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Spore Adsorption as a Nonrecombinant Display System for Enzymes and Antigens

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UNIVERSITÀ DEGLI STUDI DI NAPOLI FEDERICO II
Scuola Politecnica e delle Scienze di Base
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Dear Editor,

I would like to thank you and the Reviewers for your consideration of our manuscript and the comments aimed at improving it.
We are now submitting a revised version of the manuscript that takes into consideration all comments raised by you and the Reviewers. In this “revised version” all changes are marked in red and the essential steps of the protocol are highlithed in yellow.
A point-by-point response (rebuttal letter) to the reviewer’s comments has been provided.
We hope our manuscript is now suitable for publication on JOVE

Sincerely,

Ezio Ricca

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TITLE:

Spore Adsorption as a Nonrecombinant Display System for Enzymes and Antigens

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KEYWORDS:

Mucosal delivery, vaccines, recyclable enzymes, biocatalyst, spore formers, display platform

SUMMARY:

This protocol focuses on the use of bacterial spores as a “live” nanobiotechnological tool to adsorb heterologous molecules with various biological activities. The methods to measure the efficiency of adsorption are also shown.

ABSTRACT:

The bacterial spore is a metabolically quiescent cell, formed by a series of protective layers surrounding a dehydrated cytoplasm. This peculiar structure makes the spore extremely stable and resistant and has suggested the use of the spore as a platform to display heterologous molecules. So far, a variety of antigens and enzymes have been displayed on spores of *Bacillus subtilis* and of a few other species, initially by a recombinant approach and, then, by a simple and efficient nonrecombinant method. The nonrecombinant display system is based on the direct adsorption of heterologous molecules on the spore surface, avoiding the construction of recombinant strains and the release of genetically modified bacteria in the environment. Adsorbed molecules are stabilized and protected by the interaction with spores, which limits the rapid degradation of antigens and the loss of enzyme activity at unfavorable conditions. Once utilized, spore-adsorbed enzymes can be collected easily with a minimal reduction of activity and reused for additional reaction rounds. In this paper is shown how to adsorb model molecules to purified spores of *B. subtilis*, how to evaluate the efficiency of adsorption, and how to collect used spores to recycle them for new reactions.

INTRODUCTION:

Display systems are aimed at presenting biologically active molecules on the surface of microorganisms and finding applications in a variety of fields, from industrial to medical and environmental biotechnologies. In addition to phages[1,2] and cells of various Gram-negative and -positive species[3–7], bacterial spores have also been proposed as display systems by two approaches[8,9].

Because of its peculiar structure, namely a dehydrated cytoplasm surrounded by a series of protective layers[10], the spore provides several advantages over phage- and cell-based display systems[8,9]. A first advantage comes from the extreme robustness and stability of spores at conditions that would be deleterious to all other cells[10,11]. Spore-displayed antigens and enzymes are stable after prolonged storage at room temperature[12] and protected from degradation at low pH and high temperatures[13]. A second advantage of spores is the safety of many spore-forming species. *B. subtilis, B. clausii, B. coagulans*, and several other species are used worldwide as probiotics and have been on the market for human or animal use for decades[14,15]. This exceptional safety record is an obvious general requirement for a surface display system and is of particular relevance when the system is intended for human or animal use[16]. A third, important advantage of a spore-based display system is that it does not have limitations for the size of the molecule that has to be exposed. In phage-based systems, a large heterologous protein may affect the structure of the capsid, while in cell-based systems, it may affect the structure of the membrane or may limit/impair the membrane translocation step[17]. The protective layers surrounding the spore are composed of more than 70 different proteins[10] and are flexible enough to accept large foreign proteins without any evident structural defect or functional impairment[8]. In addition, with both spore-based display systems, the membrane translocation of the heterologous protein is not required[8,9]. Indeed, heterologous proteins are either produced in the mother cell cytoplasm and assembled on the spore that is forming in the same cytoplasm or adsorbed on the mature spore[8,9].

Spore display was initially obtained by developing a genetic system to engineer the spore surface[18]. This genetic system was based on i) the construction of a gene fusion between the gene coding for a spore coat protein (used as a carrier) and the gene coding for the protein to be displayed—the presence of the transcriptional and translational signals of the endogenous gene will control the expression of the fusion, and ii) integration of the chimeric gene on the *B. subtilis* chromosome to grant genetic stability. A variety of antigens and enzymes have been displayed by this recombinant approach, using various spore surface proteins as carriers and aiming at various potential applications, ranging from mucosal vaccine to biocatalyst, biosensor, bioremediation, or bioanalytical tool[8,13].

More recently, a different approach of spore display has been developed[19]. This second system is nonrecombinant and relies on the spontaneous and extremely tight adsorption of molecules on the spore surface[9]. Antigens[19,20] and enzymes[13,21] have been efficiently displayed and have revealed that this method is significantly more efficient than the recombinant one. This nonrecombinant approach allows the display of proteins in the native form[20] and can also be used with autoclaved, death spores[19]. The molecular mechanism of adsorption has not been fully clarified yet. The negative charge and the hydrophobicity of the spore have been proposed as properties relevant for the adsorption[13,19,22]. Recently, it has been shown that a model protein, the red autofluorescent protein (mRFP) of the coral *Discosoma*, when adsorbed to the spore, was able to infiltrate through the surface layers localizing in the inner coat[23]. If proved true for other proteins, the internal localization of the heterologous proteins could explain their increased stability when adsorbed to spores[23].

In a recent study, two enzymes catalyzing two successive steps of the xylan degradation pathway were independently displayed on spores of *B. subtilis* and, when incubated together, were able to perform both degradation steps[21]. Spores collected after the reaction were still active and able to continue the xylan degradation upon the addition of fresh substrate[21]. Even if a loss of about 15% of the final product

was observed in the second reaction[21], the reusability of adsorbed enzymes for single, as well as multi-step, reactions is an additional important advantage of the spore display system.

Pan et al.[24] reported an additional approach to display heterologous proteins on the spore surface: heterologous proteins (an endoglucanase protein and a beta-galactosidase one) produced in the mother cell during sporulation were spontaneously encased in the forming spore coat, without the need of a carrier. This additional spore display system is a combination of the two approaches described so far. Indeed, it is recombinant since the heterologous proteins were engineered to be expressed in the mother cell during sporulation, while their assembly within the coat was spontaneous and, therefore, nonrecombinant[24]. However, the efficiency of display of this additional approach remains to be tested and compared with the other two approaches by using the same heterologous proteins.

The present protocol excludes the processes of spore production and purification, which have been described extensively elsewhere[24]. It includes the adsorption reaction, the evaluation of the efficiency of adsorption by dot-blotting and fluorescence microscopy, and the recycling of adsorbed enzymes for additional reaction rounds.

PROTOCOL:

1. Adsorption reaction

1.1. Incubate 2, 5, and 10 µg of mRFP with 2 x 10^9 of *B. subtilis* wild-type purified spores in 200 µL of binding buffer, 50 mM sodium citrate, pH 4.0 (16.7 mM sodium citrate dihydrate; 33.3 mM citric acid) for 1 h at 25 °C on a rocking shaker (**Figure 1**).

1.2. Centrifuge the binding mixtures (13,000 x *g* for 10 min) to fractionate pellets (P2, P5, and P10) and supernatants (S2, S5, and S10). Store the supernatants for the indirect evaluation of the efficiency of adsorption described in step 3.2.

1.3. Wash the pellets 2x with 200 µL of binding buffer and resuspend them in 100 µL of binding buffer and use it for the next analysis.

NOTE: The binding reaction preferentially occurs at a pH value lower than the isoelectric point of the protein; typically, 1.5 M phosphate-buffered saline (PBS), pH 4.0, or 50 mM sodium citrate, pH 4.0, are used.

2. Direct evaluation of the efficiency of adsorption

2.1. Extraction of surface proteins and western blot analysis

2.1.1. Take 50 µL of mRFP-adsorbed spores' resuspension (P2, P5, and P10 of step 1.3) and add 50 µL of 2x sodium dodecyl sulfate (SDS)-dithiothreitol (DTT) (0.1 M Tris-HCl, pH 6.8; 2% SDS; 0.1 M DTT) to solubilize surface spore proteins.

2.1.2. Use the free, purified mRFP and extracts of the same amounts of spores that are not adsorbed to the protein as positive and negative controls, respectively.

2.1.3. After 45 min of incubation at 65 °C, centrifuge (13,000 x *g* for 10 min) the mixtures and analyze 10 µL of the extracted proteins (supernatant) by western blot with the monoclonal anti-His antibody recognizing the His tag present at the N-terminal of mRFP (**Figure 2A**).

2.2. Fluorescent microscopy observations

NOTE: By using fluorescent heterologous proteins or performing an immunofluorescence analysis, it is possible to localize and quantify the adsorbed molecules by fluorescence microscopy.

2.2.1. Take 5 µL of the adsorbed spores' resuspension from step 1.3 and add 95 µL of 1x PBS, pH 4.0, to obtain ~1 x 10^6 spores/µL.

2.2.2. Place 5 µL of suspension on a microscope slide, cover it with a coverslip previously treated with poly-l-lysine for 30 s, and observe it under a fluorescence microscope.

2.2.3. For each field, save the phase-contrast microscopy image and fluorescence microscopy image (**Figure 2B**).

NOTE: Alternatively, fluorescent spores can be analyzed by cytofluorimetry. Resuspend a total of 10^6 spores adsorbed or not-adsorbed with the heterologous protein in 1 mL of 1x PBS, pH 4.0, and analyze the suspension using a flow cytometer (**Figure 2C**).

2.3. Data analysis using ImageJ

2.3.1. Open the fluorescence microscopy images with ImageJ software (http://rsbweb.nih.gov/ij/) and check to make sure all images are in the 8-bit format (**Image** | **Type** | **8-bit**).

2.3.2. Adjust the contrast if necessary (**Image** | **Adjust** | **Brightness Contrast**) and verify that the scale of the image is PIXEL (**Image** | **Set Scale**).

2.3.3. From the Analyze menu, select **Set measurements**. Make sure to have **Area**, **Integrated Density**, and **Mean Gray Value** selected.

2.3.4. Draw a line around the spore of interest using any of the drawing/selection tools (i.e., circle, polygon, or freeform) (**Figure 3**).

2.3.5. Select **Measure** from the Analyze menu (or hit **cmd** + **M**). A popup box with a stack of values for this first spore appears (**Figure 3**).

2.3.6. Repeat these two steps for at least 50 other spores in the field of view chosen to be measured.

2.3.7. Select several regions without any spores (that have no fluorescence) and repeat the measurement; this will be the background.

NOTE: The size is not important. These corresponding background fluorescence values will be used to manually subtract the background.

2.3.8. Select all the data in the Results window and copy the results into a spreadsheet.

2.3.9. Calculate the mean of the **Integrated Density** and **Area** of the selected spores and of the background fluorescence values and use them to obtain the corrected total-per-cell fluorescence (CTCF) using the formula: CTCF = mean Integrated Density - (mean Area x mean Background fluorescence).

NOTE: Alternatively, if all the spores appear well separated, it is possible to analyze all the spores of the image field using the function **Analyze Particles**, following ImageJ's instruction. To avoid that the software reads a specific fluorescence or spore aggregates, the dimension of particles has to be set at pixelˆ2 = 50–200 (**Figure 4**).

3. Indirect evaluation of the efficiency of adsorption

3.1. Prepare serial dilutions for the purified protein.

3.1.1. Prepare a first 1.5 mL tube containing 250 µL of purified mRFP at a final concentration of 0.5 ng/µL, using the binding buffer. This volume is sufficient to load two lanes.

3.1.2. Perform six twofold serial dilutions of 250 µL (final volume) each, using the binding buffer.

3.2. Prepare twofold serial dilutions for the supernatant samples containing the unbound mRFP fraction of the adsorption reaction (S2, S5, and S10 from step 1.2).

3.2.1. Put 100 µL of each supernatant in a 1.5 mL tube and add 100 µL of binding buffer. Perform six twofold serial dilutions of 200 µL (final volume) each, using the binding buffer.

3.3. Cut a nitrocellulose membrane (0.45 µm cutoff), 9 cm x 10 cm in size, to cover the area of 5 (number of samples) x 6 (number of dilutions) dots. The membrane should not extend beyond the edge of the gasket of the dot blot apparatus.

3.4. Place the prewet membrane in the dot blot apparatus. Remove any air bubbles trapped between the membrane and the gasket. Cover the unused portion of the apparatus with tape or paraffin film to prevent air from moving through those wells.

3.5. Assemble the dot blot apparatus as described by the manufacturer.

3.6. If a vacuum is used during the assembling, rehydrate the membrane with 100 µL of 1x PBS per well to ensure the uniform binding of the antigen and prevent halos or a weak detection signal.

3.7. Gently remove the buffer from the wells by vacuum. As soon as the buffer solution drains from all the wells, stop the vacuum pump and disconnect it.

3.8. Load the standard in the two most external lanes and the samples in the middle (**Figure 5**). Fill the appropriate wells with 100 µL of each dilution. The same volume used for each well should ensure a homogeneous filtration of all sample wells.

3.9. Turn on the vacuum pump for 2 min, stop it, and then, allow the sample to filter through the membrane by gravity flow.

3.10. Wash all the wells with 100 µL of 1x PBS and let the vacuum pump run for another 5 min after the washing buffer has been completely drained from the apparatus.

3.11. With the vacuum on, loosen the screws, and carefully open the dot blot apparatus.

3.12. Turn off the vacuum, take the membrane, and process it following a western blot protocol.

3.13. Perform a densitometric analysis of the filter, using appropriate software, such as ImageJ.

3.13.1. Measure the integrated density of each dot by outlining them with a circle of the same area and using the **Analyze/Measure** command.

3.13.2. Make a background correction of the image, drawing a circle in an empty area and measuring its integrated density or using the **Process/Subtract Background** command.

3.13.3. Correlate the integrated density of the standard dots with the amount of loaded protein and obtain a calibration line (R^2 values for calibration curves should be over 0.95).

3.13.4. Use the calibration curve to extrapolate the concentration of mRFP of each sample dot.

3.13.5. Calculate the concentration of the mRFP remaining in the unbound fractions.

NOTE: To ensure the correct closing of the dot blot apparatus and, therefore, to subject the membrane to a uniform pressure, tighten the screws by following a diagonally crossed scheme and, then, open the vacuum pump to tighten the screws more strongly.

4. Spore collection and reuse

4.1. For the recycling of the adsorbed spore, perform two adsorption reactions of 2.0 x 10^9 purified spores with 10 µg of purified GH10-XA xylanase or 10 µg of purified GH3-XT β-xylosidase, as described in steps 1.1–1.3 (**Figure 6A**).

4.2. Collect the pellets containing the enzyme-adsorbed spores, resuspend them in 50 µL of the optimal buffer for the enzymatic reaction assay (50 mM sodium phosphate buffer at pH 6.5; 2.48689 g/L

Na_2HPO_4, 4.88991 g/L NaH_2PO_4), and mix them together to obtain a 100 µL mixture of spores adsorbing GH10-XA or GH3-XT.

4.3. Add the substrate (5 mg/mL 4-O-methyl-d-glucuronod-xylan [MGX]) and let the enzyme reactions take place for 16 h at 65 °C.

4.4. Centrifuge the reaction mixture (for 15 min at 13,000 x *g*) and store the supernatant containing the enzyme reaction product.

4.5. Resuspend the pellet in 100 µL of fresh 50 mM sodium phosphate buffer at pH 6.5 in the presence of a new substrate (MGX) (**Figure 6B**).

NOTE: To adsorb more than one enzyme, it is possible to adsorb both together or one by one independently. The latter possibility facilitates the quantitative analysis of the adsorption efficiency and of the activity of each enzyme, allowing a stoichiometric balance of each enzyme needed for the reactions.

REPRESENTATIVE RESULTS:

Successful adsorption can be assessed by western blotting. Upon the reaction, the mixture is fractionated by centrifugation and washed, and the pellet fraction (**Figure 1**) is used to extract the surface proteins. The extract is fractionated by SDS polyacrylamide gel electrophoresis (PAGE), electrotransferred to a polyvinylidene fluoride (PVDF) membrane, and reacted against primary and secondary antibodies. The presence of proteins of the expected size, only in the lane loaded with an extract of the adsorbed spores, is indicative of a successful adsorption reaction (**Figure 2A**).

The efficiency of the adsorption reaction can be evaluated by direct and indirect methods. The direct evaluation of the adsorption efficiency depends on the heterologous protein that has been used and can be performed by fluorescence microscopy (**Figure 2B**) and cytofluorimetry (**Figure 2C**) on the pellet fraction after the fractionation of the adsorption reaction. A quantification of the fluorescent signals present on spores can be performed by using the ImageJ software (**Figure 3** and **Figure 4**). An indirect analysis of the adsorption efficiency can be performed by a dot blotting analysis (**Figure 5A**) of the supernatant fraction containing the unbound protein (**Figure 1**). A densitometric analysis of the unbound protein (**Figure 5B**) will then allow scientists to calculate indirectly the amount of protein adsorbed on spores.

Two successive reactions can be catalyzed by a mixture of spores displaying either one of the two specific enzymes (**Figure 6A**). The adsorbed enzyme(s) can be collected with a simple centrifugation step, washed, and incubated with fresh substrate for a new reaction cycle (**Figure 6B**).

FIGURE LEGENDS:

Figure 1: General scheme of the adsorption experiment.

Figure 2: Direct analysis of the efficiency of the mRFP display. (**A**) Western blot with mRFP-specific antibody. C+ = free purified mRFP; C- = a protein extract from spores not adsorbed to the protein;

P2/P5/P10 = proteins extracted from *B. subtilis* spores adsorbed with 2, 5, and 10 mg of mRFP, respectively. (**B**) Immunofluorescence microscopy of adsorbed spores. Immunoreactions were performed with a primary antibody recognizing the adsorbed protein and with a fluorescent secondary antibody conjugated with fluorescein isothiocyanate (FITC). The left panel shows the red intrinsic mRFP fluorescence, the right panel shows the green fluorescence of FITC-conjugated secondary antibody. (**C**) Flow cytometric analysis of adsorbed spores. The spores reacted with mRFP-specific antibodies and with FITC-conjugated secondary antibodies and, then, were analyzed by cytofluorimetry. The analysis was performed on the entire spore population (10,000 events, ungated). In the left panel, not-adsorbed spores are shown in black, mRFP-adsorbed spores in red. The right panel shows the forward and side scatter (FSC-SSC) dot plot.

Figure 3: Manual quantification of the fluorescent signal by ImageJ. A fluorescence microscope image of spores, adsorbed with the red fluorescent protein mRFP, analyzed with ImageJ software. A yellow circle has been drawn around one spore to obtain densitometric data (enlarged image). The popup box shows the results of the densitometric analysis of the selected spore.

Figure 4: Simultaneous quantification of the fluorescent signal by ImageJ. (**A**) Popup box obtained when selecting **Analyze Particle**. An interval value of 50–200 pixel^2 has to be set for spores[23]. (**B**) Segmentation of the image of **Figure 3A** after using **Analyze Particles** (left), and the relative densitometric analysis results (right).

Figure 5: Dot blotting and densitometric analysis. (**A**) Dot blotting with serial dilutions of the purified mRFP in duplicate (Std_1 and Std_2) and the supernatant (S10, S5, and S2) of the adsorption reaction performed with 10, 5, and 2 µg of mRFP, respectively[23]. (**B**) The dot blotting of panel **A** is used for the densitometric analysis. The circles indicate the area used to quantitate the density of the signals. The panel on the right reports an example of results obtained with the densitometric analysis.

Figure 6: Conversion of xylan by reusable spores. (**A**) General scheme of xylan degradation. (**B**) Spores displaying the xylanase or the β-xylosidase enzymes, when mixed together, catalyze the two-step degradation of xylan. After the reaction, the sample is fractionated by centrifugation. The supernatant contains the reaction product, while the pellet contains spore-bound enzymes that can be reused by adding fresh substrate.

DISCUSSION:

This spore adsorption protocol is very simple and straightforward. The reaction is strictly dependent on the pH of the reaction buffer and the efficiency of adsorption is optimal at acidic pH values (pH 5.0 or lower). At neutral pH conditions, the efficiency of adsorption is low, and at alkaline pH values, adsorption may not occur. Optimal adsorption is obtained using a volume of 200 µL in 1.5 mL tubes (or keeping a similar ratio) on a rocking shaker.

Adsorption is very tight, and washes with a buffer at the same pH of the reaction buffer do not cause any release of the adsorbed proteins. Washes with alkaline buffers may result in a minimal (generally less than 15%[26]) release of the adsorbed protein.

The indirect evaluation of the efficiency of adsorption by dot blotting is reliable if several dilutions of

purified and unbound protein are analyzed and the densitometric analysis is properly performed. No evidence of degradation of the heterologous proteins has been reported[25]. The direct evaluation of the efficiency of adsorption greatly depends on the protein that is adsorbed. If the protein is autofluorescent or fluorescently labeled, an ImageJ-assisted analysis provides a quantitative determination of the fluorescence and of the amounts of fluorescent molecules present on the spore. If the protein has an enzymatic activity, a specific enzymatic assay could provide an indication of the amounts of protein present on the spores. However, it is known that the enzymatic activity associated with spores may be increased by a stabilization effect due to the interaction with the spore[13]. If the adsorbed protein is not fluorescent and does not have an enzymatic activity, the efficiency of adsorption can be evaluated by dot blotting on spores extracted under drastic conditions.

A collection of used spores can be done by a very simple procedure. A washing step with the reaction buffer may be important to remove reaction by-products, while the addition of fresh substrate is essential to initiate a new reaction[21].

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

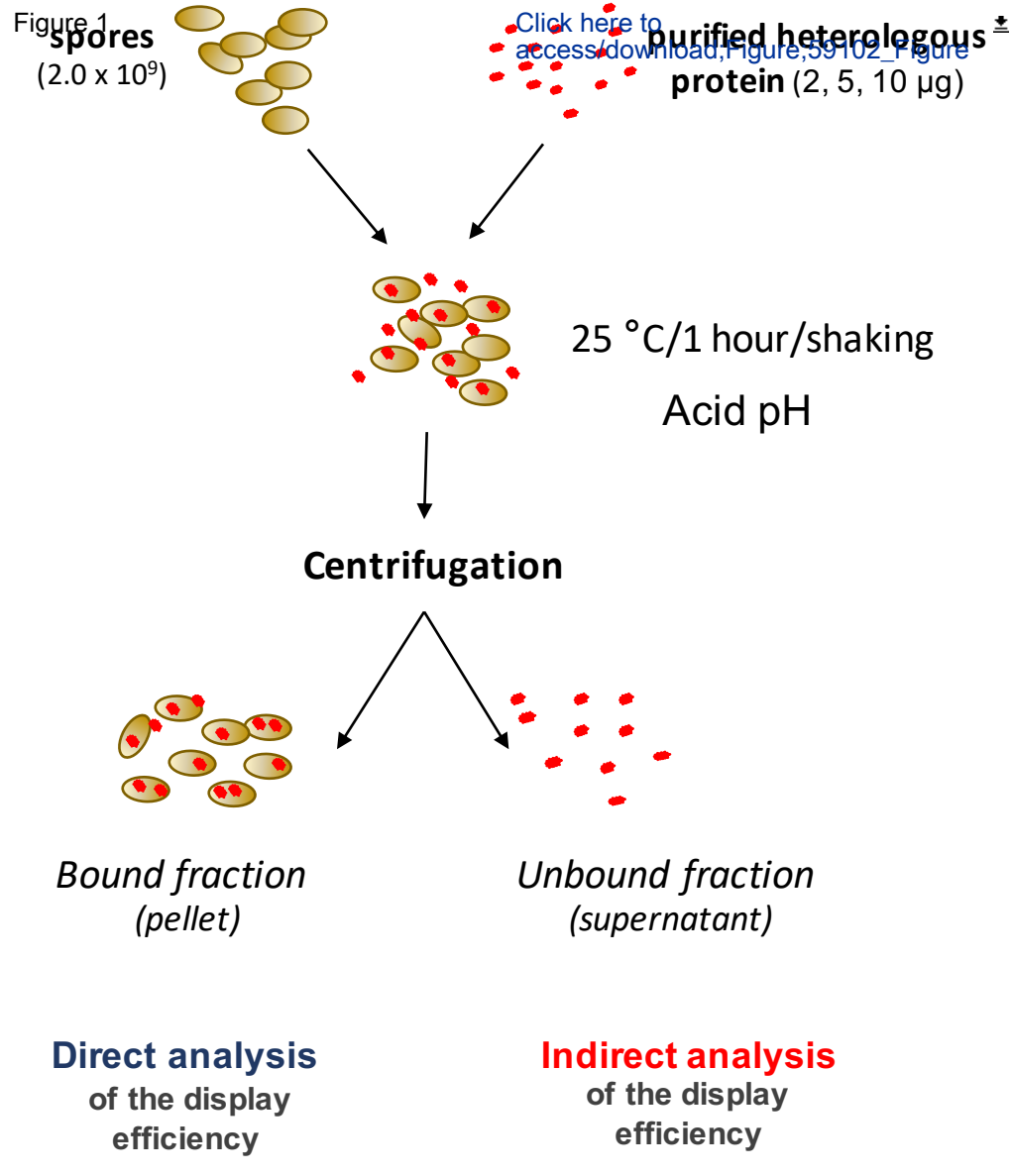


Figure 2

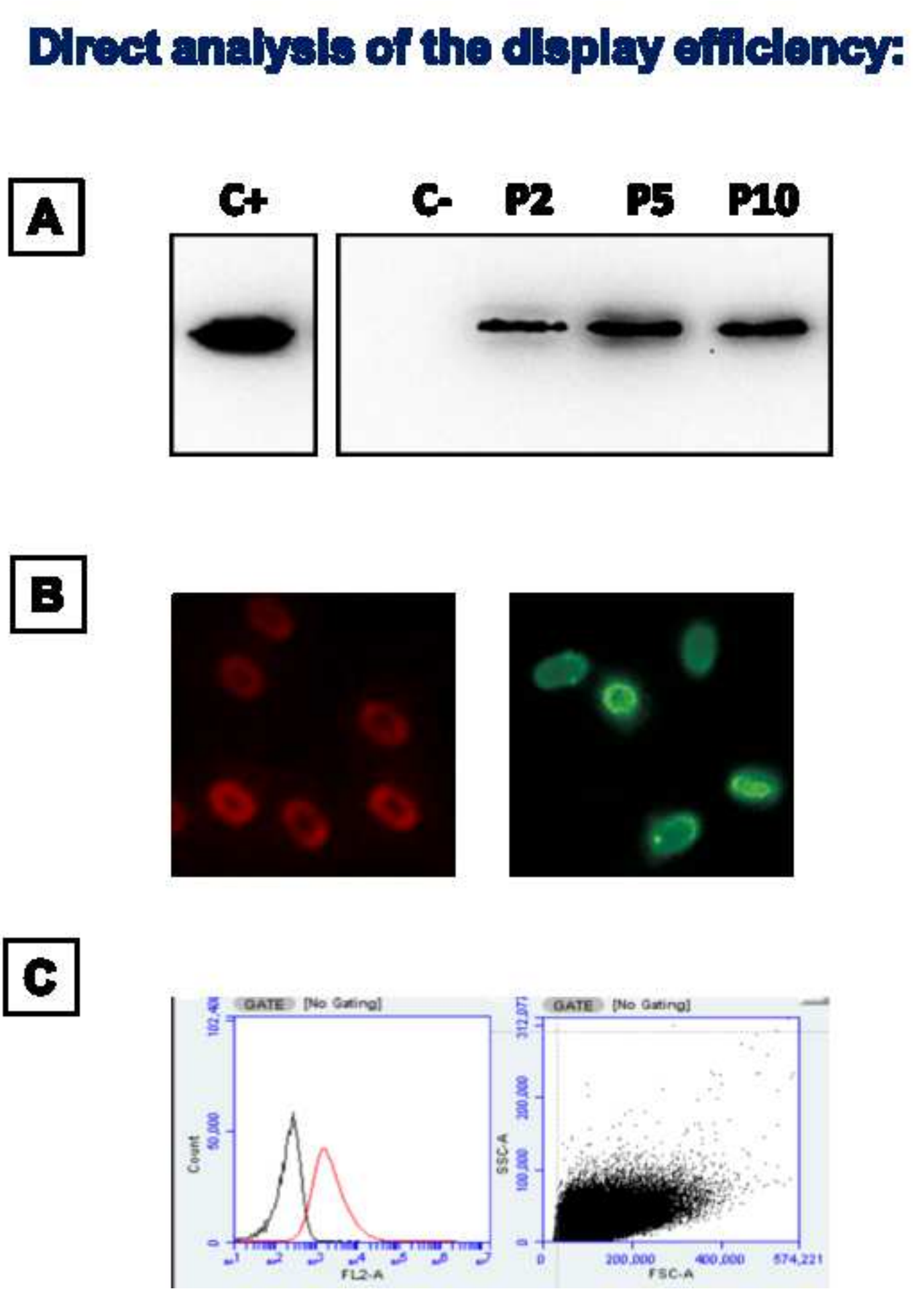


Figure 3

Click here to access/download;Figure;figure 3 jove.png

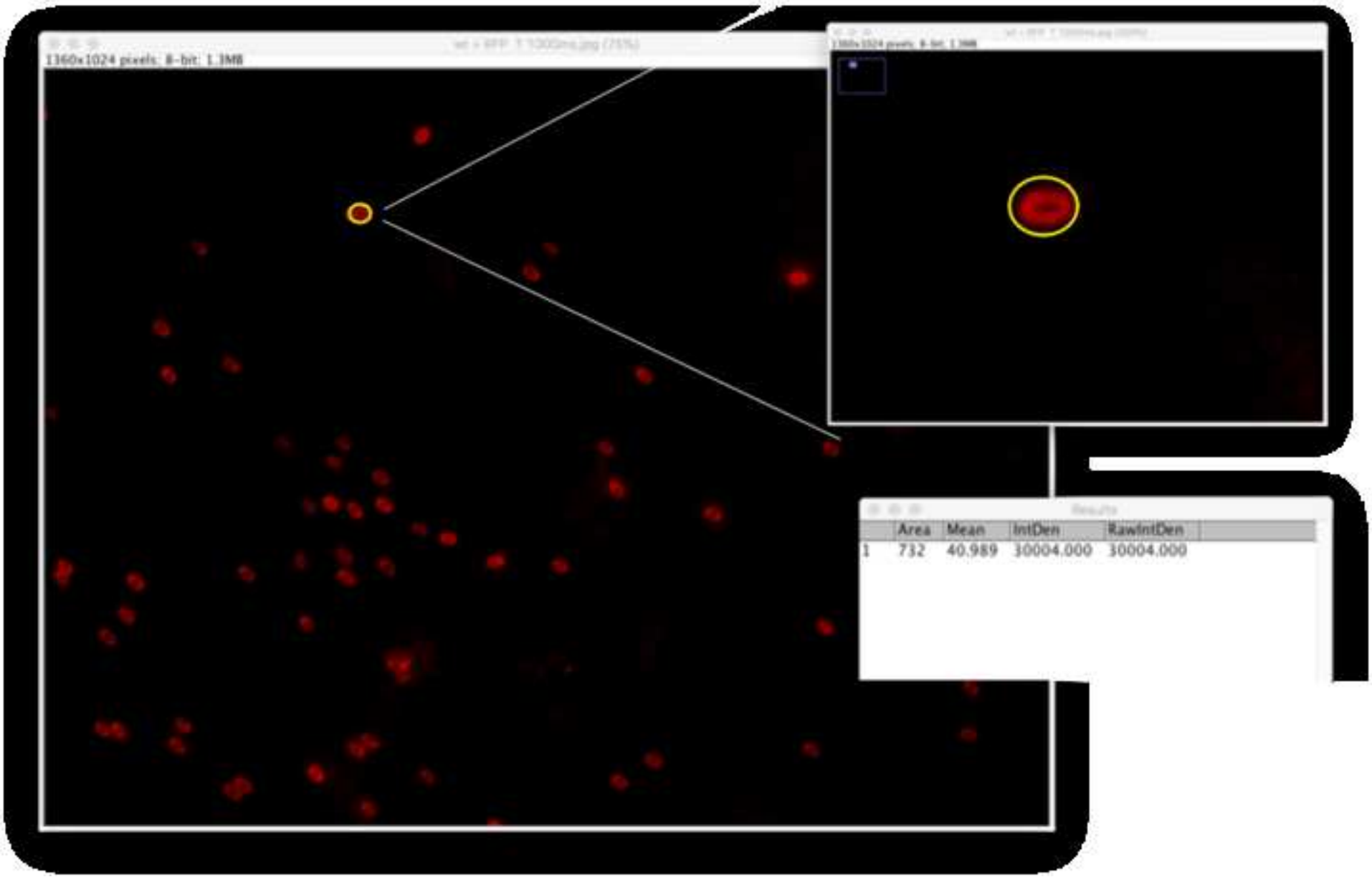
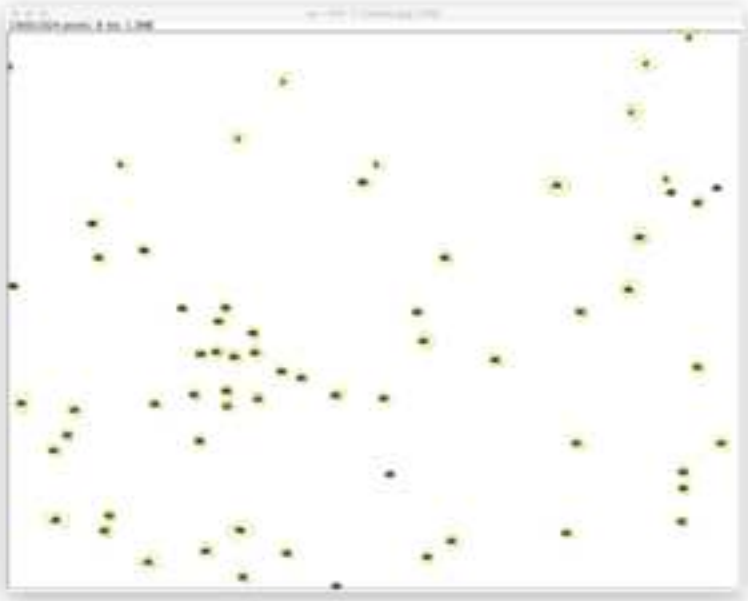


Figure 4

A

B



Results

	Area	Mean	IntDen	RawIntDen
1	0.019	77.340	1.483	59320.000
2	0.013	83.083	1.057	42289.000
3	0.003	71.696	0.247	9894.000
4	0.005	74.155	0.358	14312.000
5	0.008	85.956	0.685	27420.000
6	0.027	90.304	2.413	96535.000
7	0.004	70.094	0.298	11916.000
8	0.013	76.266	0.997	39887.000
9	0.010	85.094	0.885	35399.000
10	0.006	88.713	0.541	21646.000
11	0.008	89.131	0.697	27898.000
12	0.005	73.830	0.402	16095.000
13	0.012	122.938	1.435	57412.000
14	0.008	99.036	0.832	33276.000
15	0.108	104.061	11.228	449126.000
16	0.030	116.472	3.445	137786.000
17	0.016	98.150	1.570	62816.000
18	0.027	112.004	2.991	119620.000
19	0.005	72.837	0.357	14276.000
20	0.006	84.000	0.491	19656.000
21	0.029	102.989	3.010	120394.000
22	0.007	90.667	0.632	25296.000

Figure 5

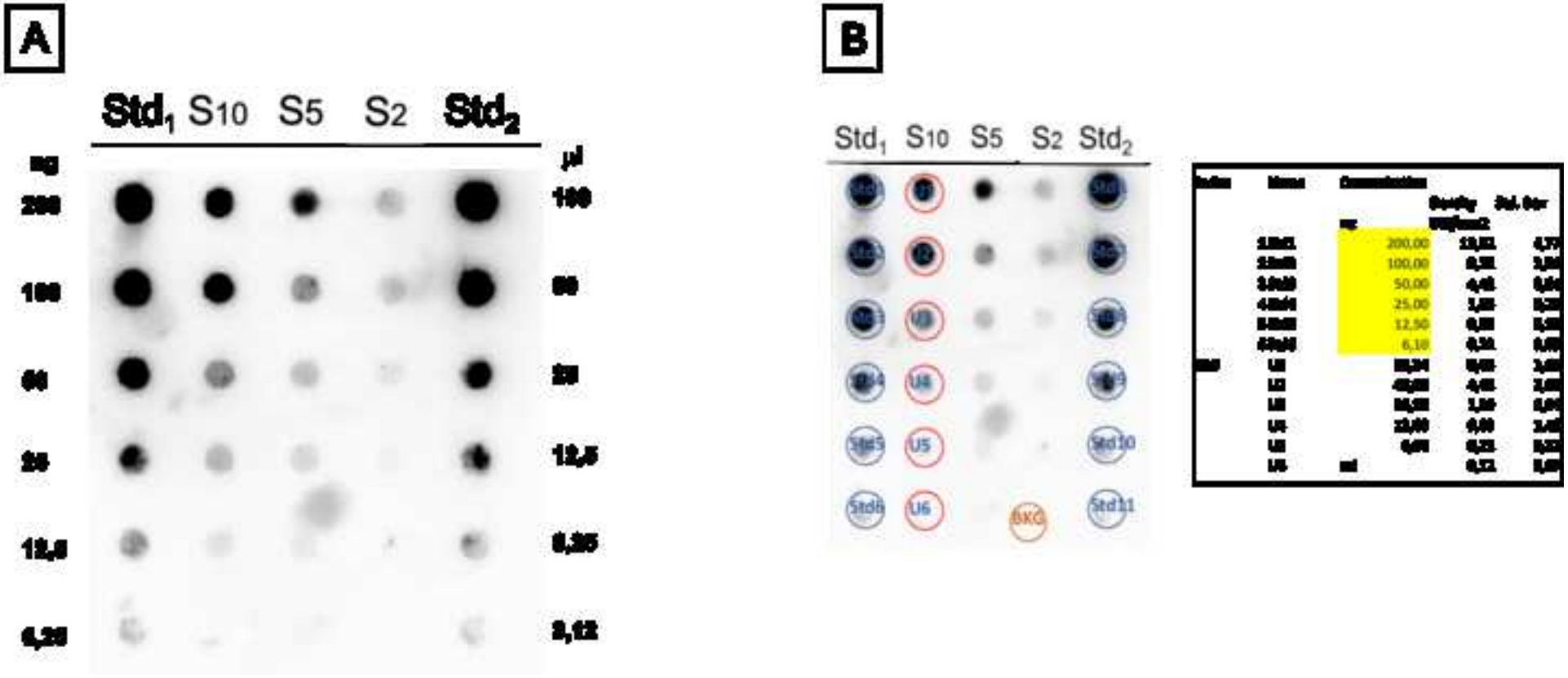
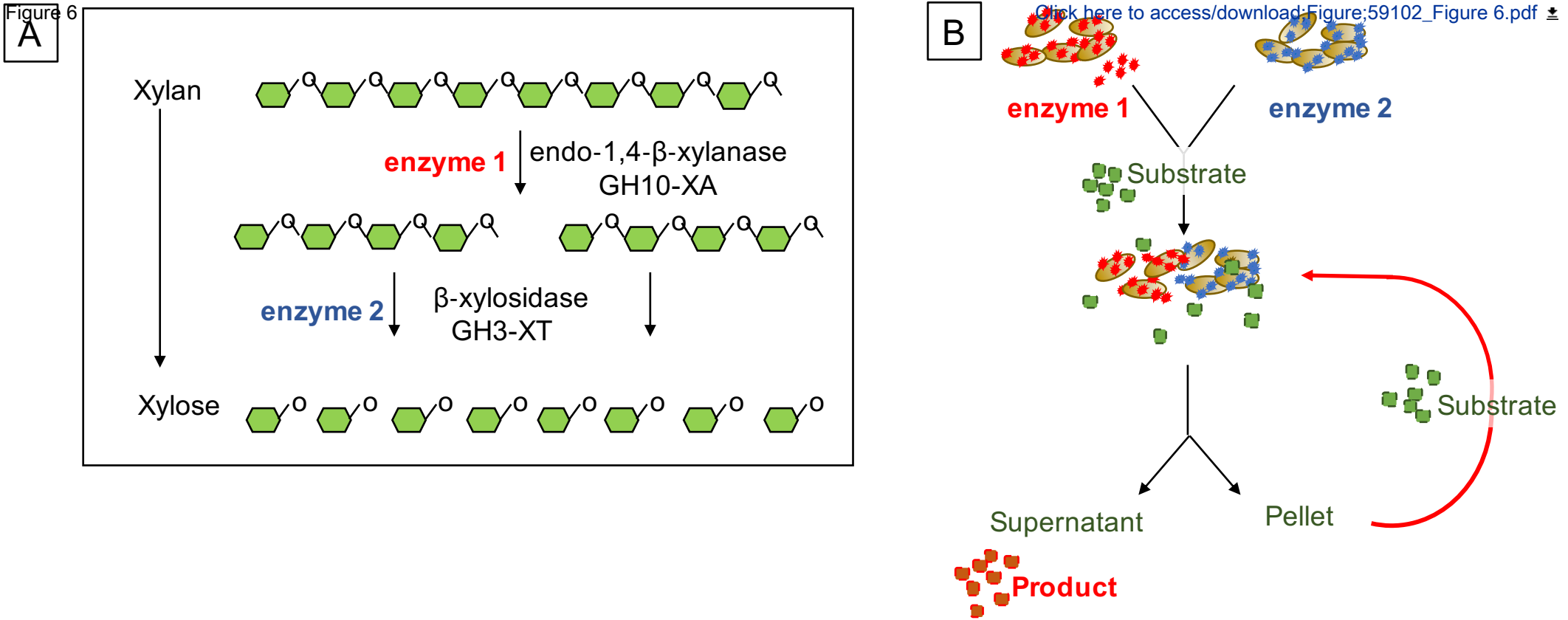


Figure 6



Name of Material/ Equipment	Company	Catalog Number
0.1% Poly-L-lysine solution	Sigma	P8920
0.45 µm Nitrocellulose Blotting	Sartorius	M_Blotting_Membranes
100× objective UPlanF1	Olympus	microscope equipment
Bacillus subtilis strain NCIB3610	Bacillus Genetic Stock	3A1
BD ACCURI C6 PLUS	BD	flow cytometer
BX51	Olympus	Fluorescent microscope
Clarity	Biorad	1705060
DP70 digital camera a	Olympus	microscope equipment
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, FITC	Thermo fisher	F-2754
Goat Anti-Rabbit IgG H&L (HRP)	Abcam	ab6721
Monoclonal Anti-polyHistidine–Peroxidase antibody	Sigma	A7058-1VL
SecureSlip glass coverslip	Sigma	S1815-1PAK
Superwhite Uncharged Microscope Slides	VWR	75836-190
U-CA Magnification Changer	Olympus	microscope equipment

	Comments/Description
	used to detect adsorbed proteins presenting a 6xhistidine-tag at C- or N- terminal

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Author(s): *Rachele Isticato, Ezio Ricca, Loredana Baccigalupi*

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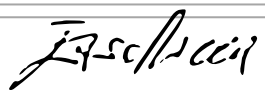
Signature:	 Date: *09/13/2018*

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After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 x 1080 pixels or 300 dpi.

Your revision is due by **Oct 24, 2018**.

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Editorial comments:
Changes to be made by the author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please revise lines 32-34, 74-77, 94-95, 170-171, 200-202 to avoid previously published text.
The lines have been changed with the sentences in red
3. Please revise the title to avoid punctuation and to represent the content included in the protocol.
we changed the title following also the suggestionof reviewer 4

4. Please define all abbreviations before use. Done

5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.
Done
6. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). Done

7. Please add more details 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. 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. See examples below:

Line 107: Please specify the proteins that will be used in the video and specify incubation temperature. Done
Line 115: Please provide the composition of binding buffer. Is it the same as acid buffer? What volume of buffer is used to wash? Done
Line 123: Please specify the centrifugation parameters (force and time) and antibodies used. Done
Line 192: Please describe how this step is actually performed. Done

Lines 239-251: We cannot film a generalized protocol; we need specific details of a specific experiment. For instance, please specify the enzymes, optimal buffer, enzyme reaction conditions used in these steps. Done

Line 251: Please specify the fresh buffer and new substrate added. Done

8. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step. Done

9. Please include single-line space s between all paragraphs, headings, steps, etc. Done

10. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol
Done

11. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense
Done

12. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
Done

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The figures are original and never published before

14. Figure 3: The yellow circle can hardly be seen. Please revise to make it more visible.
The images of figure 3 are from ImageJ software, and the yellow circle comes automatically from the program. We have now added a hard yellow circle to the original one to make it more visible.

15. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.
Other comments have been added in the discussion (lines 334-335 and 337-338)

16. References: Please do not abbreviate journal titles.
References have been corrected

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This is a nice description of use of bacterial spores to adsorb proteins, to assess the levels of adsorbed proteins, and then use of these spores with adsorbed proteins.

Major Concerns:
The English usage needs much work, as there are many examples of use of inappropriate words or grammar. However, the meaning of everything is clear. Three examples in the summary are: 1) line 21 change "expose" to "adsorb"; 2) line 22, change "activity" to "activities"; and line 23 - change "have been shown" to "are also."
Done

Minor Concerns:
1) line 53 and elsewhere - I am not sure that they spore structure is "peculiar" - perhaps "novel" is a better word. The spore is a “peculiar” cell form due to its quiescent metabolism, its dehydrated cytoplasm and surrounding layers.

2) line 96 and elsewhere - make it clear that it is the spores with adsorbed enzymes that are not recovered, not the enzymes themselves.
The Introduction has been modified clarifying this point.

3) lines 123-126 - what about analyzing the supernatant from step 4 in the Adsorbtion reaction?
This is the analysis described at point 4 and named “indirect evaluation of the efficiency of adsorption”. A sentence has been added at lines 112-113 to clarify this point.

4) lines 139, 143 - define DIC and FACS - and other abbreviations as well.
Done

5) line 187 - which supernatant?
The point has been clarified (lines 194-195)

6) line 192 - perhaps say something here about the "membrane".
Done

7) line 195 and elsewhere - "Dot blot apparatus", not "Dot apparatus".
Done

8) line 225 - software such as ImageJ?
two examples of possible software have been reported.

9) line 243 - if the pH must be maintained at ~4.0 to keep proteins adsorbed, how does this influence enzyme activity assays?
when possible, it is recommended to perform the enzymatic reaction at a pH lower than the isoelectric point of the adsorbed enzyme. If not possible, a bit release of the adsorbed protein

could occur (less than 15%, ref 26). A comment about this point has been added in the discussion (lanes 337-338)

10) line 273 - spore-free supernatant, not cell-free.
Corrected

11) Legends to Fig 2-5, make clear that *B. subtilis* spores (I presume) are used in these experiments.
We have specified in the text that spores of *Bacillus subtilis* have been used in the described protocols.

12) Fig 2 legend - what is the fluorescent protein in panel B, and the enzyme in panel C? If the assay is at pH 7, do most protein deadsorb? Would this preclude reuse of these spores?
Figure 2 has been modified and now it reports the results obtained with the adsorption of the monomeric red fluorescent protein mRFP on the *B. subtilis* spores. Also the text (paragraph "representative results") has been corrected

13) line 289 and elsewhere - fluorescent here should be "fluorescence".
Corrected

14) line 310 - recycled should be reused - any data on what % of adsorbed protein is lost after one use of the spores, especially if for a reaction at pH 7?
A sentence about the loss of product in the second reaction has been added in the introduction (lines 88-90)

15) References 12 and 13 - titles of articles are in all capitals.
Done.

16) Fig. 3 - I am not sure that panel B is needed - these numbers could be added to the inset in panel A. Perhaps note in text about the inset in panel A, that the adsorbed protein is in spores' outer layers.
Done

17) Fig. 5 - in panel B, "Surnatant" should be "Supernatant".
Done

18) Table - some of the names of material/Equipment have been cut off I presume the Superwhite uncharged microscope should be followed by slides, and something is also missing after "blotting".
Corrected

Reviewer #2:

Manuscript Summary:
The authors present several broadly-applicable protocols for adsorbing proteins onto Bacillus spores and characterising 'loaded' spores. The protocols are straightforward and clearly described, and would be of use to researchers who would like to try this approach for the first time. The MS does require copy-editing for language issues particularly in the protocol section itself.

Major Concerns:
Some references in the introduction are quite dated, especially in regard to spores as vaccine

delivery vehicles. Recent references include: Copland et al Front Immunol. 2018 Mar 12;9:346., Zhou et al J Med Microbiol. 2017 Jan;66(1):83-89., Sun H et al Parasit Vectors. 2018 Mar 7;11(1):156.
The original paper (ref. 18) and recent reviews (ref. 8 and 9) on the spore systems have been cited. The suggested references describe specific examples of spore display and we believe are not appropriate.

Emphasis is placed on the use of spores as a molecular display system akin to phage display. This can be achieved using recombinant spores but it is less clear how this would work with the in vitro adsorption techniques described here. This should be clarified.
The Introduction has been modified and it has been clarified that the non-recombinant spore display is proposed as a delivery system and not to replace phages in the screening of peptide libraries.

Although preparation of the spores is outside of the scope of these protocols, some specification of the spores used here would be helpful to allow researchers to gauge the appropriateness of the protocols for their particular application. For example, is this suitable for spores that have been autoclave sterilised, or those from all Bacillus species?
In the text (line 107) we specified that *Bacillus subtilis* spores are used in the described experiments and that autoclaved spores can also be used (lines 78-80).

Minor Concerns:

The software used for densitometric analysis should be stated. Is there appropriate open-source or freely available software that could be recommended?
Examples of open-source and freely available software have been added in the text.

Figure 2A - lane labels are misaligned.
Corrected

A cytometry dot plot of physical characteristics of the spores (FSC/SSC) and any recommended gating approaches would add to Figure 2B
The graphs of figure 2C (in the new version of figure 2) were obtained analyzing the entire spore population (10,000 events, ungated) and were intended to be only an example of the methodology.

Figure resolution is poor throughout review pdf
The resolution has been increased

l240 - 'paragraph 1' should read 'protocol 1'
Done

Reviewer #3:

Manuscript Summary:
The technical quality is fair. This topic and experimental protocol could be very interesting for readers working in the specific field. The manuscript can be acceptable for publication in JoVE. However, in its present form there are some parts to be supplemented or revised.

Major Concerns:

1. A recent study entitled "Display of native proteins on Bacillus subtilis spores" described a method of native protein display on the Bacillus spore surface that obviates the need to construct fusion proteins to display a motif. Does this display system be classified as recombinant or non-recombinant display system? In my opinion, It is necessary to add comments on this display system in the manuscript.
A comment has been added to clarify the point (lines 92-99).

2. The introduction may be shortened to the information most relevant to the work.
Done

3. All micrographs in figures 2B, 3 & 4 are of poor quality. New and improved micrographs are required. The micrographs have been replaced.

Minor Concerns:

1. Line 115: Is the binding buffer same as an acid buffer(line 107)? If not, please provide the recipe for binding buffer. The binding buffer is the acid buffer used in the adsorption reaction, as clarified in the text (lines 112-115)

2. Line 131: Replace "slides" by "slide".
Done

3. Line 137: The first letter of "Fluoresscent" written in lowercase.
Done

4. Line 321-323: Please provide references for the following statement "with small proteins or peptides adsorbed more efficiently than large".
The sentence has been deleted.

5. Line 333: What does "ad hoc" mean? Please describe in more detail
The word “ ad hoc” has been replaced with “ specific” (line 345)

6. In section "Adsorption reaction": some details is lost. For example, "in a shaker bath": how many is rotation rate? ; "Wash the pellet with the binding buffer": how many the volume?
More details have been added in the text

7. In figure 5: How to determine the adsorbed protein molecules per spore. A detailed calculation procedure would be helpful to illustrate the adsorption efficiency.
The amount of adsorbed protein is indirectly calculated determining by dot-blot the amount of unbound protein. We added the indication of the calibration curve to extrapolate the concentration of heterologous protein in each sample dots and calculate the average of the significative values (line 239)

8. References list:
Reference should be given in a reference list according to the Journal's style. So writing style of

references must be checked again.
Line 383-384: please check the article and journal name. Done
Line 392, 395, 397, 400, 406: the journal name is not abbreviated. Done

Reviewer #4:

Manuscript Summary:
The manuscript is well written and describes in detail protocols for adsortion and recycling of B. subtilis spores as a platform for adsorption of enzymes and antigens without the need to generate genetically modified strains. The protocol will find interest for those interested in the use of the platform as an alternative for the display of proteins for different purposes.

Major Concerns:
No major concern.

Minor Concerns:
Alternative title: "Spore adsorption: a non-recombinant display system for enzymes and antigens"
the title has been changed