the kinetic assay on the same plate. We observed that the citrate/acetate solution is not compatible with pSmad staining, as also seen with the lower Smad intensity level (Figure 1A). In contrary, fixation with 95% methanol was compatible with pSmad staining and did not require a permeabilization step, but it affected the actin staining. Thus, we conclude that both PFA and methanol can be used to study pSmad signaling depending on the desired readout, PFA fixation being well adapted for studies at a single time-point, while methanol can be used for kinetic studies, but it is not suited for quantitative measurement of cell spreading area.

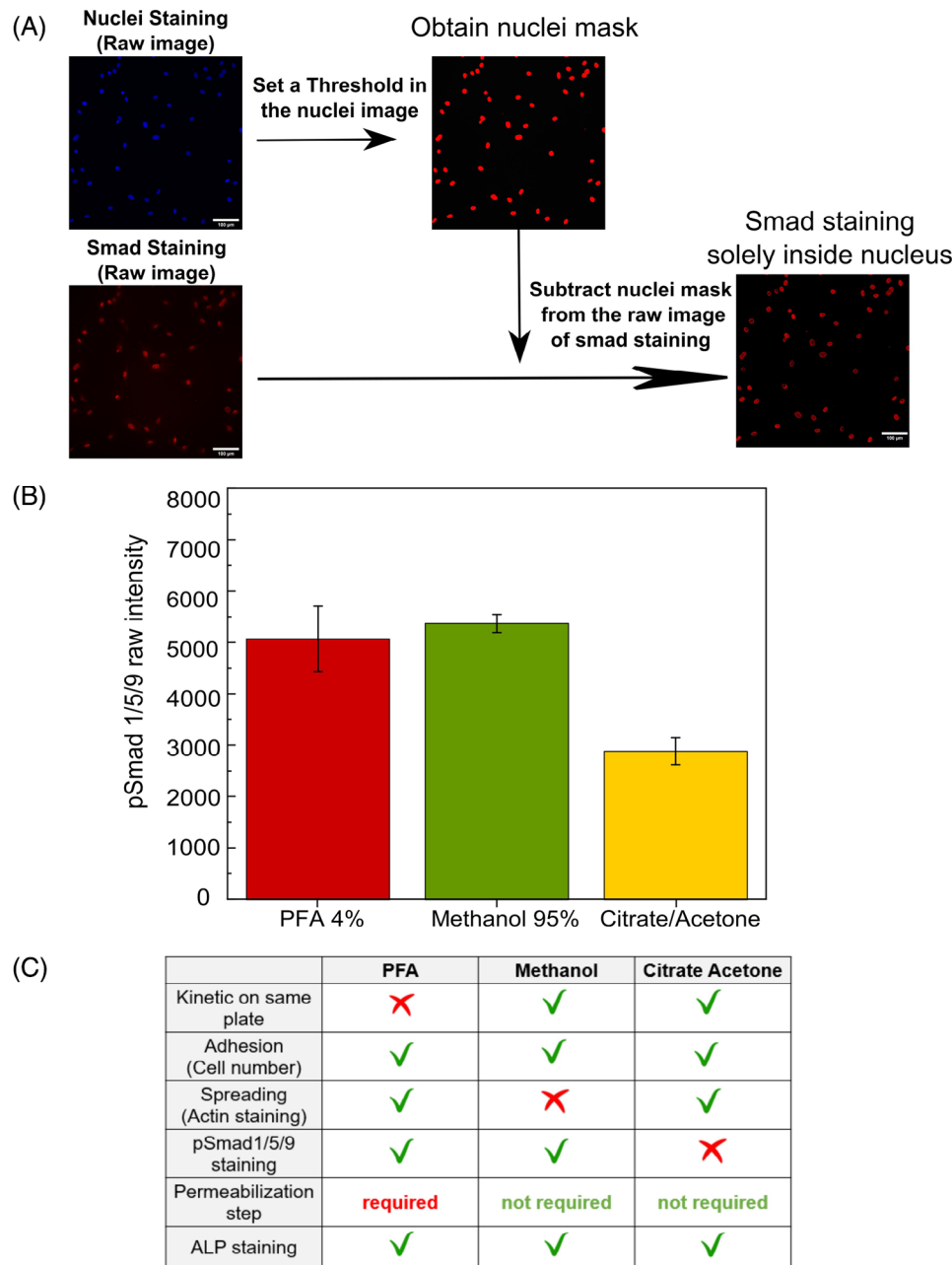
After defining the fixation conditions, we needed to optimize the imaging conditions, especially the type of antibodies for cells grown on the different types of supports (TCPS) without or with a biomimetic film coating (Figure 2). We first compared the effect of two different fluorescent primary antibodies Alexa488 and Alexa555, as well as two different supports (glass, TCPS) on the fluorescence intensity of pSmad (Figure 2A, B). pSmad 1/5/9 signaling was analyzed for cells cultured for 1 h with sBMP-2 at 400 ng mL<sup>-1</sup>. We observed a higher pSmad intensity (Figure 2A) and a lower background intensity (Figure 2B) with Alexa555 in comparison to Alexa488. Moreover, the background intensity was lower on glass plates that were selected for their image clarity. Notably, the background intensity on films was lower when Alexa555 antibody was used for pSmad staining.

As last step, we investigated the effect of the dilution level of the antibodies used for IF. We thus used five dilutions of pSmad 1/5/9 primary antibody from 1/400 to 1/1200 and two dilutions of Alexa 555 secondary antibody (1/500 and 1/700) (Figure 2C). While we observed negligible differences in secondary antibody, the concentration of primary antibody slightly impacted the signal levels. Therefore, we selected an intermediate dilution of 1/800 of primary antibody and a 1/500 dilution of secondary antibody as optimal dilutions to perform the IF test.

After selecting the preparation and imaging conditions, we performed a dose-response assay by adding sBMP-2 and sTGF- $\beta$ 1 to C2C12 cells seeded at a density of 10,000 cells cm<sup>-2</sup> and pre-cultured for 24 h (Figure 3). After 1 h of contact with sBMP-2 or sTGF- $\beta$ 1, the cells were fixed (Figure 3A). Cells in contact with sBMP-2 were stained for pSmad 1/5/9 while those in contact with sTGF- $\beta$ 1 were stained for pSmad 2. As seen in Figure 3B, a clear increase in intensity of pSmad 1/5/9 in the nucleus was visible when the concentration of sBMP-2 increased from 10<sup>-1</sup> to 10<sup>4</sup> ng mL<sup>-1</sup>. The control condition without BMP-2 (GM) was effectively negative. A representative image for pSmad2 in response to sTGF- $\beta$ 1 is also shown in Figure 51. The pSmad intensity in the nucleus was subsequently quantified and the EC50 calculated, after performing a non-linear dose-response fit (Figure 3C).<sup>[21]</sup> It is used as metric to report the drug effect on a cell culture.<sup>[22]</sup> EC50 was determined to be 0.92  $\pm$  0.06 ng mL<sup>-1</sup> for sBMP-2 (equivalent to 0.2  $\pm$  0.01 ng, when expressed in absolute mass) and 0.12  $\pm$  0.01 ng mL<sup>-1</sup> (corresponding to 0.02  $\pm$  0.002 ng) for sTGF- $\beta$ 1.

#### 3.2 | Kinetic of pSmad phosphorylation in the presence of soluble or matrix-bound BMP-2 and TGF- $\beta$ 1

After defining the optimal experimental parameters, we examined the kinetics of pSmad signaling with BMP-2 and TGF- $\beta$ 1 on both glass and biomimetic film to define the optimal time-window to perform drug experiments. The kinetic experiments were done using sBMP-2 and sTGF- $\beta$ 1 for cells cultured on glass plate, and with bBMP-2 and bTGF- $\beta$ 1 for cells cultured directly on the biomimetic films. The cells were fixed at different time points ranging from 30 min to 3 days, and pSmads were quantified at each time points (Figure 4A, B).

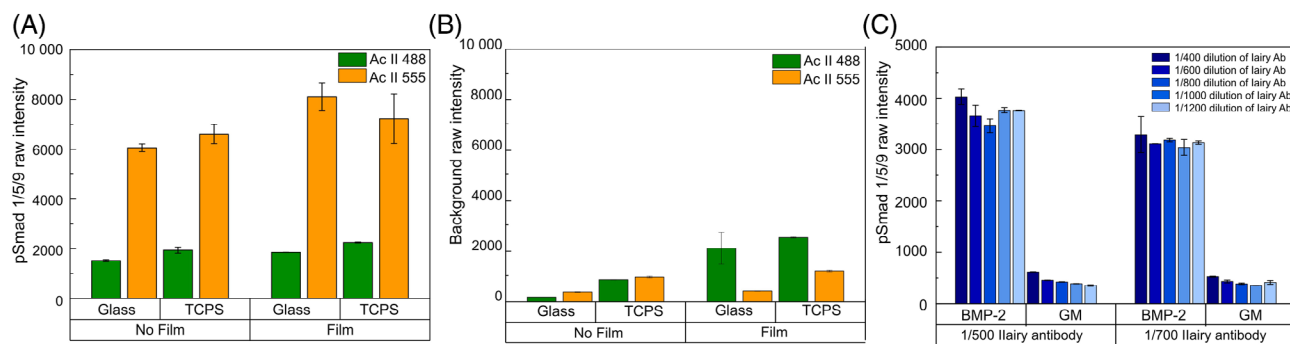


**FIGURE 1** Principles of quantifying pSmad1/5/9 staining in the nucleus and selecting an optimized fixation method for assessing parameters related to cell differentiation (pSmad, ALP) and cell adhesion (cell number and spreading area). (A) The acquisition was done using an automated microscope. The image analysis consisted of first setting a threshold on the raw image of the nuclei to create a mask image, which was then subtracted from the raw image of pSmad staining to visualize pSmad staining in the nucleus. Scale bar = 100  $\mu$ m. (B) Quantification of pSmad 1/5/9 signal in response to sBMP-2, after cell fixation with different fixatives: 4% PFA, 95% methanol, or 40 mM citrate/60% acetone. (C) A table summarizing the compatibility of the various fixation solutions with the studied output parameters: kinetics on same plate, adhesion (cytoskeleton staining), pSMAD1/5/9 staining, necessity for a permeabilization step, and ALP staining. ALP, alkaline phosphatase; PFA, paraformaldehyde; pSmad, phosphorylated Smad; sBMP-2, soluble bone morphogenetic protein-2.

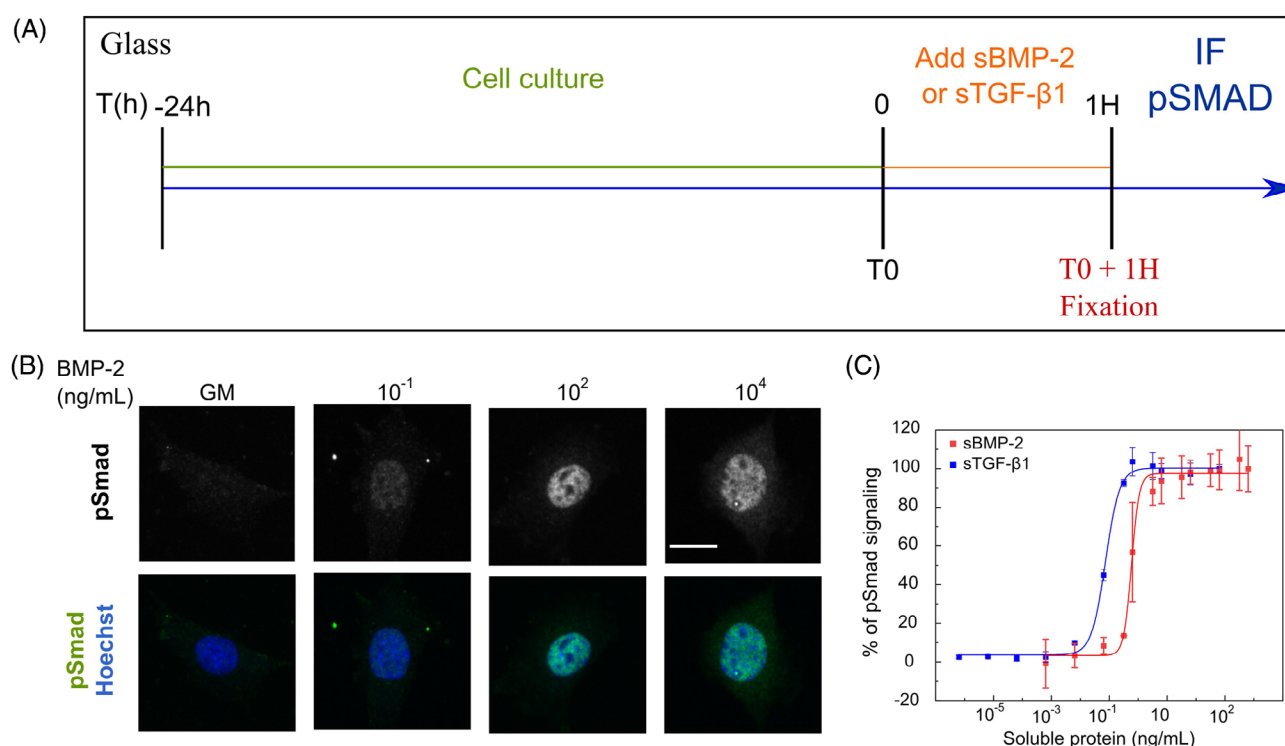
pSmad intensities exhibited differences depending on the type of ligand (BMP-2 vs. TGF- $\beta$ 1) and presentation mode of the ligand (soluble vs. matrix-bound). For sBMP-2, pSmad1/5/9 intensity was high at 30 min, increased and reached a peak intensity at 1.5 h before decreasing (Figure 4A). A second peak could be observed at 3 h. Then pSmad nuclear signal remained sustained at 40% up to 24 h, before retaining

to basal level at 48 h. For cells on bBMP-2, the pSmad peak was sharp at  $\approx$ 1 h, before decreasing to  $\approx$ 50%. The signal remained sustained up to 24 h. No second pSmad peak was observed. After 24 h, it gradually decreased until day 3 where the signal was still close to 20%.

In contrast, for TGF- $\beta$  (Figure 4B), pSmad2 kinetics was faster with an initial peak at 30 min for sTGF- $\beta$  and a second peak at 1h15 before



**FIGURE 2** Optimization of antibodies on the different types of cell culture microplates. The microplates were either made of glass, TCPS, and were without or with a biomimetic film coating. (A) The pSmad 1/5/9 intensity was measured with the secondary antibody Alexa488 and Alexa555 without and with film, on glass and TCPS. (B) The background intensity was measured in the same experimental conditions. (C) The pSmad 1/5/9 intensity was measured with 1/400, 1/600, 1/800, 1/1000, and 1/1200 dilutions of pSmad 1/5/9 primary antibody, as well as with 1/500 and 1/700 dilutions with the secondary Alexa555 antibody, in the presence and absence of BMP-2. BMP, bone morphogenetic protein; pSmad, phosphorylated Smad; TCPS, tissue culture polystyrene.

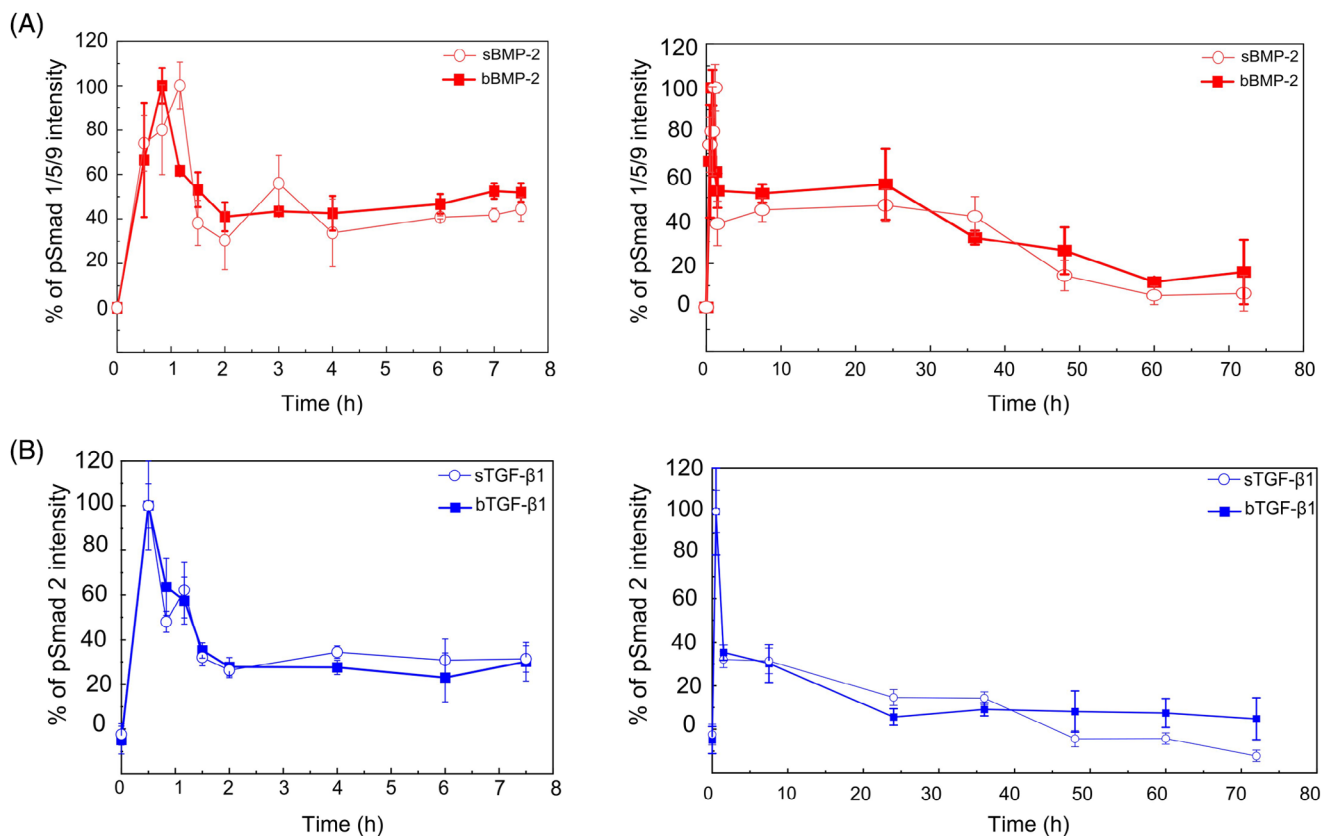


**FIGURE 3** A dose-response immunofluorescence assay for pSmad nuclear translocation. A dose-response assay was performed to quantify pSmad in the nucleus in response to soluble growth factors sBMP-2 and sTGF- $\beta$ 1. (A) The experiment consisted on seeding the cells for 24 h and then adding sBMP-2 and TGF- $\beta$ 1 at a concentration varying between  $10^{-6}$  and  $10^3$  ng/mL. After 1 h of growth factor stimulation, C2C12 cells were fixed and stained using Hoechst, and pSmad 1/5/9 or pSmad 2 antibody. (B) Cellular images of the dose-response assay with BMP-2, with pSmad 1/5/9 and nuclei staining. Scale bar = 26  $\mu$ m. (C) Dose-response curves obtained in response to sBMP-2 and sTGF- $\beta$ 1, pSmad expression being shown as a % of the maximum intensity. BMP, bone morphogenetic protein; pSmad, phosphorylated Smad; sBMP-2, soluble bone morphogenetic protein-2; sTGF- $\beta$ 1, soluble TGF- $\beta$ 1; TGF- $\beta$ , transforming growth factor.

quickly returning to a low level at  $\approx 25\%$  at 1h30. Then, the signal fully returned to its basal level at 48 h, some minor oscillations being visible over time. For bTGF- $\beta$ , the first peak was at the same time point as for sTGF- $\beta$ , but the second peak was less marked. A decrease to 25% was also fast and quickly observed at 1h30, then the signal was sustained until 24 h, after which it returned to its basal level. The kinet-

ics of pSmad1/5/9 and pSmad 2 exhibited some distinct differences. Additionally, the presence of the films appears to attenuate the second peak which is prominently observed with soluble ligands, while it also contributed to sustain signaling.

Since we observed an initial peak varying from 30 min to 1h30 for both BMP-2 and TGF- $\beta$ 1 GFs for cells either cultured on glass or on the



**FIGURE 4** Kinetic study of pSmad phosphorylation in C2C12 skeletal myoblasts in the presence of soluble and matrix-bound BMP-2 and TGF- $\beta$ 1. Data points corresponding to the cells cultured on glass are represented with an empty circle, while cells cultured on the biomimetic films are represented with a filled rectangle. (A) Kinetics of pSmad 1/5/9 after 1 h of C2C12 cell exposure to BMP-2 on glass and film. (B) Kinetics of pSmad 2 after 1 h of C2C12 cell exposure to TGF- $\beta$ 1 for cells cultured on glass and on films. BMP, bone morphogenetic protein; pSmad, phosphorylated Smad; TGF- $\beta$ , transforming growth factor.

films, we selected 1 h as common time point for the future experiments using drugs.

### 3.3 | Test of drug inhibitors against BMP receptors for cells cultured on glass and on biomaterials

After optimizing the time frame, we performed dose–response assays with soluble and matrix-bound proteins to determine the optimal concentration for the drug experiments.

As a first control, since the drugs are in dimethyl sulfoxide (DMSO), we tested the effect of DMSO on the fluorescence intensity of Smad 1/5/9 and Smad 2/3 (Figure S2A, B). We did not observe any effect on the Smad intensity signal for levels of DMSO up to 1%, which was the maximal concentration used in our assay, while 2% and 5% levels showed an important increase in Smad signaling.

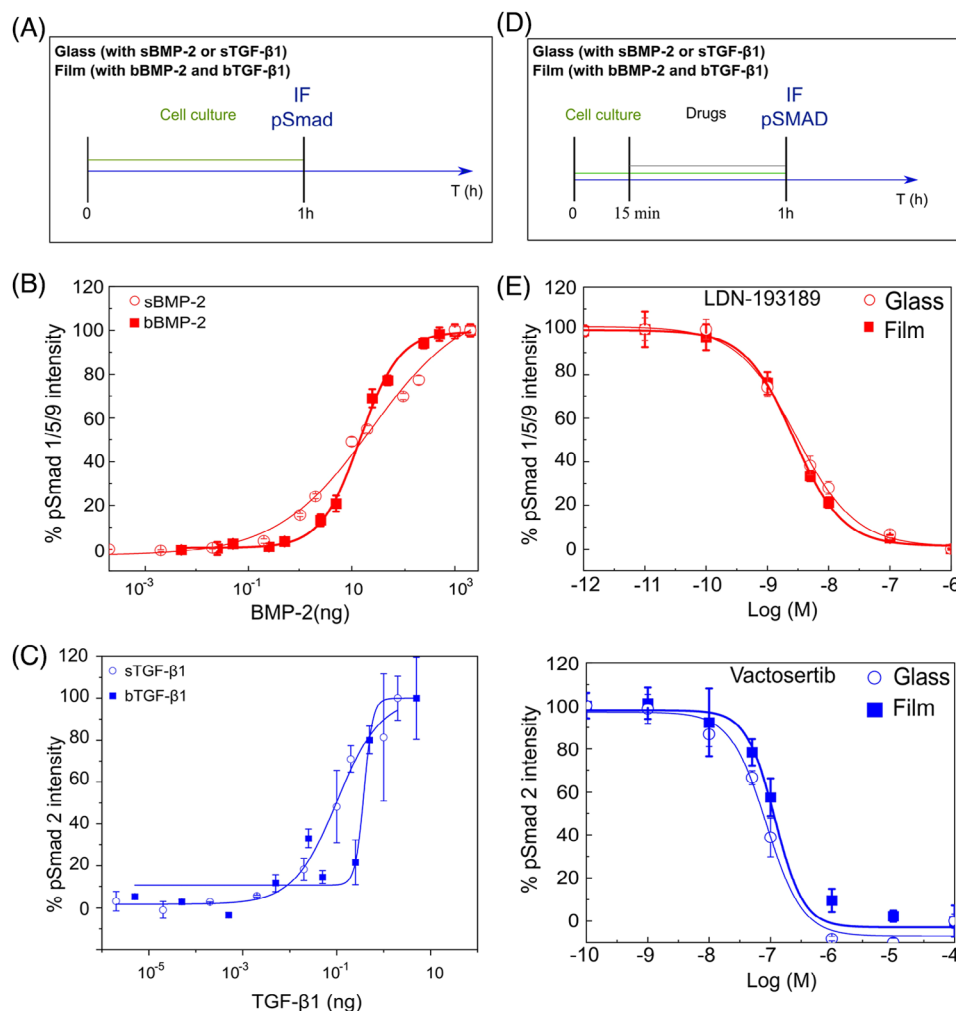
For the film condition, we seeded C2C12 cells directly on the biomimetic films loaded with bBMP-2 and bTGF- $\beta$ 1. For glass condition, the medium was supplemented with sBMP-2 or sTGF- $\beta$ 1 as described in Figure 5A, in contrast to the previous dose–response assay performed with soluble protein on glass support, after 24 h of

cell culture (Figure 3). After quantification of Smad intensity (Figure 5B, C), we determined the EC<sub>50</sub> values. The values of EC<sub>50</sub> on glass are  $120 \pm 47 \text{ ng mL}^{-1}$  (corresponding to  $24.0 \pm 9.4 \text{ ng}$ ) for sBMP-2, and  $0.5 \pm 0.16 \text{ ng mL}^{-1}$  (equivalent to  $0.1 \pm 0.03 \text{ ng}$ ) for sTGF- $\beta$ 1. For the film, the EC<sub>50</sub> values were determined to be  $288 \pm 20 \text{ ng mL}^{-1}$  (equivalent to  $14.4 \pm 1.0 \text{ ng}$ ) for bBMP-2, and  $8 \pm 2 \text{ ng mL}^{-1}$  (corresponding to  $0.4 \pm 0.1 \text{ ng}$ ) for bTGF- $\beta$ 1. So, EC<sub>50</sub> values were globally higher for cells on films in comparison to glass. EC<sub>50</sub> were about two-fold higher for sBMP-2 and about 16-fold higher for TGF- $\beta$ 1.

Also, we noted that EC<sub>50</sub> value of sBMP-2, when cell seeding and sBMP-2 are added simultaneously (Figure 5B, C), are higher than the EC<sub>50</sub> value of sBMP-2 and sTGF- $\beta$ 1 when cells have adhered for 24 h: EC<sub>50</sub> is  $24.0 \pm 9.4$  versus  $0.2 \pm 0.01 \text{ ng}$  for sBMP-2, and  $0.1 \pm 0.03$  versus  $0.02 \pm 0.002 \text{ ng}$  for sTGF- $\beta$ 1 (Figure 3C).

Interestingly, we can also observe that the slopes of the pSmad response depends on the presentation mode, either soluble or matrix-bound. The slopes are representative of the sensitivity of the assay. For sBMP-2, it was  $0.47 \pm 0.07$  while it was two-fold higher for bBMP-2 at  $1.15 \pm 0.07$ . For sTGF- $\beta$ 1, the difference was even stronger, with a slope of  $0.63 \pm 0.40$  for sTGF- $\beta$ 1 versus  $5.35 \pm 2.82$  for bTGF- $\beta$ 1, that is, a 10-fold increase.





**FIGURE 5** ALK inhibitor drug assay using soluble and matrix-bound BMP-2 and TGF- $\beta$ 1. (A) Schematics of the experimental protocol of the dose-response tests for cells cultured on glass and biomaterials, in the presence of sBMP-2/bBMP-2 and sTGF- $\beta$ 1/bTGF- $\beta$ 1. (B) A dose-response test with sBMP-2 for cells on glass and bBMP-2 for cells on films, respectively, using C2C12 cells. (C) A dose-response test with sTGF- $\beta$ 1 for cells on glass, and bTGF- $\beta$ 1 for cells on films, respectively, with C2C12 cells. (D) Schematics of the experimental protocol of the drug tests on glass and biomaterials with sBMP-2/bBMP-2 and sTGF- $\beta$ 1/bTGF- $\beta$ 1. (E) Drug assay of LDN-193189 with sBMP-2 and bBMP-2, and (F) Vactosertib with sTGF- $\beta$ 1 on glass, and bTGF- $\beta$ 1 with C2C12 cells on films. bBMP-2, matrix-bound BMP-2 loaded in the biomimetic films; BMP, bone morphogenetic protein; bTGF- $\beta$ 1, matrix-bound TGF- $\beta$ 1 loaded in the biomimetic films; sBMP-2, soluble bone morphogenetic protein-2; sTGF- $\beta$ 1, soluble TGF- $\beta$ 1; TGF- $\beta$ , transforming growth factor.

After characterizing the cellular response to BMP-2 and TGF- $\beta$ 1 on biomaterials, we chose suitable concentrations higher than the EC50 values to perform the drug experiments. We selected LDN-193189, one of the first discovered kinase inhibitor of type I BMP receptors,<sup>[23]</sup> in addition to two ALK5 kinase inhibitors being developed in cancer clinical trials: galunisertib, which was stopped at phase 2 of clinical trials by the sponsor company, and vactosertib, which is currently in phase 2.<sup>[24,25]</sup> The experiments were performed as shown in Figure 5D. The drugs were added for 45 min, followed by cell fixation.

After quantification of Smad signal, we determined the IC50, defined as the concentration of pharmacological agent required to inhibit half the maximal biological response, and used to determine a drug efficacy.<sup>[26]</sup> The results showed similar curves and IC50 values

for both the film and glass conditions (Figure 5E). The IC50 for LDN-193189 is determined at  $3.0 \pm 0.03$  and  $2.7 \pm 0.2$  nM for glass and film, respectively. The IC50 of vactosertib were found to be  $85 \pm 12$  and  $118 \pm 21$  nM, for glass and film, respectively. For galunisertib, IC50 are  $1276 \pm 79$  and  $1587 \pm 552$  nM for glass and film, respectively (Figure S3). Thus, the underlying support onto which cells are cultured does not appear to influence IC50 value.

In order to validate that IC50 are not BMP concentration-dependent, we performed a drug assay with LDN-193189 in the presence of several concentrations of BMP-2. The results showed that the pSmad intensity is independent of BMP-2 concentration (Figure S4). This result may be explained by the action mode of the inhibitors that inhibit the BMP and TGF- $\beta$  signaling at the receptor level.

In order to determine the stability of the IF assay in performing high-throughput screens, we carried out a  $Z'$  factor experiment, where LDN-193189 was added at a concentration of 100 nM and BMP-2 at 2  $\mu\text{g mL}^{-1}$  and 400  $\text{ng mL}^{-1}$  for the film and glass, respectively. The study demonstrated a  $Z'$  factor of 0.3 for glass and 0.6 for film. Since an acceptable assay has a  $Z'$  factor around and higher than 0.5,<sup>[27]</sup> we show here that our pSmad assay is adapted for high-content drug assays on both glass and biomaterials.

## 4 | DISCUSSION

In this study, we establish a new assay to quantify pSMAD pathway at high content using IF. We defined and optimized all steps of the method (Figures 1, 2) and compared the conditions where BMP-2 is delivered in solution or via a biomimetic film where it is in a matrix-bound manner. Since, IF and immunohistochemistry are considered semi-quantitative methods, research efforts have been directed toward standardization of the signal and the development of imaging analysis techniques to determine protein expression.<sup>[28]</sup> These techniques are then compared to established protein measurements methods such as mass spectrometry<sup>[29]</sup> and ELISA.<sup>[30]</sup>

Our methodology consisted on performing a dose–response assay and establishing our assay on glass support as shown in Figure 3C. The EC50 values that we obtained ( $0.92 \pm 0.06 \text{ ng mL}^{-1}$  for sBMP-2 and  $0.12 \pm 0.01 \text{ ng mL}^{-1}$  for sTGF- $\beta$ 1) are consistent with the literature data: a study reported the EC50 values with an AlphaLISA test in C2C12 to be  $0.9 \pm 0.1 \text{ ng mL}^{-1}$  for BMP-2 and  $0.052 \pm 0.002 \text{ ng mL}^{-1}$  for TGF- $\beta$ 1.<sup>[31]</sup> In addition, other studies determined using luciferase assay, the EC50 of BMP-4 to be  $0.3 \text{ ng mL}^{-1}$  in human cervical carcinoma cells,<sup>[32]</sup> and  $2.4 \text{ ng mL}^{-1}$  in human embryonic kidney cells (HEK293).<sup>[33]</sup> If compared to BMP-4, the values of EC50 for BMP-2 appears to be comparable to literature data. Our data using C2C12 are also consistent with EC50 values on the same cells obtained using Western blot.<sup>[32]</sup> The detection limit of the pSmad signal is of the same order of magnitude.

After confirmation of EC50 with the literature, we performed kinetic studies (Figure 4). These not only aided in identifying the optimal time window for the drug assay but also served as a proof of concept for studying variations in transcription factor pathways within the cells. The kinetic results showed an initial signal peak at 1 h or 30 min followed by a sustained signal for 48 or 24 h for BMP-2 (Figure 4A) and TGF- $\beta$ 1 (Figure 4B), respectively. These observations are consistent with the literature. For instance, kinetic studies of BMP-2 by Western blot demonstrated the presence of a pSmad 1/5/8 (with Smad 8 also known as Smad 9) signal after 30 min with a sustained signal up to 180 min on different types of biomaterials.<sup>[34–36]</sup> Similarly, studies with TGF- $\beta$ 1 in various cell models showed the activation of pSmad 2 signaling after 30 min of TGF- $\beta$ 1 addition, and up to 8 h after periodic pulse exposure to the ligand.<sup>[37,38]</sup>

Notably, the presentation of matrix-bound BMP/TGF- $\beta$  leads to a sustained signal with less oscillations. The presence of a transient or a sustained signal may be explained by ligand depletion.

Indeed, it was reported that it is the short stimulation of TGF- $\beta$  that causes transient activation by controlling the duration of Smad 2 phosphorylation.<sup>[38]</sup> In addition, factors, such as the mechanism of trafficking across the nuclear membrane, interactions with inhibitory Smads, and the sequestration of TGF- $\beta$  receptors by endocytosis after ligand binding to the receptors and Smads recruitment, contribute to this phenomenon.<sup>[39,40]</sup>

In the case of matrix-bound ligands, the large number of ligands available in the biomimetic and their spatial proximity ensures that the BMP/TGF- $\beta$  receptors are activated, and also, clustered at the basal side of the cells. Thus, it is likely that this receptor clustering, which is specific to the case of matrix-bound proteins, is at the origin of increased and sustained signal activation.

Dose–response studies and EC50 were also performed (Figure 5B, C). The higher EC50 values observed when BMP-2 was added to non-adhered cells compared to cells adhered for 24 h, align with previous research examining the adhesion of C2C12 on the biomimetic films containing bBMP-2.<sup>[41]</sup> They showed that when BMP is presented in a matrix-bound manner within biomimetic films, a crosstalk between the BMP receptors and integrins occurs, influencing cell adhesion and cell differentiation. The sequence of events is initiated with BMP-2, binding to its BMP receptors, activating integrins, and subsequently triggering Smad signaling.<sup>[41]</sup> Thus, it is plausible to hypothesize that BMP-2 introduced before cellular adhesion aids in focal adhesion formation, ensuring cell adhesion and activating BMPR to initiate pSmad signaling. A similar mechanism likely occurs on glass substrates, suggesting that the adhesion dynamics play a role in pSmad response. It seems that there is a need for higher quantities of GFs to activate pSmad before cells adhere, in comparison to the case where cells are already adherent.

Regarding, the drug tests, the data in the literature on IC50 values varies due to type of cells and type of assays. For instance, LDN-193189, vactosertib, and galunisertib, reported IC50 values were of 0.8, 11, and 56 nM respectively by the manufacturer, using in vitro kinase assays commonly used in drug development. These assays measure the kinase activity by quantifying the transfer of radioactive ATP to the substrate.<sup>[42]</sup> Another study on pSmad 1/5/9 signaling inhibition with BMP-4 and LDN-193189 determined an IC50 to be 4.9 nM.<sup>[23]</sup> However, IC50 for galunisertib is reported to range from 50 to 430 nM depending on the employed assay.<sup>[43]</sup> Nonetheless, our findings indicate the values of IC50 are comparable for cells cultured on film or on glass conditions. So, they are independent of the presentation mode of the GF (Figure 5E, Figure S2). This consistency in results with the glass standard condition used as reference for in vitro drug testing suggests that the three drugs act similarly and independently of the presentation mode of BMPs. Furthermore, these results highlight the reliability of the IF assay and its adaptability to various experimental conditions involving soluble and matrix-bound proteins, cells cultured on glass or on biomaterials. This opens the possibility to investigate pathologies in a model biomechanical context by modifying and tuning the biomimetic film properties, like film stiffness and presentation of GFs or peptides.

In terms of assay, the IF assay is an alternative to the reference method to study pSmad signaling in untransformed cells, the

Western blot. Besides employing IF as a validation method to confirm results obtained by Western blot and to image cells, this assay offers the advantage of being applicable in various types of cell culture microplates. Using microplates enables parallel and simultaneous assessment of numerous experimental conditions. Advantageously, it can also be combined with other quantitative labeling done on the same samples, such as cytoskeleton staining or cell surface markers. It may also be combined with other imaging modes, like phase contrast imaging or colorimetric staining, to quantify other parameters. As shown here, the IF-based method is compatible with high content assays and drug testing. Potentially, it may use to characterize cells at the single cell level or selectively identify cells, like subpopulations having certain phenotypic characteristics.<sup>[29]</sup> Furthermore, different signaling pathways may be studied simultaneously, using other markers. As potential disadvantage, one needs to use a high content microscope and to conduct image acquisition and image analysis following standardized protocols. These protocols and equipment may not be easy to handle and requires dedicated training, in comparison to Western blot experiments. In view of the higher experimental complexity than doing Western blot, cell biology labs may not be tempted to use it. However, nowadays, research institutes are equipped with microscopy platforms that can be used for this purpose. In view of the potentialities offered, the IF-based method could be easily adapted to various microscopes and to multimode microplate readers.

We also envision that the IF-based assay will be used for single cell studies. By doing so, subpopulations may be distinguished and mechanistic studies could reveal heterogeneities in the cellular responses. Last but not least, IF and immunohistochemistry could potentially have several clinical applications, notably in cancer immunotherapy. Indeed, quantitative spatial distribution of immune markers such as programmed death ligand 1 (PD-L1) and FOXP3 is already in use to predict overall survival in breast cancer during prognosis and after chemotherapy.<sup>[44,45]</sup> Thus, our quantitative approach could further facilitate applications in oncology and dermatology.

## 5 | CONCLUSION

In this work, we introduced and optimized a new method based on IF to quantify transcription factors at high content. We apply it to study pSmad pathways for cells cultured on glass, in the presence of GFs added in solution, and for cells cultured directly on biomaterials with matrix-bound GFs. We optimize the classical protocol for immunostaining to conduct kinetic tests on glass and on biomimetic films with matrix-bound GFs. We showed that the kinetics of pSmad expression in the nucleus is different when the GFs are presented in a matrix-bound manner, in comparison to when they are delivered in solution. In terms of detection limit, the IF-based method is as sensitive as the Western blot method. The dose-response to GFs highlights that sensitivity is higher when the GFs are physically bounded to the biomimetic films, compared to their soluble counterparts. Moreover, we present a proof-of-concept for a drug assay targeting pSmad activity. In the future, this

assay may be more widely used on biomimetic films containing other ECM proteins or GFs, and for mechanistic studies at the single cell level.

## AUTHOR CONTRIBUTIONS

**Valia Khodr:** Investigation; data curation; visualization; writing-original draft preparation; **Laura Clauzier:** Investigation; data curation; visualization; writing-review & editing; **Paul Machillot:** Investigation; methodology; software; writing-review & editing; **Adria Sales:** Methodology; writing-review & editing; **Elisa Migliorini:** Writing-review & editing; **Catherine Picart:** Conceptualization; data curation; funding acquisition; supervision; writing-review & editing.

## ACKNOWLEDGMENTS

The authors thank Marie-Odile Fauvarque, Emmanuelle Soleilhac, and Catherine Pillet for fruitful discussions and advices, notably regarding the drug assay experiments. The authors are also grateful to Sabine Bailly for her advices, reading of the manuscript, feedback, and suggestions. The study was supported by the Agence Nationale de la Recherche (ANR CODECIDE, ANR-17-CE13-022), and Institut Universitaire de France (IUF) to C.P. In addition to the European Research Council (ERC) under FP7 program (ERC Biomim GA259370), the ERC POC Bioactive coatings (GA 692924), the Fondation Recherche Médicale (FRM, grant DEQ20170336746), and the Ligue Régionale Contre le Cancer (R22, Isère, Savoie) to C.P. V.K. was supported by a PhD fellowship from Grenoble Institute of Technology.

## CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Khodr, V., Clauzier, L., Machillot, P., Sales, A., Migliorini, E., & Picart, C. (2024). Development of an automated high-content immunofluorescence assay of pSmads quantification: Proof-of-concept with drugs inhibiting the BMP/TGF- $\beta$  pathways. *Biotechnology Journal*, 19, e240007. <https://doi.org/10.1002/biot.202400007>