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(57) **Abrégé/Abstract:**

The present invention relates to methods for screening a polypeptide for desired activity against a target molecule. In particular, the present invention relates to methods for screening a polypeptide for desired activity against a target molecule by expressing the polypeptide in a Gram-negative bacterial cell and permeabilising the cell. The invention also relates to methods of packaging gene libraries in a bacterial cell.

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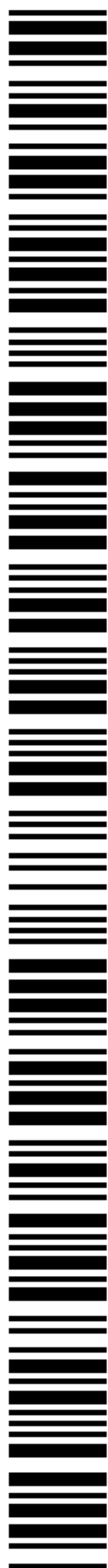


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(54) Title: METHOD OF PROTEIN DISPLAY

(57) Abstract: The present invention relates to methods for screening a polypeptide for desired activity against a target molecule. In particular, the present invention relates to methods for screening a polypeptide for desired activity against a target molecule by expressing the polypeptide in a Gram-negative bacterial cell and permeabilising the cell. The invention also relates to methods of packaging gene libraries in a bacterial cell.

METHOD OF PROTEIN DISPLAY

FIELD OF THE INVENTION

The present invention relates to methods for screening a polypeptide for a desired activity against a target molecule. In particular, the present invention relates to methods for screening a polypeptide for a desired activity against a target molecule by expressing the polypeptide in a Gram-negative bacterial cell and permeabilising the bacterial cell. The invention also relates to methods of packaging gene libraries in a bacterial cell.

BACKGROUND OF THE INVENTION

10 The earliest method of protein display is phage display (Smith, 1985), in which the protein of interest is fused to one of the outer-coat proteins of the phage where it may be present along with wild-type copies of the protein. For example, a display platform based on the M13 filamentous phage using fusions to the gIII protein.

Other display methods include '*in vitro*' display methods where the protein is expressed using a cellular translation extract, and the coupling between the protein and the coding nucleic acid is achieved through physical linkage (e.g. ribosome display, mRNA display) or through attachment to a common scaffold or encapsulation within a membrane, such as in *in vitro* compartmentalization (IVC) where the mRNA is translated within a micelle suspension that may also include a microbead (magnetic or
15
20 sepharose) capture system for both mRNA and protein.

Another method of protein display is microbial surface display which involves the targeted location of expressed proteins to the exterior of a microbial cell, either Gram-negative or Gram-positive eubacteria or yeast. The proteins are fused to anchor domains that attach them to the cell surface. The anchor domains may have motifs dictating lipidation or covalent attachment to the cell wall, or they may be a fusion to an integral membrane protein within an exposed loop region. Due to the scalability of production, microbial surface display may not only be used for screening for improved protein variants from a diverse library, but may also be used to present antigens for vaccination or as a cellular-scaffold for enzymes for industrial biotechnology.

30 Protein display methods are commonly applied to the evolution of affinity proteins, such as antibodies. Single molecule display methods are historically the most popular, but they suffer from high background and low resolution between affinity scales. Antibodies identified by *in vitro* display or by phage systems are usually reformatted for expression in the *E. coli* periplasm, even though periplasmic yields are

often extremely poor comparable to expression in the cytoplasm. When antibodies are expressed in the cytoplasm at high yield, however, in almost every instance they form insoluble inclusion bodies that must be laboriously refolded and tested for activity.

Thus, there remains a need for methods of protein display, particularly for the screening of affinity protein display libraries and enzyme libraries.

SUMMARY OF THE INVENTION

The present inventors have developed a protein display method in which a polypeptide being screened for a desired activity is produced in the cytoplasm of a Gram-negative bacterial cell, and the polynucleotide encoding the polypeptide is packaged within a lysis-defective phage that is also retained within the bacterial cell. One or more of the bacterial cell membranes are permeabilised, whereby the bacterial cell can be contacted with a target molecule in order to screen for the desired activity.

Accordingly, the present invention provides a method of screening a polypeptide for a desired activity against a target molecule, the method comprising:

- a) culturing a Gram-negative bacterial cell comprising an exogenous polynucleotide encoding the polypeptide such that the polypeptide is produced in the cell,
 - b) allowing a lysis-defective phage to package the polynucleotide encoding the polypeptide, wherein the lysis-defective phage is retained within the bacterial cell,
 - c) permeabilising:
 - i) the outer membrane of the bacterial cell, or
 - ii) the inner and outer membranes of the bacterial cell,
 - d) contacting the bacterial cell with the target molecule, and
 - e) screening the polypeptide for the desired activity,
- wherein the polypeptide is retained within the bacterial cell by the bacterial cell wall or inner membrane and/or the polypeptide is attached to the bacterial cell wall or inner membrane.

In one embodiment, the polypeptide may be expressed and retained within the cytoplasm of the bacterial cell by the bacterial cell wall. Thus, in one embodiment, the method comprises permeabilising the inner and outer membranes of the bacterial cell.

The polypeptide may be of sufficient size such that it is retained within the bacterial cell comprising permeabilised inner and outer membranes by the intact cell wall. Alternatively, if the polypeptide is sufficiently small in size it may diffuse through the intact cell wall. In order to prevent the polypeptide diffusing through the cell wall, in one embodiment the polypeptide is associated with at least a second polypeptide to

form a protein complex that is retained within the permeabilised bacterial cell and/or is attached to the bacterial cell wall. In one embodiment, the polypeptide is fused to the second polypeptide or a subunit thereof.

In one particular embodiment, the second polypeptide is selected from:

- 5 i) a polypeptide having a molecular size such that the protein complex is retained inside the permeabilised bacterial cell wall;
- ii) a DNA-binding protein;
- iii) a bacterial cell wall-binding protein; and/or
- iv) a phage coat protein of the lysis-defective phage.

10 While any suitable method may be used to permeabilise the inner and outer bacterial membranes, in one embodiment, the inner and outer bacterial membranes are permeabilised with one or more detergents or an organic solvent.

In one embodiment, the one or more detergents is a non-ionic detergent.

In another embodiment, the non-ionic detergent is selected from Decanoyl-N-
15 methylglucamide (Mega10), demithyloctylphosphine oxide (Apo8), n-octyl- β -D-thioglucopyranoside (8TGP), and a mixture of Decanoyl-N-methylglucamide (Mega10) and demithyloctylphosphine oxide (Apo8).

Alternatively, the inner and outer membranes of the bacterial cell may be permeabilised with an organic solvent such as chloroform. For example, in one
20 embodiment, the inner and outer membranes of the bacterial cell are permeabilised by incubating the bacterial cell in an aqueous solution saturated with chloroform.

In one particular embodiment, the bacterial cell is incubated in the aqueous solution saturated with chloroform for about 10 minutes at about 25°C.

In another embodiment of the invention, the polypeptide is produced in the
25 bacterial cell and attached to the inner membrane of the bacterial cell. Thus, the outer membrane of the bacterial cell is permeabilised and the cell wall is at least partially hydrolysed, while the inner membrane is left intact.

Accordingly, in one embodiment of the method of the invention:

- i) the bacterial outer membrane is permeabilised;
- 30 ii) the bacterial cell wall is at least partially hydrolysed; and
- iii) the polypeptide is attached to the inner membrane.

In one particular embodiment, the bacterial cell wall is at least partially hydrolysed with lysozyme.

In a further embodiment, the polypeptide is fused to a protein that attaches to the
35 inner membrane. In one particular embodiment, the polypeptide is attached to the outer face of the inner membrane.

In yet another embodiment, the polypeptide is associated with a bacteriophage coat protein. In one particular embodiment, the polypeptide is fused to either end of the lambda bacteriophage capsid protein, gpD. In another embodiment, the polypeptide is fused to the N-terminal end of the P2 bacteriophage capsid protein, gpL.

5 The DNA encoding the polypeptide may be genomic DNA and/or episomal DNA. In one embodiment, the polynucleotide encoding the polypeptide is a plasmid, cosmid, phagemid or phage DNA.

In one embodiment, the lysis-defective phage is a temperate phage selected from lambda phage, 186, P2, a hybrid of 186 and P2, and/or P4.

10 The lysis-defective phage may be present in the Gram-negative bacterial cell as a phage or integrated into the host cell genome as a prophage. Thus, in one embodiment, the lysis-defective phage is a prophage.

In one particular embodiment, the bacterial cell comprises lysis-defective lambda, 186, P2, a hybrid of 186 and P2, and/or P4 prophage.

15 In another embodiment, the bacterial cell comprises P2 and P4 prophage.

In one particular embodiment, the bacterial cell comprises lambda prophage.

In yet another embodiment, the bacterial cell comprises a hybrid of 186 and P2 prophage.

20 In one embodiment, allowing the lysis-defective phage to package the polynucleotide encoding the polypeptide comprises inducing activation of the prophage in the bacterial cell to produce phage, wherein the phage package the polynucleotide.

In one embodiment, inducing activation of the prophage comprises producing one or more phage activator proteins in the bacterial cell.

25 In one particular embodiment, the bacterial cell comprises P2 and P4 prophages and the method comprises producing P2 and/or P4 activator proteins in the bacterial cell.

In one embodiment, the P2 and/or P4 activator proteins are selected from one or more of P2 *cox*, P2 *ogr*, P4 δ and/or P4 ϵ .

30 In another embodiment, inducing activation of the prophage comprises inactivating one or more phage repressor proteins in the bacterial cell.

In one embodiment, the bacterial cell comprises P2 and/or P4 prophage and inducing activation of P2 prophage comprises inactivating a temperature-sensitive repressor allele of P2 protein C in the bacterial cell.

35 In one embodiment, the bacterial cell comprises lambda prophage and inducing activation of the lambda prophage comprises inactivating a temperature-sensitive repressor allele of lambda phage repressor protein cI in the bacterial cell.

In another embodiment, the bacterial cell comprises 186 prophage and inducing activation of the 186 prophage comprises inactivating a temperature-sensitive repressor allele of 186 protein cI in the bacterial cell.

5 In another embodiment, the bacterial cell comprises a hybrid of 186 and P2 prophage and inducing activation of prophage comprises inactivating a temperature-sensitive repressor allele of the hybrid phage in the bacterial cell.

In yet another embodiment, the prophage is lysis-defective due to deletion or mutation to an inactive form of either of the lysozyme or holin genes, or deletion or mutation to an inactive form of both the holin and lysozyme genes. In one particular
10 embodiment, the P2 prophage lysozyme gene comprises a sequence of nucleotides comprising SEQ ID NO:17 and the P2 holin gene comprises a sequence of nucleotide comprising SEQ ID NO:18. In another embodiment, the lambda prophage holin gene comprises a sequence of nucleotides comprising SEQ ID NO:23 and the lambda lysozyme gene comprises a sequence of nucleotides comprising SEQ ID NO:24.

15 In another embodiment, inducing activation of the prophage comprises increasing the incubation temperature of the bacterial cells. In one particular embodiment, the incubation temperature of the bacterial cells is increased from about 30°C to about 42°C to induce activation of the prophage. In one embodiment, the prophage is lambda phage. In another embodiment, the prophage is 186 or a hybrid of
20 186 and P2 prophage.

The person skilled in the art will understand the method of the present invention may be used together with other known phage display systems. In contrast to the present invention, known phage display systems do not package the polynucleotide encoding the polypeptide into a lysis-defective phage and/or do not retain the
25 polypeptide within the bacterial cell or attached to the bacterial cell wall or cell membranes.

Accordingly, in one embodiment, the method further comprises an additional screening of the polypeptide for a desired activity against a target molecule in a Gram-negative bacterial cell, wherein

30 i) the polynucleotide encoding the polypeptide is not packaged into a lysis-defective phage, and/or

ii) the polypeptide is not retained within the bacterial cell by the bacterial cell wall and/or attached to the bacterial cell wall.

While the additional screening using a known phage display system may be
35 performed prior to and/or after the method of the invention, in one embodiment the additional screening is performed prior to the method of the invention.

In one embodiment, the phage in the additional screening is performed using a lytic phage or temperate phage to package the polynucleotide encoding the polypeptide.

In another embodiment, the bacterial cell in the additional screening is lysed to release the phage.

5 In yet another embodiment, the phage in the additional screening is a lytic phage which lyses the bacterial cell.

Where the bacterial cell is lysed during the additional screening, it is desirable to attach the polypeptide to the phage particle.

Thus, in one embodiment:

10 i) the lytic phage comprises a first binding partner on the phage coat, and
ii) the polypeptide being screened for a desired activity is a fusion protein comprising a second binding partner,

wherein the fusion protein comprising the second binding partner binds to the first binding partner on the lambda phage coat.

15 In one embodiment, the lytic phage is lambda phage.

In another embodiment, the lytic phage is 186, P2, a hybrid of 186 and P2 prophage, and/or P4.

In one embodiment, the first binding partner is calmodulin and the second binding partner is calmodulin-binding peptide.

20 In yet another embodiment, the one or more prophages in the additional screening is a lysis-defective phage and the cells are lysed chemically and/or enzymatically. In one particular embodiment, enzymatically lysing the cells comprises lysing the bacterial cells with lysozyme. The lysis-defective phage may be, for example, lysis-defective lambda, 186, P2, a hybrid of 186 and P2 prophage, and/or P4.

25 While the method of the invention may be used to package any gene library, in one embodiment, the library of polynucleotides encodes polypeptides to be screened for a desired activity against a target molecule.

Also disclosed is a Gram-negative bacterium comprising a lysis-defective phage with a temperature-sensitive repressor protein.

30 Also disclosed is a Gram-negative bacterium comprising a lysis-defective phage and a polynucleotide encoding one or more phage activator proteins.

In one embodiment, the lysis-defective phage is selected from lambda, 186, P2, a hybrid of 186 and P2 and/or P4.

In an embodiment, the phage activator proteins are selected from P2 *cox*, P2 *ogr*, P4 δ and/or P4 ϵ .
35

Also disclosed is a kit comprising the Gram-negative bacterium of the invention.

In one embodiment, the kit further comprises an agent capable of permeabilising the Gram-negative bacterial cell. In one particular embodiment, the agent capable of permeabilising the Gram-negative bacterial cell is selected from one or more detergents or an organic solvent.

In an embodiment, the detergent is a non-ionic detergent selected from Decanoyl-N-methylglucamide (Mega10), demithyloctylphosphine oxide (Apo8), n-octyl- β -D-thioglucofuranoside (8TGP), polysorbate 20 (Tween20), and a mixture of Decanoyl-N-methylglucamide (Mega10) and demithyloctylphosphine oxide (Apo8).

In another embodiment, the organic solvent is chloroform.

Also disclosed is a method of screening a polypeptide for a desired activity against a target molecule, the method comprising:

- a) culturing a Gram-negative bacterial cell comprising a polynucleotide encoding the polypeptide such that the polypeptide is produced,
- b) permeabilising the inner and outer membranes of the bacterial cell with chloroform, wherein the polypeptide and polynucleotide encoding the polypeptide are retained inside the permeabilised bacterial cell,
- c) contacting the permeabilised bacterial cell with the target molecule such that it diffuses into the permeabilised bacterial cell, and
- d) screening the polypeptide for the desired activity.

Also disclosed is a method of screening a polypeptide for a desired activity against a target molecule, the method comprising:

- a) culturing a Gram-negative bacterial cell comprising a polynucleotide encoding the polypeptide such that the polypeptide is produced and attaches to the bacterial cell wall,
- b) permeabilising the inner and outer membranes of the bacterial cell with chloroform, wherein the polynucleotide encoding the polypeptide is retained inside the permeabilised bacterial cell,
- c) contacting the permeabilised bacterial cell with the target molecule, and
- d) screening the polypeptide for the desired activity.

In one embodiment, step d) comprises:

- i) determining if the polypeptide binds, and/or the extent of binding to, the target molecule, and/or
- ii) determining if the polypeptide enzymatically modifies, and/or the rate of enzymatic modification of, the target molecule.

In one embodiment, the polypeptide is associated with at least a second polypeptide to form a protein complex that is retained inside the permeabilised bacterial cell and/or attached to the bacterial cell wall.

In one embodiment, the polypeptide is fused to the second polypeptide, or a subunit thereof.

In the method of the invention, the second polypeptide may be selected from:

- i) a polypeptide having a molecular size such that the protein complex is retained inside the permeabilised bacterial cell wall;
- ii) a DNA-binding protein;
- 10 iii) a bacterial cell wall-binding protein, and/or
- iv) a phage coat protein.

Also disclosed is a method for identifying a polypeptide with a desired activity, the method comprising:

- a) screening a library of polypeptides using a method of the invention; and
- 15 b) selecting one or more polypeptides with the desired activity.

Also disclosed is a Gram-negative bacterial cell obtained by permeabilising the inner and outer membranes of the bacterial cell with chloroform, wherein the bacterial cell comprises an exogenous polypeptide associated with a second polypeptide to form a protein complex that is retained inside the permeabilised bacterial cell.

20 Also disclosed is a Gram-negative bacterial cell obtained by permeabilising the inner and outer membranes of the bacterial cell with chloroform, wherein the bacterial cell comprises an exogenous polypeptide attached to the bacterial cell wall.

Also disclosed is a kit comprising:

- a) a vector comprising
 - 25 i) a site for inserting into the vector a polynucleotide encoding a first polypeptide, and
 - ii) an open reading frame encoding a second polypeptide which associates with the first polypeptide to form a protein complex that is retained inside a permeabilised Gram-negative bacterial cell, and
- 30 b) chloroform for permeabilising a bacterial cell.

Also disclosed is a kit comprising:

- a) a vector comprising
 - i) a site for inserting into the vector a polynucleotide encoding a first polypeptide, and

ii) an open reading frame encoding a second polypeptide which associates with the first polypeptide to form a protein complex that attaches to a Gram-negative bacterial cell wall, and

b) chloroform for permeabilising a bacterial cell.

5 As will be apparent, preferred features and characteristics of one aspect of the invention are applicable to many other aspects of the invention.

Throughout this specification the word “comprise”, or variations such as “comprises” or “comprising”, will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of
10 any other element, integer or step, or group of elements, integers or steps.

The invention is hereinafter described by way of the following non-limiting Examples and with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

FIGURE 1. Detergent permeabilisation of *E. coli* cells. *E. coli* cells expressing
15 GFP were treated with detergents to determine the effectiveness of membrane permeabilisation. Cells were viewed by either brightfield (first column) or fluorescence microscopy (second and third columns). Permeabilisation was effective if GFP (column 2) was released from the cell concurrent with uptake of the membrane-impermeable DNA-binding dye, Gel Red (third column). Detergents 8TGP (0.5 %) and 0.5 % Megal0/0.5 % Apo 8 (‘Agent86’) were found to be most effective in
20 permeabilising *E. coli* cells.

FIGURE 2. SDS-PAGE of detergent supernatants. The supernatant of the detergent permeabilisation of *E. coli* cells shown in Figure 1 were loaded onto a 9 % SDS-PAGE to qualitatively judge protein release by the detergents (first lane). To
25 demonstrate retention of a subset of cellular proteins by the cell wall capsule in detergent permeabilised cells, a sample of detergent permeabilised cells was treated with lysozyme (2 mg/mL) to hydrolyse the cell wall (second lane).

FIGURE 3. Tetrameric-fusion protein expression. (A) Expression of His6::SNAP::tetramer fusion proteins in *E. coli* was examined by Western blot using an
30 α His antibody probed against total cellular protein. A high-molecular weight band of >250 kD was observed in the RhnA tetramer fusion (lane 4), in addition to a band of the expected molecular weight, which is a presumptive SDS-resistant form of the complex that migrated as a tetramer. (B) The BetB tetrameric fusion protein extract was separated into the detergent-soluble and detergent-insoluble (cell capsule pellet)
35 extracts, and examined by SDS-PAGE.

FIGURE 4. SNAP labeling of tetramer-fusion proteins. The His6::SNAP::tetramer fusion proteins were expressed in *E. coli*, and the cells were permeabilised with 8TGP, as described by Example 1. Expression of the fusion protein was detected by fluorescence microscopy of permeabilised cells labeled with the membrane-impermeable SNAP ligand BG-547 (second column), as described in Example 3. Cellular DNA was labeled with the membrane-permeable dye, Sytox Green (first column). The overlay of the SNAP and Sytox Green signal is presented in the third column.

FIGURE 5. α His antibody labeling of His6::SNAP::BetB tetramer in permeabilised cells. Fluorescence microscopy of permeabilised cells expressing the His6::SNAP::BetB tetramer probed with an α His antibody (first panel), as described in Example 3. Cells were labeled with the SNAP ligand BG-547 (second panel). The co-localisation of both α His and SNAP (third panel) indicates that the α His antibody penetrated through the cell wall of permeabilised cells.

FIGURE 6. BetB, RhnA and YdcW tetramer fusions with HALO and SNAP expression reporters. The BetB, RhnA and YdcW tetramers were separately fused to the expression reporters, HALO and SNAP. Cells expressing the fusion protein were permeabilised and the host DNA was labeled with Gel Red and the fusion protein was detected using the fluorescent ligands for HALO (G1001) and SNAP (BG-488).

FIGURE 7. Expression of the GFP5::DNA Binding Protein (DBP) fusion. The non-specific, high-affinity, DNA binding protein, ComE, from *N. gonorrhoeae* was fused to the C-terminus of GFP5 and expressed in *E. coli*. Cells were permeabilised and viewed by fluorescence microscopy for GFP (first panel) and Gel Red (second panel). Co-localisation (third panel) of the fluorescence indicates that both the fusion protein and host DNA were retained within the permeabilised cell capsule.

FIGURE 8. Retention of DNA in permeabilised cells. *E. coli* cells expressing the GFP5::DBP fusion, or a His6::eGFP fusion were either left untreated (rows 1 and 4) or were permeabilised (rows 2, 3, 5 and 6) as described in Example 1. Permeabilised cells were either stored overnight at 4°C or resuspended in TBS and shaken overnight at 37°C before being viewed by fluorescence microscopy for GFP (first column) or Gel Red (second column). Co-localisation of GFP and Gel Red is presented in the third column.

FIGURE 9. DNA extraction from permeabilised cells. *E. coli* cells expressing (A) GFP5::DBP or (B) His6::eGFP fusion proteins were permeabilised as described in Example 1. Permeabilised cells were stored overnight at 4°C or resuspended in TBS and shaken overnight at 37°C before plasmid DNA was extracted and electrophoresed

on an ethidium-bromide stained 1 % agarose gel with TAE buffer. Lane 1 is the total plasmid DNA in untreated cells. Lanes 2 and 4 are the supernatants from the permeabilisation step of cell capsules stored overnight at 4°C and shaking at 37°C, respectively, and lanes 3 and 5 are plasmid preparations from the cell capsules stored overnight at 4°C and shaking at 37°C, respectively.

FIGURE 10. SNAP labeling of the OmpF::SNAP::LPP fusion protein. *E. coli* cells expressing the OmpF::SNAP::LPP fusion protein were permeabilised as described in Example 1. Fusion protein localization was detected by labeling with the SNAP ligand BG-488 as described in Example 3. Labeled cells were viewed by brightfield microscopy (first panel) and by fluorescence microscopy (second panel). The third panel is the overlay of both brightfield and fluorescent views.

FIGURE 11. Binding of eGFP by α GFP::HALO::RhxA fusion protein. *E. coli* cells expressing the α GFP::HALO::RhxA fusion protein were permeabilised as described in Example 1. Purified eGFP protein was bound to the cell capsules as described in Example 8 and eGFP was visualized by fluorescence microscopy. First panel, brightfield view; second panel, eGFP fluorescence; third panel, overlay of brightfield and fluorescence.

FIGURE 12. Binding of eGFP by α GFP::KzPG::SNAP::DBP fusion protein. *E. coli* cells expressing the α GFP::KzPG::SNAP::DBP fusion protein were permeabilised as described in Example 1. Purified eGFP protein was bound to the cell capsules as described in Example 8 and eGFP was visualized by fluorescence microscopy by two methods, wet mount and dry mount, as described in Example 3. (A) eGFP bound to wet-mounted cell capsules. Inset panels (i) and (ii) show the cell-wall localization of the eGFP bound by the α GFP::KzPG::SNAP::DBP fusion protein. (B) and inset panel (Aiii) show the same cells prepared for microscopy by dry mount in DABCO/glycerol.

FIGURE 13. Binding of eGFP by OmpF:: α GFP::SNAP::LPP fusion protein. *E. coli* cells expressing the OmpF::SNAP::LPP or the OmpF:: α GFP::SNAP::LPP fusion protein were permeabilised as described in Example 1. Purified eGFP protein was bound to the cell capsules as described in Example 8 and eGFP was visualized by fluorescence microscopy by dry mount, as described in Example 3. (A) Cells expressing the OmpF::SNAP::LPP fusion protein lack eGFP fluorescence (second panel, top row), unlike cells expressing the OmpF:: α GFP::SNAP::LPP fusion protein (second panel, bottom row).

FIGURE 14. Demonstration of covalent attachment to the cell wall by the LPP fusion protein. *E. coli* cells expressing the OmpF:: α GFP::SNAP::LPP fusion protein were permeabilised as described in Example 1. Fusion protein localization was detected

by labeling with the SNAP ligand BG-488 as described in Example 3 and DNA was stained with Gel Red. Samples were heated for 5 minutes at 22°C (A) or at 95°C (B) before being dry mounted and viewed by fluorescence microscopy.

FIGURE 15. Outer membrane permeabilisation using a detergent/Ca²⁺ buffer.

5 *E. coli* cells expressing the OmpF::αGFP::SNAP::LPP fusion protein (external αGFP or the αGFP::HALO::FLAG::Rhna fusion protein (internal αGFP) were permeabilised as described in Example 10. Permeabilisation of the outer membrane to large ligands was assessed by binding of eGFP to the αGFP domain attached to the cell wall. Permeabilisation of the inner membrane was assessed using a large ligand (eGFP) and
10 small ligand (Gel Red). Both detergents Apo8 (A) and Tween20 (B) in Ca²⁺ buffer demonstrated selective permeability of the outer membrane to large ligands.

FIGURE 16. Outer membrane permeabilisation using a detergent/EDTA buffer.

E. coli cells expressing the OmpF::αGFP::SNAP::LPP fusion protein (external αGFP) or the αGFP::HALO::FLAG::Rhna fusion protein (internal αGFP) were permeabilised
15 as described in Example 10. Permeabilisation of the outer membrane to large ligands was assessed by binding of eGFP to the αGFP domain attached to the cell wall. Permeabilisation of the inner membrane was assessed using a large ligand (eGFP) and small ligand (Gel Red). Both detergents Apo8 (A) and Tween20 (B) in EDTA buffer demonstrated selective permeability of the outer membrane to large ligands.

20 **FIGURE 17.** FACS analysis of a mixed population of eGFP and SNAP-labeled cells. Three populations of *E. coli* cells expressing; eGFP (#1 arrow); the αGFP::KzPG::SNAP::DBP fusion protein labeled with SNAP ligand BG-488 (#2 arrow); and His6::SNAP::BetB labeled with SNAP ligand BG-547 (#3 arrow) were sorted by FACS. Sorted populations were reanalysed for purity and cell integrity
25 minutes after the first sort.

FIGURE 18. A peptide linker between the αGFP and KzPG domains enables binding of *E. coli* cells expressing a αGFP::KzPG::SNAP::DBP fusion protein to a sepharose support. Cells expressing a αGFP::KzPG::SNAP::DBP fusion protein with a 12-mer linker region, RL6, between the αGFP and KzPG domains were bound to a
30 Co²⁺-sepharose support through a His6::eGFP intermediate. GFP binding is shown in the left panel (green); SNAP ligand (red) binding of the fusion protein is shown in the middle panel; overlay of each is shown on the right.

FIGURE 19. Binding of *E. coli* cells expressing αGFP::RL6::KzPG::SNAP::DBP fusion protein to streptavidin-labeled magnetic beads.
35 (A) Biotin-labeled eGFP (middle and right panels) was bound to cells expressing the αGFP::RL6::KzPG::SNAP::DBP fusion protein which was in turn bound to

streptavidin-labeled magnetic particles. (B) Converse binding of cells expressing the α GFP::RL6::KzPG::SNAP::DBP fusion protein to streptavidin-labeled magnetic particles which had first been labeled with biotinylated-eGFP. In this example the beads are labeled green (GFP panel), the cells were labeled with the BG-547 SNAP ligand (red, SNAP red panel). (C) A domain linker, the 27th Ig domain of human titin, was also effective as a binding spacer. *E. coli* cells expressing the α GFP::I27::RL6::KzPG::SNAP::DBP fusion protein were first bound to biotinylated eGFP (green, GFP panel) and labeled with the BG-547 SNAP ligand (red, SNAP red panel) before being bound to streptavidin-labeled magnetic particles.

FIGURE 20. Expression of mouse scFv genes in the *E. coli* cytoplasm as scFv::I27::RL6::KzPG::SNAP::DBP fusion proteins. A mouse scFv library was constructed and displayed according to the method of the invention in the *E. coli* cytoplasm. Clones with detectable expression were detected via SNAP ligand binding and were categorised as misfolded (left panel), weakly expressed but soluble (middle panel) or highly expressed and soluble (right panel).

FIGURE 21. Detection of soluble and insoluble scFv expression in the *E. coli* cytoplasm. Selected clones that were found to be highly expressed and soluble in a limited screen from the mouse scFv expression library were subcloned into expression constructs as scFv::I27::RL6::FLAG and scFv::RL6::FLAG fusion proteins. Protein fractions were loaded as either soluble or insoluble onto SDS-PAGE gels, transferred to nitrocellulose membranes and detected using α FLAG antibodies. Samples are paired for soluble (S) or insoluble (P) fractions, as well as each clone being expressed with the I27::RL6 (I) or RL6 (R) linker. A fluorescence microscopy image of the original scFv clone in the I27::RL6::KzPG::SNAP::DBP display construct isolated from the library screen is also shown in the lower panels.

FIGURE 22. Permeabilisation of *E. coli* membranes using organic solvents. *E. coli* cells expressing an α GFP::RL6::KzPG::SNAP::DBP fusion protein were suspended in aqueous mixtures of organic solvents. Membrane permeabilisation was indicated by the binding of (A) a small molecular weight DNA-binding fluorescent ligand, Gel Red; and of a 30 kD protein, eGFP. Of the organic solvents tested, only chloroform permeabilised both inner and outer membranes to enable entry into the cell of the high-molecular weight eGFP (B).

FIGURE 23. Expression of α GFP:I27:gpL fusion protein in *E. coli*. Expression of the α GFP:I27:gpL fusion protein was induced by arabinose induction, as described in Example 20. Soluble protein was released from *E. coli* cells by permeabilisation with 0.5% 8TGP and the remainder of the sample was considered insoluble. Samples

were boiled in SDS loading buffer and electrophoresed on a 15% SDS-PAGE. Proteins were transferred to nitrocellulose membrane and probed with α FLAG monoclonal antibody to detect the α GFP:I27:gpL fusion protein. (1) sample 1: uninduced α GFP:I27:gpL fusion clone 1; (2) induced α GFP:I27:gpL fusion clone 1; (3) induced α GFP:I27:gpL fusion clone 2. S = soluble fraction; In = insoluble fraction.

FIGURE 24. Fluorescence imaging of mAG1-labeled encapsulated lambda phage displaying the gpD:: α -mAG1 fusion protein. *E. coli* cells induced for a lambda prophage and expressing the gpD:: α -mAG1 fusion protein were permeabilised and stained with the mAG1 protein and the DNA binding dye, Gel Red. mAG1 was observed by fluorescence microscopy to bind in a punctate pattern within permeabilised cells (left panel).

FIGURE 25. Screenshot of Influx FACS analysis of encapsulated lambda phage displaying the gpD:: α -mAG1 fusion protein. Shown is the fluorescence graph for 100 K events on an Influx FACS (BD Biosciences) with an input of ~1% of α -mAG1-positive cells. The cell population has been co-stained with the DNA binding dye, Gel Red (red; 561 nm), and the fluorescent mAG1 protein (green; 488 nm). The P2 gated population is α -mAG1-positive and the P3 gated population is α -mAG1-negative.

KEY TO THE SEQUENCE LISTING

- SEQ ID NO:1 – Nucleotide sequence of pAra3::His6::SNAP arabinose vector
- 20 SEQ ID NO:2 – Nucleotide sequence of pAra3::His6::KzPG::SNAP::DBP vector
- SEQ ID NO:3 – Nucleotide sequence of pAra3::OmpF::SNAP::LPP vector
- SEQ ID NO:4 – Nucleotide sequence of pAra3:: α GFP(R35)::HALO::FLAG::Rhna vector
- SEQ ID NO:5 – Randomized peptide spacer domain
- 25 SEQ ID NOs:6 to 12 – Peptide linker spacers
- SEQ ID NO:13 – I27::RL6::KzPG::SNAP::DBP
- SEQ ID NO:14 – I27::RL6::KzPG::SNAP::DBP coding sequence
- SEQ ID NO:15 – Library scaffold vector
- SEQ ID NO:16 – I27 spacer
- 30 SEQ ID NO:17 – Nucleotide sequence of enterobacteriophage P2 endolysin gene
- SEQ ID NO:18 – Nucleotide sequence of enterobacteriophage P2 holin gene
- SEQ ID NO:19 – Nucleotide sequence of temperature-inducible P4 δ vector
- SEQ ID NO:20 – Amino acid sequence of the α GFP::I27::gpL fusion protein
- SEQ ID NO:21 – Amino acid sequence of the gpL:: α GFP::I27 fusion protein

SEQ ID NO:22 – Nucleotide sequence of the α GFP::I27::gpL fusion protein expression vector

SEQ ID NO:23 – Nucleotide sequence of lambda phage holin gene

SEQ ID NO:24 – Nucleotide sequence of lambda phage lysozyme gene

5 SEQ ID NO:25 – Amino acid sequence of lambda lysis cluster deletion remnant

SEQ ID NO:26 – Nucleotide sequence of the lambda *cos* region

SEQ ID NO:27 – Nucleotide sequence of the lambda SR deletion (Δ SR) vector

DETAILED DESCRIPTION

10 General techniques and definitions

Unless specifically defined otherwise, all technical and scientific terms used herein shall be taken to have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in protein chemistry, biochemistry, cell culture, molecular genetics, microbiology, and immunology).

15 Unless otherwise indicated, the recombinant protein, cell culture, and immunological techniques utilized in the present invention are standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984), J. Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd edn, Cold Spring Harbour Laboratory Press (2001), R. Scopes, Protein Purification – Principles and Practice, 3rd edn, Springer (1994), T.A. Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), DNA Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996), and F.M. Ausubel et al. (editors),
20 Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present), Ed Harlow and David Lane (editors) Antibodies: A Laboratory Manual, Cold Spring Harbour Laboratory, (1988), and J.E. Coligan et al. (editors) Current Protocols in Immunology, John Wiley & Sons (including all updates until present).

30 The terms “polypeptide”, “protein” and “peptide” are generally used interchangeably herein. As used herein, the term “exogenous polypeptide” refers to a polypeptide encoded by an exogenous polynucleotide. The term “exogenous polynucleotide” as used herein refers to a polynucleotide which is foreign to the cell into which it has been introduced, or that the sequence is homologous to a sequence in
35 the cell into which it is introduced but in a position within the host cell nucleic acid in

which the polynucleotide is not normally found.

The term "antibody" as used in this invention includes polyclonal antibodies, monoclonal antibodies, bispecific antibodies, diabodies, triabodies, multibodies, heteroconjugate antibodies, chimeric antibodies including intact molecules as well as
5 fragments thereof, such as Fab, F(ab')₂, Fv and scFv and other antibody-like molecules.

The term "about" as used herein refers to a range of +/-5% of the specified value.

Lysis-defective phage

10 In one embodiment of the invention, a polypeptide is screened for a desired activity in a Gram-negative bacterial cell, wherein the polypeptide is produced in the cell and the polynucleotide encoding the polypeptide is packaged into a lysis-defective phage. By "lysis-defective phage" it is meant a lytic or temperate phage that would normally have a lytic stage in its life-cycle but which has been modified so that,
15 although it may enact all other functions of a lytic cycle, it is incapable of lysing a Gram-negative bacterial cell to release packaged phage. Thus, lysis-defective phage include temperate phage that are capable of having a lysogenic cycle in which the viral genome is integrated into the host cell DNA as a prophage, or which replicates as a plasmid (phagemid). The prophage remains dormant in the bacterial cell until host cell
20 conditions allow for the prophage to become active and initiate the reproductive cycle. Whereas initiation of the reproductive cycle of a prophage would normally result in lysis of the bacterial host cell, the lysis-defective phage in the method of the present invention has been modified so that the bacterial host cell is not lysed and the phage remains within the bacterial cell.

25 The term "lysis-defective phage" as used herein does not include reference to a phage that does not normally have a lytic stage in its lifecycle, hence the skilled person will understand that it does not include reference to phage that are released from a bacterial cell by extrusion, for example filamentous phage such as M13, f1 or f2, or that are released from a bacterial cell by budding.

30 Examples of lytic phages that may be modified to remove the lytic stage from their life-cycle so as to produce a lysis-defective phage include phiX174, T1, T2, T3, T4, T5, T6 and T7 bacteriophages. Examples of lysogenic phage which may be modified so as to remove the lytic stage of their life-cycle include lambda phage, N15 phage, P22 phage, Mu phage, P2 phage, phage 186 and the P2 satellite phage, P4
35 (Lindqvist et al., 1993; Ziermann et al., 1994; Liu et al., 1997; and Briani et al., 2001).

The skilled person will understand that some temperate phages that are capable of packaging a polynucleotide in a bacterial cell require the presence of another phage, for example, a helper phage, in order to undergo polynucleotide packaging and/or for bacterial cell lysis to occur. An example of this relationship is the P2 phage and its
5 satellite phage, P4. The requirement for the presence of a helper phage for polynucleotide packaging and/or for bacterial cell lysis is known as a helper-phage system. In a helper-phage system, the activity of a helper-phage, or of phage polypeptides (i.e. "activator proteins"), induces another phage to undergo polynucleotide packaging and/or cause bacterial cell lysis. Thus, the skilled person will
10 understand that while a polynucleotide may be packaged into one phage (i.e. one phage in a helper-phage system), the activity of another phage (i.e. a helper-phage) may be required to lyse the bacterial cell in which both the phages are present. For use in some embodiments of the method of the present invention, the phage which would normally provide the lytic activity is modified so that it is no longer capable of lysing a bacterial
15 cell. Accordingly, the term "lysis-defective phage" as used herein also refers to a phage into which a polynucleotide is packaged, wherein the phage would normally rely on a second phage to provide lytic activity, but in which the second phage has been modified so that it is no longer capable of lysing a Gram-negative bacterial cell.

The skilled person will appreciate that the polynucleotide may also be physically
20 separate from the genome of the lysis-defective phage. For example, the polynucleotide may be operably linked to sequences that are sufficient for packaging of the polynucleotide by the phage structural and replicative proteins to form an infectious unit that morphologically resembles, or is identical to, the parental strain of the lysis-defective phage.

25 As a non-limiting example, plasmid vectors of the appropriate size may contain the sequence around the *cos* region required for DNA packaging into the lambda bacteriophage. These plasmid vectors may be packaged *in vivo* by helper phage, and may also be packaged *in vitro* by purified extracts containing the phage structural and replicative proteins. A sequence sufficient for lambda packaging is provided as SEQ
30 ID NO:26. These vectors are known as cosmids (*cos* + *plasmid*), and are well-known to the skilled person for their ability to clone exogenous polynucleotides and propagate them as bacteriophage particles. Commercial kits for cloning polynucleotides within cosmids, and kits for *in vitro* packaging are known in the art.

Exemplary helper-phage system: the P2-P4 System

One non-limiting example of a helper-phage system is the P2-P4 phage system. While each phage carries the genes necessary to assure its own DNA replication and integration into the host genome, the *E. coli* P4 phage lacks the genetic information
5 necessary for tail and lysis functions, as well as the major structural protein for capsid formation (Kahn et al., 1991; Liu et al., 1997). P4 is therefore reliant on phage P2, or P2-related phage, such as phage 186, in order to make P4 phage structural components, to package its DNA and to lyse the host cell. When P4 infects a P2 lysogenic host cell (for example, *E. coli* comprising a P2 prophage in its genome), the P2 prophage is
10 derepressed by the P4 ϵ gene. Derepression results in P2 early and late-gene expression and is sufficient for the completion of the P4 lytic cycle.

P4 may also be packaged by the P2-like phage, phage 186. This phage has orthologous structural proteins to P2 (~75% identity) and hybrid P2/186 phage have been constructed that contain the P2 structural genes that are regulated by the phage
15 186 transcription factors (Younghusband et al., 1975). Crucially, the early region of phage 186, and P2/186 hybrids (Hy2 and Hy5) is not related to P2 and thus is not inhibited by the P4 ϵ protein. However, a phage 186 can be used as a P4 helper phage if the temperature-sensitive mutant of the phage 186 immunity repressor is used to induce phage 186 functions to coincide with P4 infection (Sauer et al., 1982) or activation.

20 The skilled person will understand that a P2/P4 bacteriophage system will be suitable for use in the method of the present invention. Advantageous features of a P2/P4 bacteriophage system include:

i) the preference of P2 terminase enzyme for plasmid templates, unlike other bacteriophage terminases which prefer to package concatamerised, linear
25 polynucleotide templates. Hence, this system is more suitable for *in vivo* packaging of plasmids that encode the polypeptide being screened for a desired activity, and

ii) a cosmid (i.e. a plasmid that comprises bacteriophage *cos* sequence that dictates bacteriophage packaging) that can be packaged efficiently into a P4-sized capsid (approximately 10-12 kb) is more amenable to routine cloning methods and
30 iterative mutagenesis than larger bacteriophage genomes, such as lambda (48.5 kb).

Genetic modification to produce lysis-defective phage

The life-cycle of lytic bacteriophage involves both genome replication and packaging as a phage particle, but also cell lysis for release of the particles for re-infection. Cell lysis of Gram-negative bacteria is a two-stage process, with the inner
35 membrane being first perforated, allowing a cell-wall degrading enzyme (a lysozyme)

to access the periplasmic space and act on the peptidoglycan cell wall. The cell is lysed by the difference in osmotic pressure between the cytoplasm and the surrounding solution thereby releasing phage particles into the medium.

The activities of membrane perforation (holin) and lysozyme are usually encoded by two genes in most lytic and lysogenic phage. Due to the parsimony of most phage genomes, these genes are often neighbours in the same operon. To retain the integrity of the cell wall, and to prevent the release of phage particles and proteins that are being functionally screened by a method of the invention, the gene encoding the phage lysozyme, or both the lysozyme and the holin, must be deleted from the phage genome. Due to the frequent use of overlapping reading frames and stop/start codons leading to translational coupling in many phage genes, the effects on the downstream gene/s must be carefully considered when designing these deletions. If the lysis cluster is translationally coupled to downstream structural genes, then preferably the deletion would leave a truncated ORF as the residual 'scar' at the locus having the start region of one gene and the stop region of the other.

In one embodiment, the method of protein screening of the invention uses permeabilisation of both inner and outer membranes of Gram-negative bacterium while retaining the structural integrity of the peptidoglycan cell wall. Thus, in this embodiment, the holin genes may be kept functional in the bacteriophage genome and may help contribute to the permeabilisation of the inner membrane, whereas the lysozyme gene is deleted in order to retain the cell wall structural integrity. In the instance of the lysis-defective phage being used in a screening system that is coupled to a periplasmic-targeted protein, such as described in WO 2002/034886 and WO 2005/095988, or to cell-wall binding fusion proteins, then the holin or holin/lysozyme functions must be deleted from the phage genome in order to retain the integrity of the inner membrane of the cell or spheroplast.

The person skilled in the art will understand that the lysin and/or lysozyme gene functions may be deleted by deleting the gene encoding the molecules from the phage genome, or alternatively by mutating the lysin and/or lysozyme genes such that they are defective and no longer encode a functional lysin and/or lysozyme protein.

In embodiments of the invention that utilise the P2 and P4 satellite system, the P2 genome contains both the holin (*Y*) and lysozyme genes (*K*) used by P4. Thus, a P2 prophage may be modified by deletion of the *K*, *Y* or *YK* genes. Construction of a lysis-defective P2 phage is described in Example 16 with the deletion of the *YK* genes from the genome of a P2 prophage of a K12 strain of *E. coli*. The P2 Δ *YK* prophage carrying a P4-sized cosmid may be infected by a P4 bacteriophage thereby inducing packaging

of the cosmid. The cosmid may also be induced for expression of the gene to be functionally screened. At the conclusion of induction of both cosmid packaging and gene expression, the cell membranes may be permeabilised and the cellular capsid screened by the method of the invention. Packaging of a cosmid by a lysis-defective P2 phage in an *E. coli* strain is described in Example 18 using infection of a strain carrying the P2 Δ YK prophage by a P4 phage.

In embodiments of the invention that utilise the lambda phage system, the lambda *S* and *R* genes encode the holin and endolysin (lysozyme), respectively. Deletion, or mutational inactivation, of the *R* or *SR* genes would produce a lysis-defective lambda prophage.

Construction of a lysis-defective lambda phage is described in Example 20 with the deletion of the *SR* genes from the genome of a lambda prophage of a K12 strain of *E. coli*. Packaging of a cosmid by a lysis-defective lambda phage in a *E. coli* strain is described in Example 21 by induction through inactivation of the thermolabile cI repressor.

In embodiments of the invention that utilise a lytic phage, using the T7 phage as an example, a lysis defective phage may be produced by mutational inactivation of gene 3.5 which encodes the T7 lysozyme. As the T7 lysozyme has a regulatory activity on T7 transcription via its inhibitory interaction with T7 RNAP, deletion of the lysozyme would be inadvisable. Therefore, mutants that specifically inactivate the cell-wall amidase activity of the enzyme are required to create lysis-defective mutants that retain T7 replication.

The lysin/holin systems of other lytic or lysogenic phages may be identified through comparison to known phage genomes, or through genetic analysis, and corresponding lysis-defective mutants may be created for their use in packaging libraries in the methods of the present invention.

Polynucleotide packaging by lysis-defective phage

In the methods of screening of the invention that utilise a lysis-defective phage, the method comprises culturing a Gram-negative cell comprising an exogenous polynucleotide encoding the polypeptide being screened such that the polypeptide is produced within the cell, and allowing the lysis-defective phage to package the polynucleotide encoding the polypeptide. The phrase "allowing a lysis-defective phage to package the polynucleotide" means that conditions are provided within a Gram-negative bacterial cell such that a lysis-defective phage is capable of packaging a polynucleotide.

The skilled person will understand that in some cases the culturing of the Gram-negative bacterial cell to produce the polypeptide and allowing the lysis-defective phage to package the polynucleotide may occur simultaneously. By way of non-limiting example, a Gram-negative bacterial cell comprising a helper prophage and a cosmid encoding a polypeptide of interest may be infected with a lysis-defective phage capable of packaging the cosmid, and then cultured to produce the polypeptide.

Alternatively, a Gram-negative bacterial cell comprising a helper prophage and a cosmid encoding a polypeptide of interest may be treated to induce the packaging functions of the prophage through, for example, heat inactivation of a labile repressor protein, or through co-induction of an activator protein, and then cultured to produce the polypeptide.

In this way, the polypeptide is produced in the Gram-negative bacterial cell while simultaneously the cosmid is packaged into the lysis-defective phage.

In one embodiment, the lysis-defective phage is retained (i.e. encapsulated) within a permeabilised Gram-negative bacterial cell and the polypeptide is screened for a desired activity according to the method of the invention. Specifically, a gene library encoding the polypeptide to be screened is cloned into a lysis-defective phage, or into a cosmid, and introduced into a Gram-negative bacterial cell. Both phage packaging and the polypeptide to be screened may be co-induced (i.e. induced simultaneously) and at the appropriate time point the population of Gram-negative bacterial cells is permeabilised using either a detergent or organic solvent with the polypeptide being retained within the cellular capsid, along with the phage. The population of permeabilised Gram-negative bacterial cells is then screened for the desired polypeptide activity.

The steps of culturing the Gram-negative bacterial cell to produce the polypeptide and allowing the lysis-defective phage to package the polynucleotide encoding the polypeptide may also be performed sequentially rather than simultaneously. Thus, the Gram-negative bacteria may be first cultured to produce the polypeptide, and subsequently the lysis-defective phage is allowed to package the polynucleotide encoding the polypeptide. The sequential production of the polypeptide in the bacterial cell followed by polynucleotide packaging may occur, for example, in instances where the polypeptide is encoded by a cosmid in the bacterial cell, and infecting the bacterial cell with a helper phage allows the lysis-defective phage to package the polynucleotide encoding the polypeptide. In one example of the method of the invention, the bacterial cell comprises P2 and/or P4 prophage and inducing activation of P2 prophage comprises inactivating a temperature sensitive repressor

allele of P2 protein C in the bacterial cell and/or expression of P4 activator proteins in the bacterial cell.

Alternatively, the Gram-negative bacteria may be cultured under conditions suitable for the production of the polypeptide, and allowing the lysis-defective phage to package the polynucleotide encoding the polypeptide may comprise inducing activation of a prophage in the bacterial cell to produce phage, wherein the phage packages the polynucleotide. As will be understood by the person skilled in the art, the step of inducing activation of the prophage in the cell could be performed simultaneously or sequentially with the step of culturing the Gram-negative bacterial cell to produce the polypeptide in the cell.

In light of the present specification, the skilled person will understand that there are several ways in which inducing activation of a lysis-defective phage to package a polynucleotide may be achieved. For example, inducing activation of a lysis-defective phage may comprise introducing a satellite or helper phage into a Gram-negative bacterial cell comprising a lysis-defective phage that is present as in the bacterial cell genome as prophage.

Alternatively, inducing activation may comprise producing one or more activator proteins of a prophage in a bacterial cell. For example, the Gram-negative bacterial cell may comprise a P2 and/or P4 prophage, and the P2 and/or P4 activator proteins may be, for example, selected from one or more of P2 *cox*, P2 *ogr*, P4 δ and/or P4 ϵ . As a result of activation, a prophage in a bacterial cell produces phage which package the polynucleotide. Alternatively, inducing activation of a lysis-defective phage to package a polynucleotide may comprise inactivating one or more phage repressor proteins in the bacterial cell. In one particular embodiment of the invention, inducing activation comprises inactivating a temperature sensitive repressor allele of lambda prophage in the bacterial cell. In another embodiment, inducing activation of a lysis-defective phage comprises increasing the incubation temperature of the bacterial cells.

30 Permeabilisation

In certain embodiments of method of the invention, either the outer cellular membrane alone, or both the inner and outer cellular membranes of a Gram-negative bacterial cell are permeabilised, thus allowing at least some of the soluble cellular components to diffuse through the cell wall. The polypeptide to be screened for a desired activity is retained within the bacterial cell wall, or is attached to the bacterial cell wall. As used herein, the terms "permeabilisation", "permeabilised" or

“permeabilised bacterial cell” refer to the use of a permeabilising agent or mechanical treatment, or a combination or both, to produce pores in the outer membrane, or both the inner and outer membranes, of a Gram-negative bacterium, or to solubilise the outer membrane, or both the inner and outer membranes, of a Gram-negative bacterium, while not hydrolysing linkages between peptidoglycans thereby keeping the cell wall intact. Non-limiting examples of agents capable of permeabilising a bacterial cell include detergents and organic solvents. A non-limiting example of a mechanical treatment capable of permeabilising a bacterial cell is electroporation.

Permeabilisation advantageously allows the entry of small to moderately sized proteins, for example up to 120 kDa, or other molecules of equivalent or smaller size, into the cellular capsule that remains intact. Further, by maintaining the integrity of the bacterial cell wall, the permeabilised bacterial cells are less fragile than spheroplasts that are produced, for example, by treatment of bacterial cells with Tris-EDTA-lysozyme, in which the bacterial cell wall is at least partially hydrolysed. The permeabilised bacterial cells produced in certain embodiments of the methods of the present invention are well suited to techniques such as fluorescence activated cell sorting (FACS), whereas spheroplasts are damaged by the high shear flow cytometry environment and require controlled osmotic conditions, thus limiting their potential uses.

Preferably, the permeabilisation treatment preserves the cellular proteins in their native state and interactions. Non-ionic detergents are generally less disruptive to protein folding and protein complexes than ionic detergents. Thus, in a preferred embodiment, a non-ionic detergent is used to permeabilise the bacterial cell wall. Non-limiting examples of non-ionic detergents include Triton X-100, Triton X-114, Brij 35, Brij 58, Tween 20, Tween 80, Nonidet P-40 Substitute, Octyl β Glucoside, Mega 8, Mega 9, Mega 10, BigCHAP, Deoxy BigCHAP, Apo8, and 8TGP (n-octyl- β -D-thioglucopyranoside).

Mixtures of detergents may be used to permeabilise the bacterial cell. For example, the detergent may be a mixture of two or more non-ionic detergents. In one embodiment, the detergent is a mixture of Mega 10 and Apo8.

When the polypeptide to be screened for a desired activity is attached to the bacterial cell wall, or integrated or attached to the inner cell membrane, the skilled person will appreciate that it may not be necessary to permeabilise the inner membrane of the bacterial cell. Thus, in one embodiment the bacterial cell is selectively permeabilised. By “selectively permeabilised” it is meant the outer membrane of the permeabilised bacterial cell is permeabilised to a greater extent than the inner

membrane, whereby 50% or less, or more preferably 40%, 30%, 20%, 10%, 5%, 4%, 3%, 2%, 1% or less, or none, of a membrane impermeable substance, for example the membrane impermeable DNA-binding ligand Gel Red, permeates the inner membrane of a selectively permeabilised cell when compared to a permeabilised cell in which
5 both the inner and outer membranes have been permeabilised such as by using a solution comprising 0.5% Mega 10 and 0.5% Apo8.

While the skilled person will be able to determine suitable conditions for selectively permeabilising a bacterial cell in accordance with the methods of the present invention, in one embodiment the bacterial cell is selectively permeabilised with a non-
10 ionic detergent. For example, the non-ionic detergent may be selected from Apo8 and Tween20. In one embodiment, a solution for selectively permeabilising the bacterial cell comprises the detergent at a concentration of about 0.2% to about 0.4%, or about 0.2% to about 0.3%, or at about 0.2%. Preferably, the solution for selectively permeabilising the bacterial cell comprises the detergent in a buffer comprising Ca^{2+} or
15 EDTA. Exemplary buffers suitable for selectively permeabilising a bacterial cell include 0.2-0.4% Apo8 or Tween20 in 25 mM Tris, 1 mM EDTA (pH 8.0), or 25 mM Tris, 2 mM Ca^{2+} (pH 8.0). In one embodiment, selective permeabilisation of a bacterial cell may be achieved, for example, by incubating the cell in a suitable buffer at about 25°C for about 10 minutes.

20 In another embodiment, the agent capable of permeabilising a Gram-negative bacterial cell is an organic solvent such as chloroform. By way of example, the inner and outer bacterial cellular membranes can be permeabilised by the suspension of the Gram-negative bacterial cells in an aqueous solution that has been saturated with the lipophilic solvent chloroform. To create a saturated solution of chloroform requires
25 mixing of the two immiscible phases of water and the organic solvent by agitating the two, usually by shaking, or on a mechanical vortex, until the chloroform phase is suspended as fine droplets. The two phases are allowed to settle, and a pulsed centrifugation is used to aid in the separation of the phases. A mixture of 5 % (v/v) chloroform is sufficient to create a saturated solution. An incubation time of 10 minutes
30 at 25°C is sufficient for permeabilisation of both cellular membranes. Example 19 and Figure 22 describe and demonstrate the permeabilisation of *E. coli* inner and outer membranes using organic solvent, chloroform.

Polypeptide expression

35 A polypeptide to be screened for a desired activity may be cloned into a suitable vector for expression in a bacterial cell. "Vector" as used herein refers to any vector

known in the art to be suitable for transforming a bacterial cell. Preferably, the vector is also capable of replicating within the bacterial cell independently of the host's genome. Vectors include plasmids, viruses and cosmids as well as linear DNA elements, such as the linear phage N15 of *E. coli*, and/or extrachromosomal DNA that replicates independently of a bacterial cell genome. Preferably, the vector is an expression vector. As would be understood by the skilled person, in embodiments in which the polynucleotide encoding the polypeptide is packaged into a phage, the vector will be in a suitable form, and comprise the necessary sequence (for example, such as the cos sequences in a cosmid), for packaging of the polynucleotide into the phage.

As used herein, an "expression vector" is a vector that is capable of effecting expression of a specified polynucleotide molecule in a bacterial cell. Preferably, the expression vector is also capable of replicating within the bacterial cell. Suitable expression vectors typically contain regulatory sequences such as transcription control sequences, translation control sequences, origins of replication, and other regulatory sequences that are compatible with the recombinant bacterial cell and that control the expression of polynucleotide molecules encoding a polypeptide. Transcription control sequences are sequences which control the initiation, elongation, and termination of transcription. Particularly important transcription control sequences are those which control transcription initiation, such as promoter, enhancer, operator and repressor sequences. Suitable transcription control sequences include any transcription control sequence that can function in a bacterial cell. A variety of such transcription control sequences are known to those skilled in the art.

Transformation of an expression vector into a bacterial cell can be accomplished by any suitable method by which a polynucleotide molecule can be inserted into the cell. Transformation techniques include, but are not limited to, electroporation and chemical transformation. Transformed polynucleotide molecules can remain extrachromosomal or can integrate into one or more sites within a chromosome of the transformed (i.e., recombinant) cell in such a manner that their ability to be expressed is retained.

Recombinant DNA technologies can be used to improve expression of a transformed polynucleotide molecule by manipulating, for example, the number of copies of the polynucleotide molecule within a host cell, the efficiency with which those polynucleotide molecules are transcribed, the efficiency with which the resultant transcripts are translated, and the efficiency of post-translational modifications. Recombinant techniques useful for increasing the expression of polynucleotide molecules include, but are not limited to, operatively linking polynucleotide molecules

to high-copy number plasmids, addition of vector stability sequences to plasmids, substitutions or modifications of transcription control signals (e.g., promoters, operators, enhancers), substitutions or modifications of translational control signals (e.g., ribosome binding sites, Shine-Dalgarno sequences), modification of
5 polynucleotide molecules to correspond to the codon usage of the host cell, and the deletion of sequences that destabilize transcripts.

The skilled person will be able to readily determine bacterial strains suitable for expressing polypeptides in the methods of the invention. Those skilled in the art would understand that Gram-negative bacteria suitable for use in the methods of the invention
10 include *Salmonella*, *E. coli*, *Shigella*, *Campylobacter*, *Fusobacterium*, *Bordetella*, *Pasteurella*, *Actinobacillus*, *Haemophilus* and *Histophilus*. In a preferred embodiment, the Gram-negative bacteria is *E. coli*.

Protein complexes

15 The polypeptide to be screened for a desired activity may be associated with at least a second polypeptide to form a protein complex having a molecular size such that the protein complex is retained inside the permeabilised bacterial cell. The polypeptide may be associated with the second polypeptide by, for example, covalent bonds such as disulphide bridges, or by non-covalent association. "Non-covalent association" refers to
20 molecular interactions that do not involve an interatomic bond. For example, non-covalent interactions involve ionic bonds, hydrogen bonds, hydrophobic interactions, and van der Waals forces. Non-covalent forces may be used to hold separate polypeptide chains together in proteins or in protein complexes. Thus, the polypeptide and second polypeptide may be expressed as separate polypeptides either from the
25 same or different vectors, or one or both of the polypeptides may be expressed from DNA encoding the polypeptides that has been integrated into the bacterial cell genome.

Alternatively, the polypeptide and second polypeptide which are associated in a protein complex may be a fusion protein. As used herein, "fusion protein" refers to a hybrid protein, which consists of two or more polypeptides, or fragments thereof,
30 resulting from the expression of a polynucleotide that encodes at least a portion of each of the two polypeptides and joined by a peptidic bond.

Protein complexes retained in the permeabilised bacterial cell by molecular size

The second polypeptide may be any polypeptide having sufficient molecular size, i.e. sufficient molecular weight or molecular radius, such that at least some of the
35 complex formed with the polypeptide being screened for a desired activity is incapable of diffusion from the permeabilised bacterial cell. Thus, the protein complex is retained

within the bacterial cell following permeabilisation of the cell. The person skilled in the art will appreciate that the nature of the second polypeptide, including its molecular weight and whether it is a globular or rod (filamentous) protein, will determine its ability to prevent or inhibit diffusion of the protein complex through the bacterial cell wall. In one embodiment, the molecular weight of the second polypeptide is at least about 30 kDa, or at least about 40, 50, 60, 70, 80, 90, 100, 120, 130, 140, 150 or more kDa. In one embodiment, the second polypeptide is at least about 120 kDa.

In one embodiment, the second polypeptide forms multimers having a molecular size greater than the pore-exclusion size of the permeabilised bacterial cell. As used herein, the term "multimer" and grammatical variations thereof refer to formation of a multimeric complex between two or more distinct molecules. The multimer may comprise, for example, two or more molecules of the same protein (i.e. a homomultimer) or a mixture of two or more different or non-identical proteins (i.e. a heteromultimer). Proteins that form multimers suitable for use in the methods of the invention include those that form dimers, trimers, tetramers, pentamers, hexamers, and higher order multimers comprising seven or more subunits.

Multimeric proteins include homodimers, for example, PDGF receptor α and β isoforms, erythropoietin receptor, MPL, and G-CSF receptor, heterodimers whose subunits each have ligand-binding and effector domains, for example, PDGF receptor $\alpha\beta$ isoform, and multimers having component subunits with disparate functions, for example, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, and GM-CSF receptors. Non-limiting examples of other multimeric proteins that may be utilized in the methods of the present invention include factors involved in the synthesis or replication of DNA, such as DNA polymerase proteins involved in the production of mRNA, such as TFIID and TFIIH; cell, nuclear and other membrane-associated proteins, such as hormone and other signal transduction receptors, active transport proteins and ion channels, multimeric proteins in the blood, including hemoglobin, fibrinogen and von Willabrand's Factor; proteins that form structures within the cell, such as actin, myosin, and tubulin and other cytoskeletal proteins; proteins that form structures in the extra cellular environment, such as collagen, elastin and fibronectin; proteins involved in intra- and extra-cellular transport, such as kinesin and dynein, the SNARE family of proteins (soluble NSF attachment protein receptor) and clathrin; proteins that help regulate chromatin structure, such as histones and protamines, Swi3p, Rsc8p and moira; multimeric transcription factors such as Fos, Jun and CBTF (CCAAT box transcription factor); multimeric enzymes such as acetylcholinesterase and alcohol dehydrogenase; chaperone proteins such as GroE, Gro EL (chaperonin 60) and Gro ES

(chaperonin 10); anti-toxins, such as snake venom, botulism toxin, *Streptococcus* super antigens; lysins (enzymes from bacteriophage and viruses); as well as most allosteric proteins. In one embodiment, the multimeric protein is an *E. coli* protein. Non-limiting examples of *E. coli* proteins that form multimers include L-rhamnose isomerase
5 (Rhna; for example NCBI accession CAA43002), β -galactosidase (β -gal; for example NCBI accession YP 001461520), betaine aldehyde dehydrogenase (BetB; for example NCBI accession AAA23506), glutamate-5-kinase (G5K; for example NCBI accession AAB08662), glutathione synthase (GshB; for example NCBI accession AP_003504), and a medium chain aldehyde dehydrogenase (YdcW; for example NCBI accession
10 AP_002067).

In one embodiment, the polypeptide being screened for a desired activity has a molecular size sufficient to retain the polypeptide within the bacterial cell wall. Thus, the person skilled in the art will appreciate that such a polypeptide need not necessarily be associated with a second polypeptide in order to retain the polypeptide within the
15 permeabilised bacterial cell.

Capsid display on lytic and lysogenic phage

In another embodiment, the polypeptide may be attached to a large macromolecular complex, such as a bacteriophage and/or phage coat protein. The attachment of the polypeptide to the phage may be achieved through a direct fusion of
20 the genes for the polypeptide to the gene of a phage coat protein, or it may be via a strong interaction between two separately expressed polypeptides. The attachment of libraries of proteins onto the surface of the head of lytic and lysogenic bacteriophage is known as "capsid display". An example of phage coat proteins that may be suitably adapted for fusion to the polypeptide are the genes for the 11 kD lambda D protein
25 (Sternberg and Hoess, 1995; Mikawa et al., 1996), which decorates lambda bacteriophage heads, or the 25 kD lambda V protein (Maruyama et al., 1994), which is the tail sheath protein. Other lytic phages have also used polypeptide fusions to the 9 kD SOC protein of T4-like phages (Rao et al., 2007) and fusions to the C-terminus of the 42 kD T7 capsid protein, 10B (Dai et al., 2008). In the instance of the P2/P4
30 bacteriophage system, peptides have been displayed at the N-terminus of the 21 kD P4 Psu protein (Lindqvist and Naderi, 1995).

An exemplary method of capsid display is the fusion of peptides or polypeptides to the capsid protein, gpD, of lambda bacteriophage. This method has been described extensively in the literature (Sternberg and Hoess, 1995; Mikawa et al., 1996; Gupta et

al., 2003; Vaccaro et al., 2006; Levy et al., 2007) and in US patent number 7,732,150 and US patent number 6,884,612.

The lambda gpD protein has been shown to tolerate fusions of polypeptides to either the N- or C-terminus (Mikawa et al., 1996) with a valency of up to ~400 per
5 phage, although loading of higher than ~50% of fusion protein per head decreases the phage viability. Direct comparisons of lambda capsid display against filamentous phage display demonstrated superior fusion protein expression and capture efficiencies during target panning (Santini et al., 1998; Gupta et al., 2003). Although lambda capsid display would be of great utility in screening antibody libraries, it has only been
10 cited as in use by three groups. Gupta et al. (2003) demonstrated that a single-chain antibody (scFv) productively folded and was approximately 100-fold more reactive by an ELISA assay than a filamentous-phage displayed antibody. Similarly, Vaccaro et al. (2006) found lambda to be an excellent platform display of a scFv. However, as shown by Vaccaro et al. (2006) this was due to the remarkable and rare stability of the scFv
15 that had been chosen, which was able to be folded in the cytoplasm. These authors demonstrated that for other scFv sequences there was likely to be difficulty in obtaining productive display. Levy et al. (2007) acknowledged and made use of this fact in an attempt to use lambda display as a genetic screen to select for *E. coli* cytoplasmic proteins that would enhance productive folding of scFvs in the cytoplasm. Their result
20 was only a very modest improvement in the productive folding of a scFv. The methods of the present invention can utilise a stable scFv scaffold, such as demonstrated by Gupta et al. (2003) and Vaccaro et al. (2006) in a capsid display system in a lysis-defective phage.

There are a number of advantages of using lysis-defective phage to both package
25 the polynucleotide and display the encoded polypeptide on the capsid surface. Firstly, the phage capsid serves as a stable, endonuclease-protected encapsulation for the polynucleotide in a form that, once released, enables a high-yield of recovery (nearly 100% of packaged phage can be recovered). Secondly, the phage capsid serves as a stable and numerous binding site for the encoded polypeptide within the permeabilised
30 cell. Figure 25 demonstrates that this property can be utilised to directly visualise a scFv binding to its fluorescent target where the scFv is fused to the lambda gpD protein. Thus, the polynucleotide library packaged into a lysis-defective phage, and further encapsulated by the permeabilised cell may be used in the method of protein display of the invention. An example of display using this embodiment would be to
35 screen for the binding of a fluorescently-tagged target to a capsid-displayed antibody or affinity scaffold, where the binding is detected using either fluorescence microscopy or

using FACS. Figure 25 demonstrates the positive identification of fluorescent target binding to encapsulated phage displaying a capsid-bound antibody using FACS.

A third advantage of library display and packaging using lysis-defective phage is that the failed release of the phage from the induced host cells enables the
5 concentration of phage to high titres through centrifugation of the host cells, followed by permeabilisation using either detergent or chloroform, and induced lysis using purified lysozyme. The inventors can report that the titres of lambdoid phages can be increased 100-fold compared with liquid culture titres of lysed phage when the lysis-defective mutants of the phages are used for packaging. It is routine to achieve titres of
10 $>10^{11}$ phage per mL of Readylyse (Epicentre)-lysed cells when phage are packaged using lysis-defective mutants. In order to achieve titres of this level requires laborious precipitation and ultra-centrifugation of the phage lysates, which risks the loss of surface-bound fusion protein during the long procedure.

Yet another enhancement of capsid display enabled by the method of the
15 invention is that excess soluble capsid fusion protein that is not bound to the encapsulated phage particles may be easily removed by cellular permeabilisation. This feature is important for target binding to the encapsulated bacteriophage particles, as otherwise binding may occur in solution to the fusion protein that is not capsid-bound, and which is usually in excess. Without partition of the capsid-bound and soluble
20 fusion protein the binding and/or capture of bacteriophage that display an affinity protein would be reduced.

Examples 20 and 23 describe the fusion of affinity proteins to both the Hy5
phage (a P2/186 hybrid with the P2 structural genes) and the lambda phage capsid proteins, gpL and gpD, respectively, and their demonstrated use in enrichment through
25 a matrix-bound target.

The polypeptide to be screened need not be directly fused to the phage coat protein, instead it may expressed as a separate polypeptide that is linked *in vivo* to the exterior of the phage through a stable association of protein domains. An example of such an association may be the high affinity between a protein domain and a peptide
30 ligand, such as observed between calmodulin and calmodulin-binding peptides (CBPs). Alternatively, the association could be established through a covalent interaction between two polypeptides. An example of this would be the SNAP and CLIP proteins (New England Biolabs) that would be separately fused as partners to the display protein and a bacteriophage coat protein, and a ligand that is covalently bound by both
35 proteins.

DNA binding proteins

The present inventors have found that DNA is retained within a bacterial cell following permeabilisation. Thus, in one embodiment, the polypeptide is associated with a DNA-binding protein to form a protein complex that binds DNA and that is retained inside the bacterial cell. As used herein, "DNA-binding protein" refers to any protein comprising a DNA-binding domain comprising at least one motif that recognizes double-stranded or single-stranded DNA. As would be known to the person skilled in the art, DNA-binding domains include helix-turn-helix, zinc finger, leucine zipper, winged helix, winged helix turn helix, helix-loop-helix, immunoglobulin fold recognizing DNA, or B3 domains. Associating the polypeptide with a DNA-binding protein advantageously provides for enhanced recovery of DNA, for example a plasmid, encoding the polypeptide in the screening methods of the invention.

Examples of DNA binding proteins include bacterial competence proteins such as, but not limited to, *E. coli* DNA binding proteins, *Neisseria gonorrhoeae* DNA binding proteins, for example ComE, Adenovirus E2 proteins, AraC transcription factor, basic helix-loop-helix transcription factors, basic-leucine zipper transcription factors, butyrate response factor, centromere protein B, COUP transcription factors, early growth response transcription factors, G-box binding factors, GATA transcription factors, HMGA proteins, homeodomain proteins, I-kappa B proteins, integration host factors, interferon regulatory factors, interferon-stimulated gene factor 3, Kruppel-like transcription factors, leucine responsive regulatory protein, matrix attachment region binding proteins, methyl-CpG-binding protein, MutS homolog 2 protein, myeloid-lymphoid leukaemia protein, NF-Kappa B, NF1 transcription factors, nuclear respiratory factors, oncogene protein p53, origin recognition complex, paired box transcription factors, POU domain factors, proto-oncogene factors, Rad51 recombinase, Rad52 DNA repair and recombination protein, replication protein A, replication protein C, retinoblastoma protein, Smad proteins, SOX transcription factors, T-box domain proteins, TCF transcription factors, telomere-binding proteins, Toll-like receptor 9, trans-activators, and winged-helix transcription factors. In one embodiment, the DNA binding protein is an *E. coli* DNA binding protein. In another embodiment, the DNA binding protein is a *Neisseria gonorrhoeae* protein, for example ComE, or a domain thereof.

Cell wall binding proteins

The polypeptide that is being screened for a desired activity may be associated with a bacterial cell wall-binding protein. The skilled person will understand that the

choice of a cell wall-binding protein would depend on the host cell species, as different bacteria have different cell wall compositions. While bacteria have cell walls made up of peptidoglycan (PG), chemical modifications between species can affect cross-species binding. The skilled person will readily be able to determine cell wall-binding proteins suitable for use in a particular bacterial species.

Bacterial cell wall-binding proteins include proteins known to have a domain structure, whereby part of the polypeptide chain in the native structure is able to recognise and bind specific molecules or molecular conformations on the bacterial cell wall. Thus, the term "bacterial cell wall-binding protein" includes a protein domain which is part of the protein which specifically binds to the bacterial cell wall. Examples of bacterial cell wall-binding proteins include the cell wall hydrolases as coded by bacteriophages, cell wall hydrolases of bacteria and different autolysins. Further encompassed are receptor molecules coded by the DNA of bacteriophages and other viruses. Where the bacterial cell wall-binding protein is from hydrolytic enzymes of bacteriophage origin, which are capable of specific binding to bacteria, the cell wall-binding protein maintain their binding ability but preferably have no significant hydrolytic activity.

In one embodiment, the cell wall-binding protein binds non-covalently to the cell wall of *E. coli*. For example, for an *E. coli* host cell there are endogenous PG-binding proteins with a conserved ~100 amino acid PG-binding domain occurring in PAL, OmpA, YiaD, YfiB, and MotB (Parsons et al., 2006). However, proteins from other organisms have been shown to be well expressed in *E. coli* and to bind the cell wall with high affinity, for example the ~70 amino acid PG-binding domain from *Pseudomonas* ϕ KZ phage (KzPG) (Briers et al., 2009). Thus a PG-binding domain from a protein that binds PG may be used as a bacterial cell wall-binding protein in the methods of the invention.

In an exemplary embodiment, the PG-binding domain may be fused to the polypeptide that is being screened for a desired activity and expressed in the cytosol of the bacterial cell. Upon membrane permeabilisation, the PG-binding domain gains access to and binds the cell wall resulting in the retention of the polypeptide of interest within the permeabilised cell. To potentially further enhance retention of the polypeptide of interest within the cell, the skilled person will understand that the polypeptide may be associated with a DNA-binding protein in addition to a bacterial cell wall-binding protein.

Alternatively, the polypeptide of interest may be associated with a protein that is capable of linking covalently to the bacterial cell wall. Preferably the protein comprises

a periplasmic-targeting signal. Thus, the polypeptide is expressed in the cytosol of the bacterial cell, but targeted to the periplasm where it is linked to the cell wall before membrane permeabilisation.

By way of non-limiting example, the bacterial cell wall-binding protein that
5 attaches to the cell wall covalently may be a lipoprotein capable of binding to the cell wall and which lacks a functional N-terminal signal sequence necessary for outer membrane attachment. For example, the lipoprotein may be *E. coli* LPP. LPP is an abundant *E. coli* protein that forms a trimeric coiled-coil. In its native form, one end is tethered to the outer membrane via lipidation and the other is covalently bound to the
10 cell wall via a C-terminal lysine. The lipoprotein may further comprise a sequence which targets the lipoprotein to the periplasm, for example an OmpF periplasmic targeting sequence. In one embodiment, the lipoprotein is *E. coli* lipoprotein lacking a functional N-terminal signal sequence necessary for outer membrane attachment.

In light of the teaching of the present specification, the person skilled in the art
15 will be able to identify or design proteins that attach covalently to the bacterial cell wall and that are suitable for use in the methods of the present invention.

In one embodiment of the invention, the polypeptide being screened for a desired activity is a fusion polypeptide comprising a KzPG domain and one or more other domains selected from a spacer, SNAP and/or DBP. In one particular
20 embodiment, the fusion polypeptide comprises one or more spacers and the KzPG, SNAP and DBP domains.

Spacers

In one embodiment, the polypeptide being screened for a desired activity may be expressed as a fusion polypeptide which comprises one or more spacers. A "spacer" as
25 used herein refers to a peptide or polypeptide that may be included in a fusion polypeptide to enhance expression of the polypeptide in a bacterial cell or to decrease steric hindrance such that the polypeptide being screened for a desired activity may assume its desired tertiary structure and/or interact appropriately with its target molecule. Thus, the fusion protein may comprise one or more spacers before, after, or
30 between one or more polypeptide domains in the fusion polypeptide. For spacers and methods of identifying desirable spacers, see, for example, George, et al. (2003).

In one embodiment, the spacer comprises one or more amino acid sequences that are between 1-50 amino acid residues in length, or about 1-25 residues, or about 5-
35 15 residues in length. For example, the spacer may be selected from one or more of I27, RL1, RL2, RL3, RL4, RL5 and/or RL6. The person skilled in the art will understand

that a limited number of amino acid substitutions, for example, 1, 2, 3, 4 or 5 amino acid substitutions may be introduced into the spacer without affecting its ability to function as a spacer. In one particular embodiment, the one or more spacers are selected from any one of SEQ ID NOs:6 to 12 or 16. Thus in one embodiment, the polypeptide being screened for a desired activity is a fusion polypeptide comprising I27, RL6, KzPG, SNAP and DBP.

In another embodiment, the spacer region may comprise a peptide sequence that is a high-affinity binding site for a protein domain. For example, calmodulin has a Ca^{2+} -dependent affinity for a number of peptide sequences from protein ligands that have been mapped to short peptide regions. These CBPs (Calmodulin Binding Peptides) have been mutated for even higher affinity binding to calmodulin (K_d 's between ~ 1 nM to 1 pM) (Montigiani et al., 1996), enabling a Ca^{2+} switchable, high affinity interaction between two proteins, one having a CBP spacer region and the other fused to calmodulin.

Screening methods and protein evolution

The present invention provides methods for screening polypeptides for a desired activity against a target molecule. As used herein, the term "desired activity" refers to any potential useful activity of a polypeptide and includes, but is not limited to, binding, enzymatic modification, folding stability and/or thermal stability.

The term "target molecule" refers to a molecule that binds to and/or is modified by the polypeptide and may be for example an antibody, a receptor, an antigen, an enzyme etc. Thus, "target molecule" can be used to refer to a substrate such as an enzymatic substrate or a molecule that is being evaluated for binding (e.g., a ligand, epitope, antigen, multimerization partner such as a homo or hetero dimeric partner, etc., or any combination thereof).

It will be appreciated that polypeptide activities may be screened for or selected in the context of a single type of cell expressing a single polypeptide, or in the context of a library of cells each expressing a different polypeptide or polypeptide variant. Thus, the methods of the present invention may be used for *in vitro* protein evolution. *In vitro* protein evolution allows for a large number of protein functions and characteristics to be investigated and typically comprises two main steps: diversification and selection. Diversification relies on the ability to generate diverse libraries of nucleic acids coding for polypeptides. Selection can be achieved by screening the libraries for a desired activity and linking the activity to the genotype, for

example, by identifying the member of the library that comprises the genotype that is responsible for the observed activity.

DNA libraries are a collection of recombinant vectors containing DNA inserts (DNA fragments) that encode a polypeptide. The origin of the DNA inserts can be genomic, cDNA, synthetic or semi-synthetic. The polypeptide may have any desired activity, for example the polypeptide of interest may be a binding protein, for example an antibody, or an enzyme for example, a polymerase, ligase, restriction enzyme, topoisomerase, kinase, phosphatase, metabolic enzyme, catalytic enzyme, or a growth factor hormone, antimicrobial peptide, antigen, receptor, reporter protein, immunomodulatory protein, neurotransmitter, structural protein, transcription factor or transporter. In one embodiment, the polypeptide is an antibody or an enzyme. Thus, the methods of the present invention can be used for screening for variants of a polypeptide having a desired activity.

The cloning and construction of DNA libraries of, for example, binding proteins or enzymes, can be performed using methods known in the art. For example, Lutz and Patrick (2004) have reviewed methods of generating library variability and strategies for gene recombination for use in protein engineering. For screening of displayed polypeptide variants, the strategies used for surface-displayed libraries could be adopted and adapted for the methods of the present invention (Becker et al., 2004; Daugherty et al., 2000; Kenrick et al., 2007; Miller et al., 2006).

A library of nucleic acids can be introduced into a plurality of bacterial cells resulting in the expression of a member of the library in each of the bacterial cells. In addition to being expressed, the polypeptides are retained within the permeabilised bacterial cell, or attached to the cell wall, in order to evaluate their function or characteristic. Nucleic acid libraries of a polypeptide, for example, a binding protein such as an antibody, or of an enzyme, can be generated through a variety of methods including through the introduction of mutations such as point mutations, deletions, and insertions, or through recombination events. Methods for the generation of libraries of variants are known in the art and include error-prone PCR, synthesis of DNA in DNA repair compromised bacteria, and chemical modification of DNA. Methods for the generation of libraries through recombination are known in the art and include gene shuffling, assembly of DNA in highly recombinogenic bacteria, synthetic nucleic acid library assembly, etc., or any combination thereof. In this way a library of polynucleotides encoding polypeptides can be introduced into a plurality of bacterial cells resulting in the expression of one or members of the library in each of the bacterial cells.

In some embodiments, a library comprises two or more variants of a polypeptide wherein each variant comprises a unique polypeptide with a minor change in amino acid sequence. In other embodiments, a library comprises two or more unrelated sequences. For example, to identify a candidate polypeptide that can inhibit an enzyme, a library of random sequences or predetermined sequences may be interrogated. A library can have at least 2, at least 5, at least 10, at least 50, at least 100, at least 1000, at least 10,000, at least 100,000, at least 1,000,000, at least 10^7 or more members.

Binding protein display

In one embodiment, the methods of the present invention are applied to the evolution of binding proteins, such as for example antibodies. Thus, in one embodiment, the polypeptide that is screened for a desired activity is a binding protein, the target molecule may be any molecule to which the binding protein may bind, and the desired activity is binding, and/or the extent of binding to the target molecule. The methods of the invention may comprise, for example, culturing a bacterial cell comprising a polynucleotide encoding a binding protein so that the protein is produced in the cell. The cell is subsequently permeabilised and the permeabilised cell contacted with a target molecule. Any suitable method in the art may be used for determining if the polypeptide binds, and/or the extent of binding to, the target molecule.

The methods of the invention are particularly suited to the screening of binding protein display libraries. Unlike other methods of *in vivo* surface display, which absolutely require the targeting of the protein to an extracellular space as the cellular membranes prevent interaction with the labeled target presented to the display protein, the methods of the invention can express and fold the affinity proteins in the cytoplasm of the host cell. Thus, the screening parameters can include the high yield and productive folding of the affinity variant protein in the cytoplasm of bacteria.

Furthermore, as cytoplasmic protein expression and folding is in a reducing environment, the methods of the invention can be applied to select for variants of antibodies, or other proteins that have disulphide bonds in their native form, that can productively fold in a reducing environment. The variants selected would be expected to be more stable as they would not be reliant on intra- or inter-domain disulphide bonds for folding stability. This approach has application towards the development of antibodies that could be used for intracellular binding of targets, to either neutralize or label.

The methods of the invention can therefore be used as a platform for the display and selection of a variety of binding proteins, including those scaffolds known to the

art, such as single-chain antibodies (scFv), domain antibodies, Fab, and the non-antibody scaffolds such as lipocalins, FN3, ubiquitin, γ -B-crystallin.

Polypeptide screening in combination with phage display

Conventional phage display, with the polypeptide being screened attached to the
5 surface of a filamentous phage, generally in only one or a few copies, enables the screening of large numbers of clones in parallel for affinity to a target molecule. However, the background of low- and moderate-affinity clones is high and unique clones cannot be distinguished without subcloning and sequencing, and the determination of the properties of each unique clone (for example, expression levels,
10 solubility and affinity) usually requires a change in the format of expression. Thus, in prior art methods a substantial amount of work lies downstream from the initial phage display screen.

In comparison, the methods of the invention allow the use of FACS to characterise polypeptides within, or attached to, Gram-negative bacterial cells. FACS
15 enables the binding parameters to be defined resulting in clones with the desired characteristics being highly enriched. However, the screening of individual clones is sequential and even with sort rates of 10^4 clones per second there is a comparatively low upper limit on the number of individuals which may be processed in one screen. For example, screening 10^8 clones could take over 2 hours.

20 Thus, in certain circumstances it may be desirable to combine the parallel screening of conventional phage display systems with the clonal characterisation of cellular display analysed by FACS as provided by the present invention. Hence, early screens may be performed by conventional phage display, with the output clones being subsequently analysed by the display system of the present invention in which the
25 polypeptide is retained within the bacterial cell by the cell wall and/or by attachment to the cell wall, or by attachment to an encapsulated lysis-defective phage.

Thus, a gene library can be expressed and displayed on the surface of a lytic phage or filamentous phage using fusions to phage proteins, or through stable association of two polypeptides as described herein. These phage can be screened for
30 activity of the polypeptide ('panning') using standard techniques for bacteriophage display. Phage displaying the fusion protein that adhere to a target molecule, such as an affinity substrate, can be produced and recovered as, for example, cosmids by infection into the helper strain containing the prophage lysogen. This cycle can be iterated until the library is dominated by enriched clones. At this point, the library could be

subcloned into a vector system that performs encapsulated display according to the method of the invention.

Accordingly, in one embodiment of the present invention, an additional screening step is performed before and/or after the screening method of the invention.

5 As outlined above, the additional screening involves a conventional phage display system, i.e., a phage display system in which: i) the polynucleotide encoding the polypeptide being screened for a desired activity is not packaged into a lysis defective-phage, and/or ii) the polypeptide is not retained within the bacterial cell by the bacterial cell wall and/or attached to the bacterial cell wall. Such additional screening may
10 involve known methods of phage display such as the use of lytic lambda phage or filamentous M13 phage.

In a further embodiment of the method of the invention, the conventional phage display method can be combined with the use of the lysis-defective phage and encapsulated cellular display to enable the facile switching between the two forms of
15 protein display, without further subcloning of the DNA of the enriched library.

In this embodiment, the protein to be displayed may be encoded as a fusion to a phage capsid protein by a polynucleotide cloned into a lysis-defective bacteriophage or phagemid, or into a cosmid that is packagable by a prophage. Methods for constructing a bacteriophage vector for capsid display such as a lambda vector are described in
20 Mikawa et al. (1996), Sternberg and Hoess (1995), and Vaccaro et al. (2006). Methods for the construction of phagemid and cosmid vectors are also well known in the art. For example, phagemid vectors based on the pUC and pBR322 origins were described by Yankovsky et al. (1989) and King et al. (1982), respectively. For lambda cosmids, Sambrook and Russell (2001), and for P2/P4/186 cosmids, Kahn et al. (1991), both
25 provide good general descriptions and details of vectors. The cosmid will be transformed into a bacterial cell line containing a lysis-defective prophage and induced for both expression of the capsid fusion protein, and packaging of the cosmid vector.

Alternatively, the lysis-defective bacteriophage vector is transformed/infected into a host cell and similarly induced for expression of capsid fusion protein and the
30 bacteriophage vector genome.

Following packaging of the bacteriophage/cosmid vector the cells may be lysed to release the packaged phage by the combined treatment of a permeabilisation step using detergent or an organic solvent such as chloroform, and an enzymatic lysozyme activity, such as available commercially as ReadyLyse (Epicentre). The display library
35 may now be 'panned' for binding to a target by methods commonly used for phage display selection. Following panning, the library may be recovered by re-infection into

the bacterial host containing the lysis-defective prophage. Cycles of panning and re-infection may be iterated until the proportion of binding phage is substantially enriched in the library population at which point the next cycle of phage production the cells are permeabilised, but the enzymatic lysis step is omitted, thus producing a sub-library of
5 encapsulated phage. A fluorescently-labelled target may be bound to these encapsulated phage which may then be sorted by FACS, as described in Example 23 and observed for Figure 25.

The person skilled in the art will appreciate that this embodiment, provides for the display library to be moved between two different modes of display; 1) panning of
10 free phage to immobilised target which is a highly-parallel screen with low clonal selectivity; and 2) FACS characterisation and purification of encapsulated individual clones, which is a highly-selective but low-throughput screen. Thus, this embodiment of the method of the invention enables the ability to utilise the most powerful elements of each system without the user intervention for reformatting which would otherwise be
15 required. Such an embodiment is therefore highly amenable to a robotic, high-throughput, workflow.

In another embodiment of the method of the invention, soluble antibodies may be identified and utilised as scaffolds in gene libraries that can be switched between phage display and -Gram-negative bacterial cellular display by the method of the
20 invention.

Enzyme display

The methods of the invention can be used for the display of enzymes and enzyme libraries and for the evolution of enzyme properties. Thus, in one embodiment, the polypeptide that is screened for a desired activity is an enzyme, the target molecule
25 is a substrate of the enzyme, and the desired activity is binding to and/or enzymatic modification of the target molecule. The skilled person will understand that methods for the development of assays for enzyme activities using other surface display technologies could be equally applied as assays to the methods of the invention.

The methods of the invention would also be well suited in the use of enzyme
30 libraries that are expressed in the host cell, which is permeabilised and then suspended as a water-in-oil-in-water emulsion (w/o/w). Aharoni et al. (2005) demonstrated the utility of using cell surface-displayed enzyme libraries in a w/o/w emulsion by FACS for the improvement of paraoxonase. The advantages of encapsulation in a non-permeable oil membrane are that a diffusible substrate and product can be kept in
35 proximity to the enzyme activity and coding nucleic acid sequence. However, the

screen described by Aharoni et al. (2005) requires that the enzyme be displayed on the exterior of the host cell. Using the methods of the invention, intracellular expression and folding of enzyme libraries could be used for the improvement in enzyme function.

In the methods of the invention, a bacterial cell comprising a polynucleotide
5 encoding an enzyme is cultured in order to produce the enzyme. Following permeabilisation of the bacterial cell, the cell is contacted with a substrate of the enzyme and known methods may be used to determine if the enzyme modifies, and/or the rate of enzymatic modification of, the substrate.

In some instances, it may be desirable that the target molecule (for example an
10 enzyme substrate) is linked to the bacterial cell. The skilled person will understand that the target molecule may be linked to any component of the permeabilised bacterial cell, either directly or indirectly. Direct linking may be achieved, by way of non-limiting example, by linking the target molecule to the bacterial cell wall. Indirect linking of the target molecule may be achieved, for example, by linking the target molecule to the
15 second polypeptide that is associated with the polypeptide being screened for a desired activity to form a protein complex. For example, the target molecule may be linked to the polypeptide having a molecular size sufficient to retain the protein complex inside the permeabilised bacterial cell, or it may be linked to the DNA-binding protein, or to the bacterial cell wall-binding protein as used in the methods of the invention. Linking
20 the target molecule to the bacterial cell advantageously enables the isolation of bacterial cells presenting active enzymes using technologies such as, for example, FACS or by magnetic bead selections.

The person skilled in the art will readily be able to determine a coupling chemistry suitable for linking a target molecule to a bacterial cell. Suitable coupling
25 chemistries include cysteine labelling with thiol coupling reagents such as acrydite and maleimide, amine labeling, and carboxyl labeling which are commercially available from suppliers including Pierce Protein Research Products and Invitrogen.

Flow cytometry analysis

The cellular display technology of the present invention may present many
30 thousands of molecules of a polypeptide of interest at once and, unlike molecular display technologies such as ribosomal/mRNA display or phage display, may be screened using flow cytometry techniques, for example using fluorescence activated cell sorting (FACS) machines. Not only can positive events in the library be captured but parameters such as protein expression, enzymatic activity or target affinity can be
35 simultaneously defined for each positive member, thereby improving the output of the

screen. Instruments for carrying out flow cytometry are known in the art and include FACS Star Plus, FACScan and FACSsort (Becton Dickinson), Epics C, and MoFlo. Flow cytometric techniques in general involve the separation of cells in a liquid sample. Typically, the purpose of FACS is to analyse the cells for one or more characteristics, for example, the presence of a target molecule. Methods for performing flow cytometry analysis are well known in the art. For example, a review of methods using FACS for assaying enzyme activity is described by Farinas (2006).

For the present invention, flow cytometry is useful for multiple rounds of screening that can be carried out sequentially. Cells may be isolated from an initial round of sorting and immediately reintroduced into the flow cytometer and screened again to improve the stringency of the screen. Since flow cytometry is essentially a particle sorting technology, the ability to culture cells is not necessary. Techniques for the recovery of nucleic acids from non-viable cells are well known in the art and may include, for example, template-dependant amplification techniques including PCR.

After a Gram-negative bacterial cell has been identified that produces a polypeptide having a desired activity, DNA encoding the polypeptide may be isolated from the bacterial cell using any suitable known technique. Thus, the DNA encoding the polypeptide may be isolated and sequenced using conventional procedures. If desired, the polynucleotide may go through another round of diversification in order to generate another library of variants to be screened for the desired activity. In this way it is possible to use an iterative process to optimise the desired activity of a polypeptide.

In embodiments where the polynucleotide is packaged by a lysis-defective phage, the polynucleotides encoding the polypeptide members of the library having a desired activity may be easily and rapidly recovered from the post-FACS screen for further iterative enrichment or for clonal analysis by recovery of the packaged phage library, or cosmid library, by treatment of the permeabilised bacterial cells with a lysozyme, for example, ReadyLyse (Epicentre), to degrade the cell wall and release the phage for subsequent infection.

Thus, this embodiment of the invention allows for the concurrent characterisation of expression and binding parameters with FACS screening, together with the facile recovery and handling of phage-packaged gene libraries. Iterative rounds of FACS screening using lysis-deficient phage libraries are therefore simplified and do not rely on PCR-amplification of positive clones with both the attendant mutational error and handling required.

Packaging gene libraries using lysis-defective phage

The present inventors have found that using an inducible lysis-defective prophage allows for high efficiency cloning and packaging of a gene library in Gram-negative bacteria. An inducible prophage is one that is present in the genome of a Gram-negative bacterial cell, wherein upon inducing activation of the prophage, the prophage is activated as a phage in the bacterial cell. The phage in the cell may then be capable of packaging a polynucleotide.

The polynucleotides encoding the proteins to be screened by the display methods of the invention may be packaged into phages that have a lytic stage, or alternatively into a lysis-defective phage. Both lytic and lysogenic phage genomes generally have a region that is dispensable for either lifecycle, and may be replaced with a cloned gene and associated regulatory regions. Dispensable regions may include regions containing genes for DNA recombination (for example, the lambda bacteriophage *bet* and *exo* genes and *Nin5* region), or regions that provide host cell survival functions to a lysogen (for example, the lambda phage *Ea47*, *Ea31*, *Ea59*, *Lom* and *bor* genes; the P2 phage *Old*, and *Fun* genes). Alternatively, there may be a tolerance for packaging of genomes that are fractionally larger than normal (for example, Lambda bacteriophage will package up to 105% of the wild-type length of 48.5 kb) enabling cloning of short regions directly into the genome without replacement of nonessential regions, such as in the T7 Select Phage display system (Novagen). When the gene library is to be cloned into a lysis-defective bacteriophage vector, the lysis genes also represent a dispensable region.

Where possible, the polynucleotide encoding the polypeptide being screened for a desired activity should be cloned into a region of the phage genome such that it does not disrupt the transcription and translation of essential operons of the bacteriophage. Thus, the skilled person will understand the polypeptide may be expressed using its own transcriptional regulatory regions such as, for example, its own promoter and/or terminator.

Gene libraries of the polypeptides to be screened for a desired activity may also be constructed using elements that instruct a helper phage to package a plasmid as an infectious phage particle. For example, the *cos* regions of bacteriophages Lambda, P2, P4 and of other lambdoid phages are <500 bp elements that have the binding and cleavage sites for the terminase enzyme, which cuts and packages a plasmid containing these regions as a linear element within a phage capsid head. These *cos* regions can be cloned into a plasmid that is then referred to in the art as a cosmid. In general, the size of a cosmid must be close to the size of the wild-type genome, usually within 80 to 105

% of the wild-type phage genome, to be packaged efficiently. Alternatively, it may be a unit fraction of the wild-type genome (1/2, 1/3, 1/4) with multimers of the cosmid being packaged within a single phage head. The multimers may be formed within the cell by recombination between cosmids in a *recA*⁺ cell, or may be formed during the
5 replication cycle of bacteriophage (e.g. rolling circle replication by lambda bacteriophage).

The P2 phage, or the related 186 phage or hybrid of 186 and P2, and its satellite, P4, advantageously provide a cosmid of a manageable size for plasmid-based cloning techniques (~11 kb) and the P2 terminase protein has a preference for packaging
10 plasmid substrates containing a single *cos* region, rather than linear multimers with adjacent *cos* regions, as the Lambda terminase prefers. Thus a cosmid library can be constructed with ease and packaged *in vivo* with high efficiency. Methods for the packaging of gene libraries using a P2 phage are described in Kahn et al. (1991).

Phage libraries made using lytic bacteriophage (for example, T7 Select) may be
15 packaged *in vivo* following infection of a host cell. Where the phage is lysis-defective, the cell will remain intact until permeabilised and screened by the method of the invention. The infectious phage may then be recovered from selected cells by treatment with lysozyme to degrade the peptidoglycan and release the phage particles.

Phage libraries made using lysogenic bacteriophage or their cosmids are
20 packaged *in vivo* by inducing activation of an integrated prophage or by infection of a helper phage to produce phages and to enter the lytic pathway. Prophage induction is commonly achieved using temperature sensitive mutants of the phage immunity repressor protein. Libraries may be established in the host cell at the low temperature, and then bacteriophage packaging induced by an upward temperature shift. For
25 example, the *cI857* allele of the repressor gene of the lambda phage supports establishment and maintenance of lysogeny at 30°C, but is inactive at temperatures higher than 37°C, forcing prophage excision and entry into the lytic pathway. However, the P2 phage, which is known as a non-inducible phage, can not be induced by standard methods such as UV or a temperature-sensitive repressor. Instead, P2 functions can be
30 induced as a helper phage for cosmid packaging by using infection of the P4 satellite, in particular, *vir* mutants of P4 that prevent establishment of a P2/P4 co-lysogen, but instead activate P2. However, the P2-related phage, 186, does not have a temperature-sensitive repressor, *cI_{ts}*, that can induce phage replication and packaging upon inactivation (Woods and Egan (1974)). Furthermore, hybrid phage of P2 and 186 were
35 obtained by coinfection that contain the temperature-inducible replication control of

phage 186 with the structural genes of P2. One such phage is known as Hy5 (Younghusband et al., 1975).

Elements of P2 and P4 that regulate the P2 lytic pathway may also be cloned and induced to trigger lysis. Example 17 details the use of the transcription factors P4 δ , P2 *cox* and P2 *ogr* genes to induce lysis and cosmid packaging in an *E. coli* C strain P2/P4 co-lysogen. The lambda phage *cI857* repressor was used with its endogenous promoter and the operator region of the *cro* gene to regulate the expression of the P2 and P4 transcription factors. The δ gene was the most rapid activator of lysis, followed by the *ogr* and *cox* genes, in that order. Cellular lysis was accompanied by the release of infectious P4 phage and cosmid particles.

Other P2 control genes that may induce P2 activation include the entire P4 *sid- δ -*psu* operon and the P4 ϵ anti-repressor, or combinations thereof.*

A cosmid library transformed into cells containing a P2 helper phage with a co-lysogenic P4 helper phage, and/or the P4 control regions described above, may be packaged *in vivo* following induction of P2 activation. Where the phage is lysis-defective, the cell will remain intact until permeabilised and screened by the method of the invention. The infectious cosmid phage particles may then be recovered from selected cells by treatment with lysozyme to degrade the peptidoglycan and release the phage particles.

Kits

The necessary components for performing the methods of the invention may conveniently be provided in the form of a kit. As will be understood to a person skilled in the art, the various components in the kit may be supplied in individual containers or aliquots, or the solution components may be combined in different combinations and at different concentrations to achieve optimal performance of the methods of the invention. It is within the knowledge of the skilled addressee to determine which components of the kit may be combined such that the components are maintained in a stable form prior to use.

In one embodiment, the kits of the invention will typically at a minimum contain a vector which comprises a site for inserting into the vector a polynucleotide encoding a first polypeptide, and an open reading frame encoding a second polypeptide which associates with the first polypeptide to form a protein complex that is retained inside or attaches to the cell wall of a permeabilised bacterial cell. Preferably, the kit also contains an agent for permeabilising a bacterial cell. In one embodiment, the kit further comprises bacterial cells, preferably Gram-negative bacterial cells. Other additional

components may be included with the kit, or other components supplied by the end user, if required.

The invention also provides kits suitable for use in methods of screening a protein for a desired activity which utilise a lysis-defective phage. Such kits will typically comprise at a minimum a Gram-negative bacterium comprising a lysis-defective phage together with a temperature-sensitive phage repressor protein and/or a polynucleotide encoding one or more phage activator proteins. In one embodiment, the kit comprises a lysis-defective phage selected from P2, 186, Hy5 and/or P4. In another embodiment, the kit comprises a lysis-defective lambda phage. The kit may optionally
10 comprise an agent for permeabilising a Gram-negative bacterial cell.

EXAMPLES

Example 1. Screening for detergents that permeabilise *E. coli*

To identify detergents that would permeabilise *E. coli* cells, we screened a number of detergents, both ionic (n-dodecyl- β -iminodipropionic acid; decyltrimethylammonium chloride; sodium dodecanoyl sarcosine; anzerger 3-10) and
15 non-ionic (dimethyloctylphosphine oxide [Apo8]; dimethyldecylphosphine oxide; n-octyl- β -D-thioglucopyranoside [8TGP]; sucrose monododecanoate; Megal0; Tween 80; Triton X100; Triton X114), both for the uptake of the membrane-impermeable dye, Gel Red (Biotium, cat. no. 41002) and for the release of GFP. The detergents tested for
20 permeabilisation were purchased from Anatrace.

The *E. coli* host strain used in all reported experiments was the K12-derived *Argentum* (Alchemy Biosciences) cell line ($\Delta mcrA$ $\Delta(mrr-hsdRMS-mcrBC)$ $\Delta endA$ $lacZ\Delta M15$). However, the method of the invention was also tested, with comparable results, with the B-strain-derived BL21 (F- *dcm ompT hsdS(r_B- m_B-)* *gal*) and with the
25 K12 cloning strain DH5 α (F' *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* $\Phi 80dlacZ\Delta M15 \Delta(lacZYA-argF)U169$, *hsdR17(r_K m_K⁺)*, λ^-).

GFP was cloned into an arabinose-inducible, high copy-number vector (pAra1::GFP5). Expression was from a culture heavily inoculated from a plate with freshly-streaked colonies. The culture was grown at 37°C until an OD600 of ~0.3
30 when expression was induced by the addition of arabinose to a final concentration of 0.2%. The induced culture was shaken at 25°C for 2 hours before harvesting.

Cells were pelleted from 1 mL of induced culture by centrifugation and permeabilised by suspension in 300 μ L of 0.5 % detergent in LB and incubated at 25°C for 10 minutes. The permeabilised cells were pelleted and resuspended in 1 x Gel Red
35 in water for 2 minutes before being pelleted and washed once in 300 μ L of TBS. They

were suspended in 300 μ L of TBS and processed for fluorescence microscopy by the addition of DABCO/glycerol (0.0325 g DABCO dissolved in 900 μ L glycerol + 100 μ L PBS).

Samples were visualized on either an Olympus Provis AX70 Light Microscope with a Slider Camera (SPOT RT 2.3.0 Software v4.6), or a Leica TCS SP2 Confocal Scanning Laser Microscope/Leica DM IRE2 Inverted Microscope (Leica Confocal Software v2.0).

Figure 1 shows the result of detergent permeabilisation with GFP-expressing *E. coli*. Whereas untreated cells are green (GFP), cells that have been permeabilised lose their internal GFP and take up the DNA-binding Gel Red dye to be stained red. While nonidet-40 shows some permeabilisation, Apo8 and Mega10 display a higher proportion of cells that have been permeabilised. A blend of these two detergents at 0.5% each, named Agent 86, demonstrated almost complete permeabilisation, as did another detergent, n-octyl- β -D-thiogluco-pyranoside (8TGP). Mega10, Apo8 and 8TGP are all non-ionic detergents, which are less disruptive than ionic detergents to protein folding and function.

As the cell wall remained intact following permeabilisation, soluble protein extracts of the supernatant from the detergent permeabilisation described above were analysed by SDS-PAGE. Hen egg-white lysozyme (Boehringer Mannheim; 837 059) was also added to a final concentration of 2 mg/mL to a sample of the cells being permeabilised to remove the cell wall and release the total cellular proteins. SDS-PAGE loading dye with β -mercaptoethanol was then added to the samples, which were denatured at 95°C for 2 minutes. 20 μ L of samples were loaded onto a 9% SDS-PAGE and stained/fixed with Coomassie Brilliant Blue/methanol/acetic acid.

Figure 2 shows that the release of soluble protein directly correlates to the release of GFP and intake of Gel Red as observed by microscopy. Significantly, there were differences between the release of protein from cells with intact cell walls compared to those whose cell walls were removed using lysozyme, with the cell-wall encapsulated cells releasing soluble protein up to a size of ~120 kD. This is presumably the cut-off size above which globular proteins are unable to leave the cell through the pores of the peptidoglycan lattice that constitutes the cell wall of Gram-negative eubacteria.

Example 2. Screening for permeabilisation solutions that retain host DNA

If the method of the invention is to be used for screening gene libraries for protein variants with improved properties, there must remain a linkage between the

expressed protein and its coding nucleic acid. As the membrane permeabilisation step removes the barrier that prevents DNA loss through the cell wall, conditions for permeabilisation were examined that might reduce or prevent host DNA loss.

Permeabilisation of cells using 0.5 % 8TGP was conducted in different media and the loss of DNA was examined by fluorescence microscopy using the DNA-binding dye, Gel Red.

Compositions of permeabilisation media tested (all media with 0.5 % 8TGP):

- LB media (10 g tryptone, 5 g yeast extract, 10 g NaCl per Lt)
- 10 LB [-salt] media (10 g tryptone, 5 g yeast extract per Lt)
- 50 mM Tris, pH 7.5
- 50 mM Hepes, pH 7.0
- 170 mM NaCl
- 250 mM NaCl
- 15 25 mM Tris, pH 7.5 + 1.5 % PEG 6000 (w/v)
- 50 mM Tris, pH 7.5 + 3 % PEG 6000 (w/v)
- 50 mM Tris, pH 7.5 + 170 mM NaCl
- 50 mM Tris, pH 7.5 + 250 mM NaCl

- 20 An optimal media for permeabilisation was identified as LB bacterial media. Accordingly, permeabilisation was henceforth conducted using either 0.5 % 8TGP in LB or Agent 86 in LB (0.5 % Mega10 and 0.5 % Apo8 in LB).

Example 3. Protein fusions to a tetramer scaffold

- As was observed by the experiments reported in Example 1, proteins larger than
- 25 ~120 kD in size were retained within permeabilised *E. coli* cells by the cell wall. Therefore, it was reasoned that a protein of interest that was smaller than 120 kD would be retained within the cell wall capsule if, by fusion to a protein partner, the total size could be made to exceed 120 kD.

- Accordingly, we cloned 6 different tetrameric proteins from *E. coli* for use as
- 30 fusion partners. These were β -gal, BetB, G5K, GshB, RhnA, and YdcW, that had monomeric sizes of 116 kD, 52 kD, 39 kD, 35 kD, 47 kD and 50 kD respectively.

An arabinose-inducible high copy vector was built for tetrameric expression. The SNAP tag (NEB/Covalys), a 20 kD domain that covalently binds a fluorescent substrate, was cloned upstream of the tetramer genes and used as a expression reporter.

A 6 x His epitope was also included at the N-terminus of the fusion protein to facilitate purification or detection.

The sequence of the arabinose vector, pAra3::His6::SNAP, is provided as SEQ ID NO:1.

- 5 Fusion protein expression was induced with the addition of 0.2% arabinose, and the culture incubated at 25°C for 2 hours.

To permeabilise the cells for protein display by the method of the invention, the protocol was as follows:

- 10
1. Pellet 1 ml of cells by centrifugation
 2. Resuspend cells in 300 µL of 0.5 % 8TGP/LB
 3. Incubate at 25°C for 10 minutes
 4. Pellet cells by centrifugation
 5. Resuspend cells in 200 µL of TBS or LB

15

To label the SNAP expression reporter domain with the membrane-impermeable SNAP dyes (Covalys/New England Biolabs), the protocol was as follows:

- 20
1. Dissolve 20 nmol of BG-488 (green dye) or BG-547 (red dye) in 300 µL DMSO as a 200 x stock
 2. Add 1 µL of 200 x stock to 200 µL of permeabilised cells suspended in TBS or LB
 3. Incubate at 25°C for 15 minutes
 4. Wash cells twice by pelleting by centrifugation and resuspending in 300 µL
- 25 TBS

To view the tetrameric fusion proteins by fluorescence microscopy for retention within the permeabilised cellular capsule, the protocol was as follows:

- 30
1. Drop 20 µL of cell suspension onto a glass microscope slide, cover with coverslip and seal edges with nail polish (wet mount); alternatively, allow the cell droplet to almost dry, drop 20 µL of DABCO/glycerol on top, cover with coverslip and seal edges with nail polish (dry mount)
 2. Visualise sample using either Olympus or Leica fluorescence microscope

35

Expression of the full-length fusion protein was confirmed by Western blot of protein extracts run on SDS-PAGE gels and probed with α -His6 antibody. All tetrameric constructs expressed in *E. coli* at detectable levels (Figure 3A).

Fluorescence microscopy of the tetrameric fusion proteins expressed in *E. coli* found that β -gal and G5K had significant inclusion bodies and low fluorescence, presumably due to difficulties in folding of the fusion protein. However, as shown by Figure 4, expression of the fusion protein, as judged by SNAP fluorescence, was good for GshB, and excellent for RhnA, BetB and YdcW. It was noted that the distribution of the fusion protein in the permeabilised host cell was not homogeneous, with foci evident both by bright-field microscopy and fluorescence. However, as the fluorescent SNAP substrate would not be bound by a misfolded domain, and as the signal was very intense, it is thought that these bodies are likely to be aggregates of folded protein, and not inclusion bodies of unfolded protein which are frequently observed when over-expressing proteins in *E. coli*.

The SNAP::tetramer fusions also had a His6 N-terminal epitope. To test whether a large molecule such as an antibody would be able to penetrate through the lattice structure of the *E. coli* cell wall permeabilised cells were probed with α His antibody to detect the SNAP::tetramer fusion.

1. Expression and permeabilisation of the His6::SNAP::BetB scaffold fusion was performed as described above.
2. Labeling with the BG-547 SNAP ligand was performed as described above.
3. 200 μ l of permeabilised, SNAP-labeled cells were washed three times in LB and allowed to settle onto a polyethyleneimine (PEI)-coated coverslip. Excess cell media was removed by aspiration and the slides allowed to air dry.
4. Cells were blocked for one hour in blocking buffer (1% BSA, 1% cold-water fish gelatin (Sigma, G7765), 0.02% Azide in PBS-Tween20).
5. Cells were incubated overnight at 25°C in α His primary antibody (Abcam, AB9136-100), diluted 1:10 in blocking buffer.
6. Cells were washed 3x in PBS-Tween20 (10 min each).
7. Cells incubated in secondary antibody diluted 1:2,000 (Molecular Probes, A11015) in blocking buffer for 1 hour at room temp.
8. Cells washed 3x in PBS-Tween20.
9. Mounted in DABCO/glycerol and viewed under the confocal/Olympus microscope.

Figure 5 shows that the α His antibody co-localised with the SNAP fluorescent ligand within the cell wall capsule, indicating that the pores of the cell wall are wide enough to allow diffusion of a relatively large protein into the inner capsule volume. Thus, even quite large protein ligands may be used as affinity substrates for affinity proteins expressed in the cytoplasm according to the method of the invention.

The SNAP fusion partner and expression reporter was compared with the HALO protein (Promega) in an attempt to see if the formation of the sub-cellular bodies was altered. The HALO protein covalently binds a membrane-impermeable fluorescent substrate (Alexa fluor 488; G1001, Promega) similarly to SNAP. The HALO reporter gene was cloned in frame directly into the place of the SNAP gene in the tetrameric expression constructs. Expression of the HALO::tetrameric scaffold proteins was compared to the SNAP variants. Labeling of the permeabilised HALO cells was conducted essentially as described for SNAP, and following the manufacturer's instructions. Figure 6 shows that the expression patterns of the HALO::tetramers and the SNAP::tetramers was found to be similar, with the exception that the HALO::Rhna fusion protein was fractionally more soluble than the SNAP::Rhna fusion, with fewer cells containing fluorescent foci.

Therefore, expressing a protein as a fusion to a tetrameric scaffold (in this example, SNAP or HALO), and then permeabilising the *E. coli* host cell with a suitable detergent enables retention of the protein of interest inside the cell wall.

Example 4. DNA binding proteins as a cellular scaffold

To couple the phenotype to genotype, the host cell must retain at least some episomal DNA following permeabilisation and throughout the functional screen. Having identified permeabilisation conditions that retained the host genomic DNA, as well as plasmid DNA, we reasoned that DNA could be used as a retaining scaffold for the expressed protein of interest.

We therefore cloned a small (80 aa) high-affinity helix-hairpin-helix DNA binding protein (DBP) from the *Neisseria gonorrhoeae* ComE gene (Chen and Gotschlich, 2001) and fused it to the C-terminus of GFP in an arabinose-inducible construct (pAra3::GFP::DBP; seq 2).

Expression by arabinose induction was conducted as described for Example 1. Cells were permeabilised and prepared for fluorescence microscopy as described for Examples 1 and 3.

Figure 7 shows that the GFP::DBP fusion (green) was retained in permeabilised cells and co-localised with the DNA-binding dye, Gel Red (red).

Therefore, expressing a protein as a fusion to a high-affinity, non-specific DNA-binding protein, and then permeabilising the *E. coli* host cell with a suitable detergent enables retention of the protein of interest within the cellular capsule.

Example 5. DNA retention in permeabilised cells

5 To demonstrate the retention of DNA, both genomic and episomal plasmid, within the cellular capsule following permeabilisation, we prepared cells expressing GFP5::DBP and His6::eGFP for fluorescence microscopy and plasmid DNA extraction.

Following induction, cells were permeabilised then either frozen or left in TBS at 37°C with shaking overnight. All samples were processed the following day for
10 either fluorescence microscopy, to visualize GFP and the capsule DNA content by the DNA-binding dye Gel Red, or a plasmid DNA preparation was conducted.

Fluorescence microscopy was performed as described for Example 3. Figure 8 shows that both the host cell DNA (red) and the GFP5::DBP (green) were retained in the cellular capsule immediately following permeabilisation and also with overnight
15 incubation at 37°C, without any apparent loss. The His6::GFP protein was lost from cells following permeabilisation, but the host cell DNA (red) was still retained both following permeabilisation, and also overnight, again without apparent loss.

To confirm that the plasmid DNA, and not just the host genome, was retained within the permeabilised cells, plasmid mini-preparations were conducted on
20 identically prepared samples.

Plasmid DNA from 1 mL of detergent-treated or untreated cells was prepared by a plasmid mini-preparation alkaline lysis protocol. Plasmid DNA released into the supernatant from the detergent extraction was extracted using a Perfectprep Gel Cleanup (Eppendorf; 955152051) column and solution, following the protocol of the
25 manufacturer.

The entire amount from each sample was loaded onto a 1% agarose gel and imaged on a FujiFilm LAS-3000 Intelligent Darkbox using Image Reader LAS-3000 software and Multi Gauge v3.0 software.

Figure 9 shows an ethidium-bromide stained 1 % agarose gel with TAE buffer
30 with samples of plasmid DNA from both cell lines.

Lane 1 of Figure 9 is the total plasmid DNA in untreated cells. Lane 2 is the supernatant from the permeabilisation step and Lane 3 is the plasmid retained in the cell capsule following permeabilisation. It is observed that there is very little plasmid release into the supernatant with permeabilisation, despite the complete loss of soluble
35 His6::GFP protein observed in Figure 8. Therefore, plasmid DNA is almost completely

retained by the cell wall and may be used in the method of the invention for the linkage of genotype to phenotype in screens for improved protein variants.

Confirming the microscopy data, the overnight incubation did not reveal any loss of plasmid DNA following overnight incubation at 37°C of permeabilised cells
5 suspended in TBS (lane 5).

Example 6. Peptidoglycan-binding scaffold

Another cellular structure that is retained following membrane permeabilisation is the cell wall, which is composed of a latticed polymer of peptidoglycan (PG).

To bind PG non-covalently, we cloned a 70 aa PG-binding domain from the
10 *Pseudomonas* ϕ KZ phage (KzPG) that was previously shown to be well expressed in *E. coli*, and to bind to the cell wall with high affinity ($K = 3 \times 10^7 \text{ M}^{-1}$) (Briers et al., 2009). As a screen for affinity proteins would hopefully identify variants that have even higher affinities for their targets than the KzPG-binding domain for PG, we needed to increase the affinity of the scaffold-binding protein. To increase the affinity of the
15 scaffold-binding moiety we linked both the *ComE* DNA binding domain (DBD) and the PG-binding domain in the same fusion protein. Therefore, the final dissociation constant of the fusion protein from both scaffolds (PG or DNA) should be the close to a multiple of each rate constant.

We therefore constructed an expression vector
20 pAra3::His6::KzPG::SNAP::DBP (SEQ ID NO:2). Expression was induced as described in Example 1 and cells were prepared for fluorescence microscopy as described in Example 3. Expression and distribution of the fusion protein was monitored by SNAP labeling, as described in Example 3.

Fluorescence was observed at the periphery of the cell, in the area of the cell
25 wall, and at a lower level in a diffuse area within the cell wall-bounded volume of the capsule.

Another embodiment of the invention would be to covalently attach the protein of interest to a cellular scaffold before permeabilisation. To achieve this, we used a protein fusion to LPP, an abundant *E. coli* protein that forms a trimeric coiled-coil in
30 the periplasm. In its native form, one end is tethered to the outer membrane via lipidation and the other is covalently bound to the cell wall via a C-terminal lysine.

We constructed an expression construct that fused the OmpF periplasmic-targeting signal sequence to the SNAP expression reporter, followed by the 57 aa *E. coli* LPP sequence lacking the N-terminal signal sequence and cysteine required for
35 outer membrane attachment. The expression vector, pAra3::OmpF::SNAP::LPP (SEQ

ID NO:3) was induced with arabinose, as described by Example 1, and cells were prepared for fluorescence microscopy as described by Example 3. Expression and distribution of the fusion protein was monitored by SNAP labeling, as described in Example 3.

- 5 Figure 10 shows the distribution of the LPP fusion protein was uneven across the surface of the cell wall, with areas of intense fluorescence and areas absent of any fluorescence. However, in almost all instances, the poles of the cells were labelled.

Example 7. Display of an α GFP affinity protein using a tetrameric protein scaffold

- 10 To demonstrate the method of the invention as applied to affinity proteins, a single-domain antibody generated from a Llama immunized against eGFP was cloned into the cellular scaffold vectors. It should be noted that, of the two sequences listed in the patent application for the α GFP antibody (WO 2007/068313), only the R35 variant was found to be functional (α GFP-R35; Protein Database ID 3K1K). Therefore, this
15 sequence was used all experimental testing.

The α GFP-R35 gene was cloned as an N-terminal fusion to the pAra3::HALO::FLAG::Rhna tetrameric scaffold to create the pAra3:: α GFP(R35)::HALO::FLAG::Rhna vector (SEQ ID NO:4).

- A pAra3::His6::eGFP vector was also constructed to produce a His6::eGFP
20 fusion protein as the target substrate of the antibody. The His6::eGFP protein was induced as described in Example 1. Soluble protein was released from cells using 0.5 % 8TGP, was purified by IMAC using Ni-NTA agarose resin (Qiagen; 30230). His6::eGFP was eluted from the Ni-NTA resin in NTTW buffer + imidazole (500 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1 % Tween20 + 200 mM imidazole).

- 25 Expression of the antibody::tetrameric fusion protein and permeabilisation of host cells was conducted as described in Example 1 and 3.

- For binding of α GFP to eGFP in permeabilised cellular capsules, the capsule pellet was suspended in 300 μ L of eGFP and allowed to equilibrate for 20 minutes at 25°C, at which point the capsules were pelleted by centrifugation, washed once in 300
30 μ L TBS, and then resuspended in TBS. Florescence microscopy on α GFP /eGFP capsules was conducted as described in Example 3.

- Figure 11 shows that the permeabilised capsules expressing α GFP::HALO::Rhna fusion protein bound eGFP throughout the cell, although there appeared to be foci of more intense staining that may correlate to the foci observed in
35 Figure 5 with HALO ligand labeling.

Therefore, the Llama α GFP antibody is functionally expressed in the cytoplasm and, furthermore, is retained within the capsule following detergent permeabilisation.

The α His antibody labeling described in Example 3 and observed in Figure 5 already demonstrated that a larger protein of ~150 kD is capable of diffusing through the permeabilised cell wall into the interior of the capsule. However, native antibodies are irregular-shaped proteins with 3 approximately equal-sized domains separated by a flexible hinge region. Thus, the effective radius that these proteins may present may be of a much smaller globular protein. However, GFP, which has a β -barrel structure and a molecular size of ~27 kD, is a symmetrical protein with a radius proportional to its size, was able to pass through the cell walls of the permeabilised capsule to be bound by the internal α GFP antibody.

Thus, the method of the invention may be used to express affinity proteins in the *E. coli* cytoplasm for the use in display of affinity libraries for binding symmetrical targets of at least 30 kD.

15 **Example 8. Display of an α GFP affinity protein using a PG- and DNA-binding protein scaffold**

The method of the invention was further demonstrated using the α GFP camelid antibody fused to PG- and DNA-binding domains.

Expression of the antibody::*KzPG*::SNAP::DBP fusion protein, permeabilisation of host cells and labeling with His6::eGFP was conducted as described for Example 6.

Both wet and dry mounts were used to image the binding of eGFP by the α GFP fusion protein. Figure 12 shows that there were significant differences with the GFP fluorescence between the two different imaging methods. Dry mounted (DABCO/glycerol) cells had mostly internal fluorescence, with a merge between the brightfield and eGFP labeling showing that the region around the cell wall was no more intense than the internal volume (Figure 12B). Cells mounted directly in TBS, however, had a distinctive pattern of an outer border of strong fluorescence that appears to be the cell wall-bound eGFP with a weaker internal signal (Figure 12A). Without being bound by theory, we speculate that the DABCO/glycerol solvent environment, being viscous and non-aqueous, prevented the interaction between the *KzPG* domain with the peptidoglycan cell wall, but did not prevent the binding of α GFP to eGFP, or the DBP to DNA.

However, as the screening procedures for affinity proteins or enzymes will almost always be conducted in aqueous environments, the distribution of the affinity fusion protein will approximate the observed cell wall-bound wet mount of Figure 12A.

Example 9. Display of an α GFP affinity protein through covalent attachment to the cell wall

The method of the invention was further demonstrated by covalently linking the α GFP antibody to the cell wall.

5 The α GFP antibody was cloned as an arabinose-inducible fusion downstream from the OmpF signal sequence and upstream from the SNAP and LPP sequences.

Upon induction of expression by arabinose, the OmpF signal sequence will direct the nascent protein through the inner cell membrane into the periplasm and will be cleaved off as it passes through the membrane pore.

10 In the periplasm, the LPP domain is expected to form a trimeric coiled-coil with two other partners, either wild-type LPP or with other α GFP fusion proteins. The C-terminal residue of the LPP domain is a lysine that is covalently linked to the *E. coli* cell wall through the ϵ amine group, most probably by the YbiS L,D- transpeptidase (Magnet et al., 2007).

15 Expression of the OmpF:: α GFP::SNAP::LPP fusion protein, cellular permeabilisation and eGFP labeling was performed as described for Example 8.

Figure 13 shows that eGFP was bound unevenly, but intensely, around the cell wall (Figure 13B). eGFP was not bound by cells expressing the OmpF::SNAP::LPP fusion without the α GFP domain (Figure 13A).

20 Covalent attachment of the OmpF::SNAP::LPP fusion to the cell wall was demonstrated by first labeling permeabilised cells expressing the fusion protein with a SNAP ligand before heating a sample of the labelled cell capsules to 95°C for 5 minutes. Figure 14 demonstrates that the fluorescence from the SNAP ligand labeling the cell wall was unchanged between the heat-treated sample, and a control that was not
25 heated. Gel Red staining also demonstrated that the genomic DNA was still retained in the cell, even in the heat-treated sample.

Example 10. Outer membrane permeabilisation experiments

In a further embodiment of the invention, the outer membrane may be selectively permeabilised for ligand targets, such as for example enzyme substrates or
30 polypeptides, while retaining the polypeptide that is being screened either within, or attached to, the cell wall.

To identify conditions that would selectively permeabilise the outer membrane, a range of detergents and buffers were screened. Both large (eGFP) and small (Gel Red) ligands were used to determine if the permeabilisation of the outer/inner
35 membranes generated either large or small membrane pores.

E. coli strains expressing arabinose-inducible OmpF:: α GFP::SNAP::LPP (cell wall attached) or α GFP::HALO::FLAG::RhnA (cytoplasmic) were grown and induced as described for Example 1.

1 mL of induced culture was washed once in 50 mM Tris (pH 8) before being
5 suspended in permeabilisation buffer variants containing 0.2 – 0.4 % detergent in either 25 mM Tris + 1 mM EDTA (pH 8) or 25 mM Tris + 2 mM Ca^{2+} (pH 8) and incubated at 25°C for 10 minutes.

Permeabilised cells were washed once in appropriate buffer and then stained with Gel Red (1 x in water) and washed with TBS. They were then incubated with
10 purified His6::eGFP for 1 hour at 25°C before being pelleted by centrifugation and resuspended in TBS and viewed by fluorescence microscopy as a wet mount.

Figures 15 and 16 demonstrate that 0.2 % Apo8 (A) or Tween20 (B) in either a Tris/ Ca^{2+} or Tris/EDTA buffer selectively permeabilised the outer membrane allowing the permeation of a large ligand (eGFP) through the outer membrane but not through
15 the inner membrane. The smaller, membrane impermeable, DNA-binding ligand Gel Red was partially permeable to the cytoplasm in most samples, indicating that some degree of poration of the inner membrane was occurring in some cells. However, the degree of Gel Red binding was much reduced compared to samples that had been treated with the detergents 0.5 % 8TGP or Agent86 where both the outer and inner
20 membranes were fully permeable to eGFP.

Example 11. Fluorescence sorting and analysis of encapsulated display

As a cellular display platform, the method of the invention is ideally suited for fluorescence-activated cell sorting (FACS) to identify ligand-binding clones. To test the stability of permeabilised *E. coli* cells for sorting by FACS, three populations were
25 induced for expression: i) eGFP; ii) α GFP::KzPG::SNAP::DBP; and iii) His6::SNAP::BetB.

The eGFP-expressing cells were not permeabilised, and were a positive control for fluorescence in intact *E. coli* cells. The α GFP::KzPG::SNAP::DBP expressing cells were permeabilised according to the method of the invention, and were labeled with the
30 SNAP BG-488 ligand (green). The His6::SNAP::BetB expressing cells were permeabilised according to the method of the invention, and were labeled with the SNAP BG-547 ligand (red).

Cells were suspended in PBS and mixed in approximately equal numbers for sorting of mixed populations or sorted separately for signal calibration. Cell sorting was

performed on a Becton Dickson Influx FACS. Data analysis was performed on FlowJo software. Parameters for *E. coli* sorting were determined by the operator.

Figure 17 demonstrates that the three populations were identifiable by fluorescence. Reanalysis of the sorted populations showed that the sorting provided relatively pure populations of each. The signals present in the low-fluorescence region of the graph were later shown to be inherent noise in the signal and later removed by the operator by instrument corrections.

Example 12. Spacer region selection for solid support binding

Cells expressing the α GFP::KzPG::SNAP::DBP fusion protein were permeabilised using 8TGP media, and cells bound to HisPur Co²⁺ sepharose beads (Thermo Scientific) via an intermediate, His6-tagged eGFP. Either cells or beads were first incubated with an excess of His6-eGFP before being washed in TBS and then incubated together for 30 minutes at 25°C. Unbound cells were then washed away from the beads before the extent of bead binding was assessed by fluorescence microscopy.

Initially no binding of the α GFP::KzPG::SNAP::DBP fusion protein to sepharose beads was detected. It was theorized that the α GFP binding domain may be in too close proximity to the cell wall to reach the cobalt-complexed eGFP on the sepharose resin. Accordingly, a 12-residue peptide spacer domain with randomized codons was cloned between the α GFP binding domain and the kzPG peptidoglycan binding domain (GGT ACC gcy gcy gkk wtb gck wtb gkk gkk gck gkk gcy gcy GGT CTG (SEQ ID NO:5))

A small library (~2,000 members) of the spacer variants was expressed and then bound to Co²⁺ sepharose, as described above. A proportion of the library was observed to bind to the beads. These clones were then PCR amplified, re-cloned and a dozen clones were tested individually for binding and sequenced. A variety of peptide spacers were found to be both resistant to proteolytic cleavage (maintaining high levels of α GFP in the fusion protein) as well as enabling binding of the detergent-treated cells to the sepharose beads as demonstrated by Figure 18. Spacer sequences that were found to be functional for support binding are listed in Table 1.

30

Table 1. Random linker (RL) spacers for solid support binding

Linker	Amino acid sequence
RL1	GSNSNNQSKPSS (SEQ ID NO:6)
RL2	GGPRNPQRHTGS (SEQ ID NO:7)
RL6	SGTRHHNSHNSS (SEQ ID NO:8)

RL9	SSNRTHKSNNSS (SEQ ID NO:9)
RL10	SGHRTTERKHSS (SEQ ID NO:10)
RL13	GGHRHTQRHNGG (SEQ ID NO:11)
RL14	GGPRTPQSQPSG (SEQ ID NO:12)

One spacer sequence, RL6, was chosen for further binding studies. Other factors contributing to strong binding to solid support matrixes were examined. The length of time for incubation of the cells with the matrix and the salt (NaCl) concentration of the binding solution were both found to have positive effects on binding. Incubation lengths of 30 minutes and a range of NaCl concentrations from ~200 mM to 500 mM were found to be effective although 300 mM was considered optimal. Binding was effective in a range of buffers, including Tris, phosphate and MOPS buffered solutions with 300 mM salt.

Conditions of binding for cells expressing the α GFP::RL6::KzPG::SNAP::DBP fusion protein to streptavidin magnetic nanoparticles (MagneSphere; Roche diagnostics) via biotinylated eGFP were also confirmed as being within the ranges identified for sepharose bead binding and demonstrated by Figure 19.

In addition to the 12-residue spacers, protein domains were also considered for use as spacer domains. The small, stable and highly-expressed 27th immunoglobulin domain from the human titin gene (I27) was cloned upstream from the RL6 spacer. This domain was also found to enable high and stable expression of the N-terminal α GFP domain as well as excellent solid matrix binding (Figure 19).

Example 13. Construction of a mouse scFv library for encapsulated display

The final domain structure for the intracellular display of a single-chain antibody (scFv) library was: scFv::I27::RL6::KzPG::SNAP::DBP. The protein and DNA sequences of the fusion protein without the scFv domain are provided as SEQ ID NO:13 and SEQ ID NO:14. This protein fusion has the scFv at the N-terminus, followed by the two spacer domains, I27 and RL6, then the peptidoglycan binding domain, KzPG, the SNAP reporter domain and, finally, the DNA binding domain (DBP).

Random-primed cDNA was produced from mouse spleen total RNA using the Superscript III (Invitrogen) enzyme. From this cDNA, the scFv light (V_L) and heavy (V_H) chain variable domains were amplified using Vent DNA polymerase (New England Biolabs) and degenerate oligonucleotide primers for the mouse antibody family sequences, as described by Schaefer et al. (2010). The oligonucleotide primers

used for library cloning differed from those described by Schaefer et al. in that they had appropriate ends for cloning via *Bsm* BI into our library scaffold vector (SEQ ID NO:15). The VL and VH domains were joined using overlapping extension PCR. The final scFv band had been subjected to a total of 60 PCR amplification cycles (30 first round, 30 second round).

For library cloning, 900 ng of the display construct was cut with *Bsm*BI, precipitated using Sureclean (Bioline) according to the manufacturer's instructions, and ligated using T4 DNA ligase to 400 ng of similarly-treated scFv product. The ligase was inactivated by incubation at 65°C for 10 minutes and the ligation electroporated into the *E. coli* *Argentum* strain (Alchemy Biosciences). The electroporated cells were recovered in SOC media and incubated for 1 hour at 37°C before pooling and then spread across 20 x 150 mm LB agar plates with 75 ug/mL ampicillin. The plates were incubated overnight at 30°C. The library size was estimated at 4×10^5 independent clones. 20 out of 20 colonies were found to contain an insert of the expected size.

15 **Example 14. Screening of an encapsulated display mouse scFv library**

Single chain antibodies isolated from phage display libraries are often difficult to express in *E. coli*, with either low levels of expression in the periplasm, or are completely insoluble in the cytoplasm due to the lack of disulphide bond formation between the B-sheets of the Ig fold. To determine whether encapsulated display could be used to select for a mouse scFv scaffold that would be soluble in the *E. coli* cytoplasm, it was necessary to determine whether scFv solubility was correlated with the behavior of the fusion protein.

It was predicted that a useful soluble scFv would have low levels of aggregation and at least a moderate level of expression. This could be judged visually as a clone that allowed binding of the KzPG domain in a permeabilised cell to the cell wall (and not therefore, localized to an inclusion body within the cell) and that showed at least moderate expression of the SNAP reporter domain.

To screen for these parameters, single colonies were picked and induced for fusion protein expression using arabinose as described previously for Example 1. Following permeabilisation they were labelled with SNAP ligand and viewed using fluorescence microscopy. We characterised the library clones into four categories based on their expression and cellular distribution of SNAP reporter, examples of which can be seen in Figure 20.

35 1) no expression of SNAP

- 2) moderate/high expression of SNAP in aggregated inclusion bodies (Figure 20, left panel)
- 3) weak expression of SNAP with cell wall localization (Figure 20, mid panel)
- 4) high expression of SNAP with cell wall localization (Figure 20, right panel)

5

Only clones with both high expression and solubility were analysed further. However, as the weak expression of the SNAP reporter could be due to inefficient expression of a protein not optimized for *E. coli* expression it is expected that a proportion of these clones would prove to be excellent for soluble cytoplasmic library display if their codon usage were optimised.

10

Clones with high expression of the SNAP reporter and an even distribution around the cell wall of permeabilised cells were sequenced to confirm the presence of a scFv insert that was in the correct translation frame with the remainder of the fusion protein. In all 21 clones analysed, the scFv insert was found to be full length, with the correct length of the glycine/serine linker region, and in the correct reading frame for translation of the entire fusion protein. This suggested that the method of screening of the invention was correctly identifying mouse scFv genes that were expressed in a soluble form in the cytoplasm of *E. coli* cells. To confirm that the scFv proteins isolated from the library were soluble in the *E. coli* cytoplasm they were shuttled from the library construct to an arabinose-inducible expression vector with a C-terminal FLAG epitope with an intervening spacer region of either I27-RL6 or RL6.

15

20

Following induction of protein expression by arabinose, the soluble scFv::I27::RL6::FLAG or scFv::RL6::FLAG fusion proteins were extracted with 0.5% 8TGP. The insoluble cellular material was pelleted and resuspended in SDS-PAGE loading buffer with β -mercaptoethanol by sonication of the sample and heated to 95°C for 5 minutes. Equal volumes of each fraction were loaded onto 10% SDS-PAGE gels and electrophoresed. Separated proteins were transferred to nitrocellulose membranes, which were then blocked with 5% skim milk powder. Recombinant protein expression was probed using a 1:1000 dilution of a sheep α FLAG antibody (Sigma) followed by an anti-mouse-HRP conjugated secondary antibody. Detection was using chemiluminescence.

25

30

Figure 21 demonstrates that the method of the invention is capable of identifying scFv genes that are expressed in a mostly soluble form within the bacterial cytoplasm. The Western blot of the expression profiles is matched in each sample with the fluorescence microscopy detected by SNAP ligand for the scFv::I27::RL6::FLAG construct.

35

Example 15. P2 lysogen generation in Argentum strain *E. coli*

A P2 lysogen of Argentum (K12; $\Delta mcrA$ $\Delta(mrr-hsdRMS-mcrBC)$ $\Delta endA$ $lacZ\Delta M15$) was created by outgrowth from a single plaque of P2 on a lawn of Argentum cells. Phage infection was conducted as described by Kahn et al. (1991).

5 Example 16. P2 ΔYK knockout*a. Generation by homologous recombination*

P2 bacteriophage has genes for a putative holin and lysin system, similar to the lysis system characterized for many lytic and lysogenic bacteriophages. The holin provides access through the inner membrane to the periplasmic space for the lysin enzyme to degrade the murein cell wall.

The P2 K gene (SEQ ID NO:17) and Y gene (SEQ ID NO:18) encode putative lysozyme and holin, respectively. These genes were deleted using homologous recombination similarly as described by Hamilton et al. (1989). Regions of flanking homology were chosen from the P2 genome (Genbank sequence NC_001895.1) and cloned between a FRT-flanked kanamycin selection cassette. The region of replacement of the P2 genome was 6,721 to 7,487 bp.

Following replacement of the targeted YK genes, the kanamycin cassette was removed by FLP recombinase expressed from the pCP20 plasmid, as described by Cherepanov and Wackernagel (1995). The resultant strain had a deletion of the YK genes with a short 20-mer peptide remaining as the only ORF.

The K12 P2 ΔYK strain was functionally tested by infection with P4 bacteriophage. Argentum (P2) and Argentum (P2 ΔYK) cultures were infected with 10^3 pfu of P4 bacteriophage and poured into top agar plates. Plaques were observed to form on lawns of Argentum (P2) but not Argentum (P2 ΔYK).

25 b. Testing using P4 vir1 and Ready-Lyse

To test the functionality of the P2 bacteriophage YK deletion (P2 ΔYK) to replicate and package an infecting P4 bacteriophage, the P2 ΔYK strain was infected with the P4 mutant, P4 *vir1*, that has a mutation that increases transcription of the P4 control region and has a clear-plaque phenotype.

P2 ΔYK cells were grown to early-log phase and supplemented with 1 mM $CaCl_2$. 1 μ L of a lysate supernatant containing 10^9 pfu/mL of P4 *vir1* bacteriophage was added to 1 mL of P2 ΔYK culture and incubated for 80 minutes at 37°C. The suspension was centrifuged to pellet the cells, the supernatant discarded and the pellet washed three times in LB media containing 0.08 mM EGTA and 2.5 mM $MgCl_2$ to remove unbound P4 *vir1*. The cells were resuspended in 1 mL LB media and then

divided into three samples. One sample was retained without further treatment (Sample 1; unpermeabilised). Samples 2 and 3 were treated further by pelleting and resuspension in LB supplemented with 0.5% of the detergent 8TGP to permeabilise both inner and outer membranes. The cells were then pelleted and washed twice in
5 unsupplemented LB media. The permeabilised, washed cell pellets were resuspended in LB media. Sample 2 (permeabilised) was retained without further treatment. Sample 3 (permeabilised; lysozyme) was further treated with 0.5 μ L Ready-Lyse (Epicentre, USA), which is a recombinant lysozyme. The rapid decrease in turbidity indicated that the peptidoglycan cell wall was degraded by Ready-Lyse and that any
10 packaged P4 *vir1* particles would now be released into the lysate.

10 μ L of each of Samples 1 and 2 and 0.1 μ L of Sample 3 (a dilution of the raw lysate) were then added to 200 μ L of fresh K12 (P2) cells supplemented with 1 mM CaCl_2 . The cells were incubated at 37°C for 20 minutes, then 7 mL of top agar (LB media, % agar) was added and poured over pre-warmed LB plates. The plates were
15 incubated overnight at 37°C and the presence of P4 *vir1* plaques in the K12 lawn determined the next morning.

Sample 1, which represented P4-infected cells that had not been permeabilised, produced 83 plaques in the top-agar plate. Sample 2, which represented P4-infected cells that had been permeabilised by detergent, produced 34 plaques. Sample 3, which
20 represented P4-infected cells that had been both permeabilised by detergent and then the cell wall degraded by lysozyme, produced 168 plaques.

Adjusting for sample dilution, the permeabilised, lysozyme-treated P2 Δ YK cells (Sample 3) had 200-fold more P4 *vir1* bacteriophage than Sample 1 and 500-fold more P4 *vir1* bacteriophage than Sample 2.

25 The presence of replication-competent P4 *vir1* bacteriophage in infected K12 P2 Δ YK cells demonstrates that the deletion of the YK lysis genes did not prevent replication of the P4 *vir1* genome, or assembly of functional bacteriophage particles. Deletion of the YK lysis genes did, however, prevent release of the assembled bacteriophage particles from infected cells. Permeabilisation of the inner and outer
30 cellular membranes, achieved by detergent treatment, did not result in release of bacteriophage particles into solution. However, treatment of the permeabilised cells with a lysozyme released infectious bacteriophage into solution.

Example 17. P2 Δ YK /P4 co-lysogen with inducible activator*a. Generation of a P2/P4 co-lysogen in C1a cells*

Historically, the strain used for experimenting with the P2 bacteriophage and its satellite, P4, is the C strain of *E. coli* (Wiman et al., 1970). Using a derivative of C strain, C1a (Sasaki and Bertani, 1965), we established a P2 lysogen through subcloning
 5 of lysogenised cells from P2 plaques, as described above. Similarly, we established P4 co-lysogens of the C1a P2 strain.

A P2/P4 colysogen strain has both prophages under transcriptional repression. To use this strain to inducibly package a cosmid library plasmid both phages need to be
 10 activated. For the other well-characterised temperate phage, lambda, release of repression occurs with inactivation of the repressor protein, cI, either through RecA/LexA-mediated cleavage or using a thermolabile mutant repressor, cI857. However, P2 is known as an uninducible phage in that it is unresponsive to depression by inactivation of its repressor, presumably because it is unable to coordinate excision
 15 from the genome with replication and structural gene transcription (Bertani, 1968). However, the infecting P4 satellite phage has mechanisms of activating repressed P2 prophage upon entry. The P4 ϵ (epsilon) protein acts as an anti-repressor through binding to the P2 repressor protein. In addition, the P4 δ (delta) protein is a potent activator of P2 structural operons, being a fused tandem duplication of the P2 *ogr*
 20 transcription activator. However, a P4 prophage has a complex and stringent control of both its own and P2's activation.

The P4 prophage uses the interplay between a transcriptional repressor, the Vis protein, on its own promoter and the downstream *Eta* and *cI* genes that rely on transcription and translation coupling to produce an inhibitory complex based on the *cI*
 25 RNA. Ultimately this complex acts to block expression of the P4 ϵ protein, which is a binding antagonist of the P2 repressor protein.

Derepression of P4 and P2 prophages requires inhibition of the P2 repressor by the P4 ϵ protein. Activated P2 in turn produces the Cox and Ogr transcription activators that act in *trans* to promote transcription of the P4 δ gene, which further activates P4
 30 via the Vis promoter and also acts in *trans* on P2 structural gene operons.

In such a complex system, with many elements interacting to reinforce their combined effects a cell containing both repressed prophages, P2 and P4, must tightly control expression of all the activator genes to prevent a positive feedback effect occurring. Potential activators of the prophages include the P2 *cox*, P2 *ogr* and P4 δ
 35 transcription activators, as well as the P4 ϵ anti-repressor.

The three transcription activators were cloned under the tight transcriptional control provided by the temperature-sensitive allele of the λ phage repressor, cI857. The low-copy pACYC184 plasmid origin of replication compatible with the pUC origin enables maintenance of the inducible activator alongside a pUC-based library plasmid.

The C1a P2/P4 co-lysogen was transformed with the inducible expression constructs and grown at 30°C. The cultures were grown to early log phase before induction through temperature shift to 42°C for 20 minutes, before growth at 37°C until lysis occurred.

All three activators were capable of inducing lysis in a colysogen, although the P4 δ gene demonstrated the earliest lysis, followed by *ogr*, and then *cox*. The polynucleotide sequence of the temperature-inducible P4 δ is provided in SEQ ID NO:19.

Production of infectious bacteriophage P4 particles was confirmed by titration of the lysate against cultures of C1a P2 lysogen. The P4 titre was determined to be $> 10^9$ pfu/mL.

Example 18. P2 ΔYK with cosmid transmission

To utilise the P2/P4 system for gene library screening and transmission, a vector was constructed that contained the P4 *cos* region. A 389 bp region from P4 starting from the *psu* gene, spanning over the *cos* cleavage site, and to the *gop* gene (11461 bp to 225 bp of the P4 genome; NCBI accession number NC_001609) was amplified by PCR and cloned into a high-copy pUC-origin plasmid vector. The identity of the P4 *cos* region was verified by sequencing. The vector also contained the *araC* gene and arabinose-inducible promoter controlling expression of a library intracellular display screening system as described by patent application PCT/AU2010/001702.

As with all cosmid vectors, whether for P2, P4 or λ bacteriophages, there is a minimum size for the packaging into the capsid head for producing a viable transmissible unit. For P4, this has been determined to be approximately 9.2 kb (Kim and Song, 2006). To achieve this minimum size for packaging in a P4 capsid head the total size of the cosmid vector was increased to 10.7 kb, closer to the wild-type P4 genome size of 11.6 kb, by cloning in a 4.3 kb 'stuffer' fragment of *E. coli* genomic DNA.

To demonstrate co-packaging of the cosmid vector resident in a C1a P2 ΔYK /P4 co-lysogen the strain was transformed with the pUC-backbone, ampicillin-resistant

cosmid vector as well as the pACYC184-backbone, chloramphenicol-resistant vector with the temperature-inducible P4 δ gene.

The strain with both colysogens and both plasmids was grown at 30°C to early-log phase before the P4 δ protein was induced by temperature shift to 42°C for 20 minutes, followed by growth at 37°C for 1 hour. As a control, a strain containing a library plasmid without the P4 *cos* region and the stuffer fragment was also induced for P4 capsid packaging.

To release the packaged cosmids and P4 bacteriophage, the induced cells were pelleted and resuspended in permeabilisation media (LB media + 0.5% 8TGP) for 10 minutes at room temperature (~25°C). Following permeabilisation, they were pelleted and resuspended in LB and 0.5 μ L of Ready-Lyse lysozyme added to digest the cell wall. Lysis was confirmed by the drop in turbidity. It was also confirmed that chloroform was also effective in permeabilising the cell for the action of Ready-Lyse on the cell wall. Packaged cosmids and P4 bacteriophage were titred by infection of C1a and C1a P2 lysogens, respectively.

It was confirmed that the library cosmid was packaged at approximately equal levels as the resident P4 prophage in induced P2 ΔYK cells as approximately equal numbers of antibiotic resistant colonies from the cosmid recovery were obtained compared to P4 plaques. No colonies were obtained from infection with a lysate prepared from the strain carrying the library plasmid that lacked a P4 *cos* region and stuffer fragment.

It was also noted that, unlike the poor stability of P4 bacteriophage or packaged cosmids in raw lysates from lysed C1a P2 cells (stored at 4°C in LB media with Mg/EGTA), presumably due to the action of cellular proteases also in the lysate, the P4 bacteriophage and packaged cosmids released from P2 ΔYK cells that were first permeabilised and washed, before lysed by the action of exogenously added lysozyme (Ready-Lyse) were stable at room temperature with only a minor drop in titre over 2 days. This is presumably due to the release of the aforementioned proteases from the permeabilised cells which are then washed away from the infectious particles that are retained by the cell wall during the pelleting and media change steps. Thus, a high-titre of cosmid particles could be easily produced by temperature induction, permeabilisation and media change, and kept at a stable titre without requiring long ultra-centrifugation purification steps as per standard bacteriophage protocols.

Example 19. Permeabilisation of *E. coli* using organic solvents

In addition to the permeabilisation of Gram-negative cells using detergents, another chemical agent for disrupting membrane integrity might be the lipophilic organic solvents. Organic solvents have been used substantially in the prior art in cell permeabilisation and fixation for immunolabelling for microscopy (Harlow and Lane, 5 Antibodies: A Laboratory Manual, Cold Spring Harbour Laboratory, 1988). In the method of the invention, the cell membrane is permeabilised for the entry of large immunoglobulin complexes which bind to intracellular targets.

In particular, the organic solvent chloroform has also been used to selectively 10 kill bacterial cells in a cell/bacteriophage suspension, presumably through permeabilisation of the cellular membranes (Sambrook et al. 2001). Chloroform has also been used in lytic bacteriophage genetics to enable rescue of holin mutants that were unable to permeabilise the inner membrane to release lysozyme from the cell cytoplasm for bacteriophage release (Ziermann et al., 1994). Similarly, it was used to 15 rescue lysozyme mutants that were unable to hydrolyse the peptidoglycan cell wall by permeabilising the outer membrane to enable active exogenous lysozyme entry to the periplasm (Ziermann et al., 1994). Therefore, chloroform was demonstrably able to allow at least small (~15 kD) lysozymes passage through both the inner and outer membranes of the Gram-negative *E. coli* cell.

20 To test organic solvents for permeabilisation of the cellular membranes for use in intracellular display described by the method of the invention, *E. coli* cells expressing the α GFP::RL6::KzPG::SNAP::DBP fusion protein (expression induced as described for Example 8) were suspended in aqueous mixtures of organic solvents. Membrane permeabilisation was indicated by the binding of a small molecular weight 25 DNA-binding fluorescent ligand, Gel Red, and of a 30 kD protein, eGFP.

Although some organic solvents remain miscible in water (e.g. the shorter-chain alcohols) others are largely immiscible and the mixture partitions into aqueous and non-aqueous phases (for example, chloroform and butanol). The phase portioning represents the saturation of the low solubility of the organic solvent in the aqueous 30 phase.

Cells expressing the α GFP::RL6::KzPG::SNAP::DBP fusion protein were collected by centrifugation and permeabilised with one of the following solvent compositions for 10 min at 25°C. LB growth media was used for the aqueous component of the mixture. Tris-buffered controls were also performed.

35

10% ethanol; 20% ethanol; 30% ethanol

- 10% methanol; 20% methanol; 30% methanol
- 10% isopropanol; 20% isopropanol; 30% isopropanol
- 10% DMSO; 20% DMSO; 30% DMSO
- 10% acetone; 20% acetone; 30% acetone
- 5 Butanol (1:5)
- Chloroform (1:5)
- 50 mM Tris/LB (pH 7.0); 1 M Tris/LB (pH 7.0)

Following solvent treatment, cells were pelleted by centrifugation, washed once
 10 with LB media by suspension, pelleted by centrifugation and then suspended in LB
 media containing either the small-molecular weight DNA-binding fluorophore, Gel
 Red (1:10,000 dilution in water), or eGFP. Following a 20 minute incubation at 25°C in
 labeling media, cells were pelleted by centrifugation, washed in LB by resuspension,
 then viewed by fluorescence microscopy. Figure 22 demonstrates that, of the organic
 15 solvents tested, chloroform and butanol permeabilised the *E. coli* cellular membranes to
 allow a small molecular weight ligand entry into the cytoplasm (A), but only
 chloroform permitted entry of a large molecular weight protein (~30 kD).

Example 20. Capsid display using the P2 gpL decoration protein

The P2 bacteriophage gpL protein was detected as a structural component of
 20 mature virions by mass spectrometry (Chang et al., 2008) and is presumed to be the
 functional equivalent of the gpD capsid protein of lambda bacteriophage, even though
 the two proteins do not demonstrate any regions of significant homology by a pairwise
 BLAST alignment (NCBI). The lambda gpD protein is 110 residues in length, whereas
 the P2 gpL protein is 169 residues in length.

25 To test whether the P2 gpL protein would function for capsid display the
 α GFP:I27 sequence was fused to the N- and C-terminal ends of P2 gpL to create the
 fusion proteins listed as SEQ ID NO:20 and SEQ ID NO:21. The fusion protein also
 included a FLAG epitope tag interspacing the gpL and α GFP:I27 domains. Expression
 of the fusion protein was made arabinose-inducible by cloning the gene sequence
 30 downstream from the *araBAD* promoter, with an upstream *araC* transcriptional
 regulator. The DNA sequence of the α GFP:I27:gpL expression vector is listed as SEQ
 ID NO:22.

The plasmid encoding the α GFP:I27:gpL fusion protein was transformed into an
E. coli K12 host containing a Hy5 prophage. The Hy5 phage is a hybrid of the related
 35 phages P2 and 186 containing the P2 structural genes under 186 transcriptional control

(Bradley et al., 1975; Younghusband et al., 1975). Furthermore, the Hy5 (186) *cI* repressor is temperature sensitive, allowing temperature induction of phage growth.

Expression of the α GFP:I27:gpL fusion protein by arabinose and analysis by SDS-PAGE produced an upper band of approximately 55 kD, which was higher than the expected size of 44 kD that was in both the soluble and insoluble fractions, and a lower band that was solely in the soluble fraction (Figure 23).

To demonstrate that the α GFP:I27:gpL fusion protein was bound to the phage capsid and was functional for binding by phage display, the prophage strain with the expression construct was heated to 45°C for 15 minutes to trigger Hy5 replication. Following heat-shock the samples were shifted downwards in temperature for growth at 32°C. Fusion protein expression was induced 30 minutes after the temperature downshift with the addition of arabinose to a concentration of 0.2%. Cultures were incubated for a total time of 70 minutes at 32°C for maximal phage release.

For capture of Hy5 phage displaying the α GFP:I27:gpL fusion protein streptavidin-coated Dynal beads (M-270, cat. no. 653-05; Life Technologies) were first labelled with biotinylated His6-GFP and thoroughly washed with TBS. Labelling of the Dynal beads with eGFP was confirmed by fluorescence microscopy.

Table 2 lists the results of the gpL fusion protein expression on Hy5 phage capture by Dynal beads. These data demonstrate that fusion of the α GFP antibody to the P2 gpL capsid protein results in 82-fold enrichment by the Dynal beads over phage that are packaged using the wild-type gpL protein. Furthermore, even the uninduced sample with an undetectable level of fusion protein expression (Figure 23, sample 1) was still affinity-purified at a significant level above the control, suggesting that even a very low level of display was resulting in phage capture.

Table 2. Enrichment of Hy5 phage displaying the gpL capsid fusion protein over Hy5 control.

	Stock titre (PFU/mL)	Panned output (PFU)	Enrichment (fold over Hy5)
Hy5	3.5×10^9	93	(reference sample)
Hy5 + α GFP:I27:gpL (not induced)	1.2×10^9	202	6.5 ×
Hy5 + α GFP:I27:gpL (induced)	1.1×10^9	2,550	82 ×

Example 21. Deletion of the lambda phage SR lysis genes

The lambda phage lysis genes are located on the right arm of the genome in a cluster containing the S'/S (holin (SEQ ID NO:23)/anti-holin), R (endolysin (SEQ ID NO:24)), Rz/Rz1 (required for lysis in certain media) genes. The lysis cluster is within a larger transcriptional unit transcribed from the pR promoter that is responsible for transcription of all lambda structural and lytic genes. The pR' mRNA is a single transcript that therefore covers approximately half of the lambda genome. To inactivate the lysis genes it was decided to delete the genes using homologous recombination. To enable facile selection for the lambda mutants the lysis genes were replaced with a kanamycin resistance gene (KanR). However, to ensure that neither promoter or transcription terminator sequences were inserted that would result in prophage structural gene expression that might be detrimental to cellular viability, the neighbouring non-essential *bor* gene was also deleted. The *bor* gene, which confers serum resistance to the host *E. coli* cell, is constitutively expressed in the prophage in the opposite direction to the pR' under its own promoter (Barondess and Beckwith, 1995). Using synthetic gBlocks fragments (IDT) we designed a truncation of the lysis cluster with a fusion of the start codon of the KanR gene to the start codon of the *bor* gene. The only sequence remaining of the lambda lysis cluster from this deletion was a truncated peptide of sequence MKMPEKQLEGTKYNEQCR (SEQ ID NO:25). The DNA sequence of the lysis deletion construct with synthetic arms and KanR cassette is listed as SEQ ID NO:27.

The synthetic homology arms and the KanR cassette were cloned into the pUC-based PCR cloning vector, pAcquire (Alchemy Biosciences, Melbourne, Australia), and were verified by sequencing.

To effect the deletion of the lambda lysis cluster, the construct was transformed into a lambda *cI857sam7* lysogen of *E. coli* strain ED8739 (F⁻, *metB*, *hsdS*, *supE*, *supF*) and phage lysis was induced by temperature induction (42°C, 10 mins) followed by growth at 37°C for 1 hour. 1 mL of supernatant containing phage was clarified by centrifugation and 1 drop of chloroform added. A culture of ED8739 with supplemented magnesium (10 mM) and maltose (0.1 %) was then infected with dilutions of the phage lysate and lysogens were recovered by outgrowth at 30°C for 2 hours before plating on LB + kanamycin (15 µg/mL) agar plates which were grown for 16 hours at 30°C. As the targeting plasmid was small enough for lambda::plasmid recombinants to be packaged as viable phage the kanamycin-resistant prophage colonies were therefore screened for the loss of the ampicillin resistance gene (i.e. Kan^R/Amp^S), which would indicate a homologous

recombination event between both homology arms of the targeting construct, excising the lysis cluster and replacing it with the kanamycin cassette as desired. Kan^R/Amp^S prophage were identified and to confirm that the deletion was effected without undesired mutations, the region was amplified by PCR and sequenced. All clones were
5 found to be correct as designed.

Example 22. Packaging of the lambda Δ SR genome

To demonstrate that deletion of the lysis cluster still produced the same number of viable packaged phage per cell (i.e. that the modified pR' transcript didn't effect production of the phage structural proteins), the lambda *cl857sam7 Δ SR* prophage was
10 grown alongside the lambda *cl857sam7* prophage at 30°C to an identical cellular density and phage production was induced by temperature induction (42°C, 10 mins) followed by growth at 37°C for 1 hour. As expected the lambda *cl857sam7* culture lysed to completion whereas the lambda *cl857sam7 Δ SR* failed to lyse. The lambda *cl857sam7 Δ SR* culture was collected by centrifugation and resuspended in LB + 0.5 %
15 8TGP and incubated at 25°C for 10 minutes. The permeabilised cells were then collected by centrifugation, washed once with LB + 10 mM MgSO₄ and resuspended in the original 1 mL volume of LB + 10 mM MgSO₄ and lysed using 0.5 μ L of ReadyLyse (Epicentre). A droplet of chloroform was added to each lysate to kill any remaining viable cells and the phage were titred using serial dilutions infected into
20 ED8739 cultures and plated on LB top agar supplemented with 10 mM MgSO₄ and 0.1 % maltose. The plates were grown for 16 hours at 37°C before the plaques were counted. Both the lambda *cl857sam7* and lambda *cl857sam7 Δ SR* prophage gave phage titres of $\sim 1 \times 10^9$ pfu/mL demonstrating that the deletion of the lambda Δ SR lysis cluster, and the corresponding insertion of a kanamycin gene in the opposite
25 transcriptional direction, did not perturb the structural genes' transcription or translation.

Example 23. Capsid display on lambda Δ SR phage

Capsid display using the lambda gpD gene has been well documented in the literature, as have methods of phage panning for target binding using gpD display.
30 However, the combination of use of capsid display with lysis-defective phage has not been proposed prior to this application. Furthermore, the combination of capsid display with lysis-defective phage in permeabilised cells according to the method of the invention enables screening for target binding to the phage capsid by FACS detection, which is a high-throughput method of clonal characterization.

To demonstrate the binding of target to lysis-defective phage retained within permeabilised cells we fused a sequence encoding a single-chain antibody (scFv) that binds to a GFP-related fluorescent protein, mAG1 (Karasawa et al., 2003), to the 3'-terminus of the lambda gpD gene. The α -mAG1 scFv is a rare class of antibody that is
5 soluble and stable when expressed in the bacterial cytoplasm in a reduced state. A FLAG epitope was fused to the C-terminus of the gpD:: α -mAG1 fusion protein and full-length soluble protein was demonstrated to be expressed in the cytoplasm of *E. coli* cells using an α FLAG monoclonal antibody.

A lambda cosmid was constructed that expressed the gpD:: α -mAG1 fusion
10 protein from the araBAD promoter, under repression by the araC protein and inducible by arabinose. The cosmid also contained features common to other cosmid vectors available commercially, and privately, of the lambda *cos* region (SEQ ID NO:26), bacterial plasmid origin of replication and antibiotic resistance genes (AmpR and ChlR). It also contained a stuffer fragment to enable *in vivo* packaging of phage. An
15 example of a commercially-available cosmid vector is pFOS1 (New England BioLabs (NEB)).

The gpD:: α -mAG1 cosmid was transformed into an *E. coli* ED8739 strain containing the λ *cI857 Δ SR* prophage and grown at 30°C for vegetative growth. To induce the phage functions the strain was cultured in LB media to a low density, then
20 heated to 42°C for 15 minutes, before growth at 31°C for 75 minutes. Induction of the gpD:: α -mAG1 fusion protein was initiated immediately following the 42°C incubation by addition of arabinose to 0.2 % w/v. At the completion of phage growth and packaging, the cells were permeabilised by the method of the invention by centrifugation and resuspension in 0.3 $\times$ volumes of LB + 0.5% 8TGP for 10 minutes at
25 25°C. The cells were then re-centrifuged and washed in 1 $\times$ volume of TBS + 10 mM MgSO₄ (TBS/Mg), before being pelleted and suspended in TBS/Mg with excess mAG1 protein for 20 minutes. Following mAG1 binding, cells were washed clean of unbound mAG1 with TBS/Mg before being suspended for viewing by microscopy or for FACS analysis.

30 Figure 24 demonstrates that the polyvalent lambda display, when encapsulated in permeabilised cells and probed with fluorescent target, generated a sufficiently strong signal for visual detection by fluorescent microscopy. Each bacterial cell demonstrated a punctate labeling of between 10 and 30 foci. These foci were only observed in cells expressing the gpD:: α -mAG1 fusion protein and induced for phage.
35 Foci were not observed within cells not expressing the fusion protein, or not induced for phage when probed with mAG1 protein. Similarly, the gpD:: α -mAG1-labeled

phage did not bind the related fluorescent protein, GFP. Given that a wild-type lambda phage burst size is 100 – 200 copies per cell, and assuming that the phage are concentrated within just a few regions of the cell, then each foci may contain between 3 and 20 phage. This estimate may be conservative as the burst size from lysis-defective phage may be larger given that the replication is allowed to persist beyond the normal timing of lysis. Therefore, the polyvalent display of the encapsulated, lysis-defective phage, as described by the method of the invention, allows the direct detection of fluorophore-labelled protein binding by light microscopy.

As the sensitivity of FACS instrumentation is superior to conventional microscopy imaging then it was to be expected that detection and collection of cells containing labeled phage, from those which were unlabeled, would be easily performed given the strength of the signal already observed by light microscopy. Figure 25 demonstrates the fluorescence graph for 100 K events on an Influx FACS (BD Biosciences) with an input of ~1% of α -mAG1-positive cells. The cell population has been co-stained with the DNA binding dye, Gel Red, and the fluorescent mAG1 protein. The P2 gated population is α -mAG1-positive and the P3 gated population is α -mAG1-negative.

The post-FACS output was recovered by the addition of ReadyLyse enzyme followed by infection into ED8739 λ *cI857 Δ SR* cells. Recovery was recorded at about 10 phage particles per positive event.

Therefore, high-throughput FACS screening of encapsulated capsid-display phage is made possible using the method of the invention.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

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CLAIMS:

1. A method of screening a polypeptide for a desired activity against a target molecule, the method comprising:
 - a) culturing a Gram-negative bacterial cell comprising an exogenous polynucleotide encoding the polypeptide such that the polypeptide is produced in the cell,
 - b) allowing a lysis-defective phage to package the polynucleotide encoding the polypeptide, wherein the lysis-defective phage is retained within the bacterial cell,
 - c) permeabilising:
 - i) the outer membrane of the bacterial cell, or
 - ii) the inner and outer membranes of the bacterial cell,
 - d) contacting the bacterial cell with the target molecule, and
 - e) screening the polypeptide for the desired activity,
 wherein the polypeptide is retained within the bacterial cell by the bacterial cell wall or inner membrane and/or the polypeptide is attached to the bacterial cell wall or inner membrane.
2. The method of claim 1, wherein the polypeptide is associated with at least a second polypeptide to form a protein complex that is retained within the permeabilised bacterial cell and/or is attached to the bacterial cell wall.
3. The method of claim 2, wherein the polypeptide is fused to the second polypeptide or a subunit thereof.
4. The method of any one of claims 1 to 3, wherein the inner and outer bacterial membranes are permeabilised with one or more detergents or an organic solvent.
5. The method of claim 4, wherein the detergent is a non-ionic detergent.
6. The method of claim 4, wherein the organic solvent is chloroform.

7. The method of claim 1, wherein:
 - i) the bacterial outer membrane is permeabilised;
 - ii) the bacterial cell wall is at least partially hydrolysed; and
 - iii) the polypeptide is attached to the inner membrane.
8. The method of any one of claims 1 to 7, wherein the polynucleotide encoding the polypeptide is a plasmid, cosmid, phagemid or phage DNA.
9. The method of any one of claims 1 to 8, wherein the lysis-defective phage is a temperate phage selected from lambda phage, 186, P2, a hybrid of 186 and P2, and P4 and/or wherein the lysis-defective phage is a prophage.
10. The method of claim 9, wherein allowing the lysis-defective phage to package the polynucleotide comprises inducing activation of the prophage in the bacterial cell to produce phage, wherein the phage package the polynucleotide and/or wherein inducing activation of the prophage comprises producing one or more phage activator proteins in the bacterial cell.
11. The method of claim 10, wherein inducing activation of the prophage comprises inactivating one or more phage repressor proteins in the bacterial cell.
12. The method of claim 11, wherein the phage is lysis-defective due to deletion or mutation to an inactive form of the lysozyme gene, or deletion or mutation to an inactive form of the holin and lysozyme genes.
13. The method of claim 11, wherein the bacterial cell comprises lambda prophage and inducing activation of the prophage comprises inactivating a temperature-sensitive repressor allele of protein cl in the bacterial cell.
14. The method of any one of claims 1 to 13, wherein the method further comprises

an additional screening of the polypeptide for a desired activity against the target molecule in the Gram-negative bacterial cell, wherein

- i) the polynucleotide encoding the polypeptide is not packaged into the lysis-defective phage, and/or
- ii) the polypeptide is not retained within the bacterial cell by the bacterial cell wall and/or attached to the bacterial cell wall.

15. The method of claim 14, wherein the additional screening is performed using a lytic or temperate phage to package the polynucleotide encoding the polypeptide.

16. The method of claim 15, wherein the bacterial cell in the additional screening is lysed to release the phage.

17. The method of claim 16, wherein the phage in the additional screening is a lytic phage which lyses the bacterial cell.

18. The method of claim 17, wherein

- i) the lytic phage comprises a first binding partner on the phage coat, and
- ii) the polypeptide being screened for a desired activity is a fusion protein comprising a second binding partner,

wherein the fusion protein comprising the second binding partner binds to the first binding partner on the lytic phage coat.

19. The method of claim 18, wherein the lytic phage is lambda phage.

20. The method of claim 18 or claim 19, wherein the first binding partner is calmodulin and the second binding partner is calmodulin binding peptide.

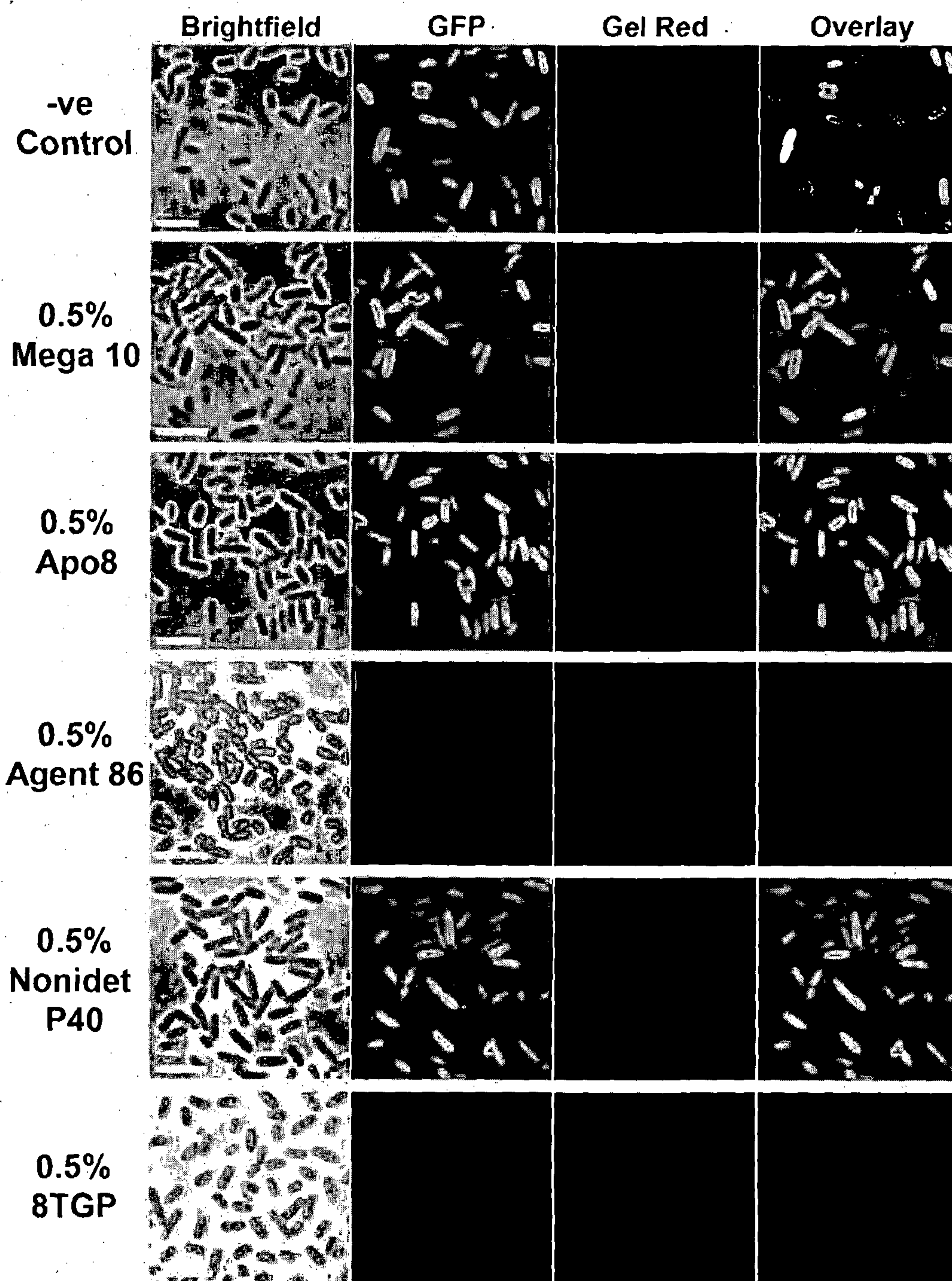


Figure 1

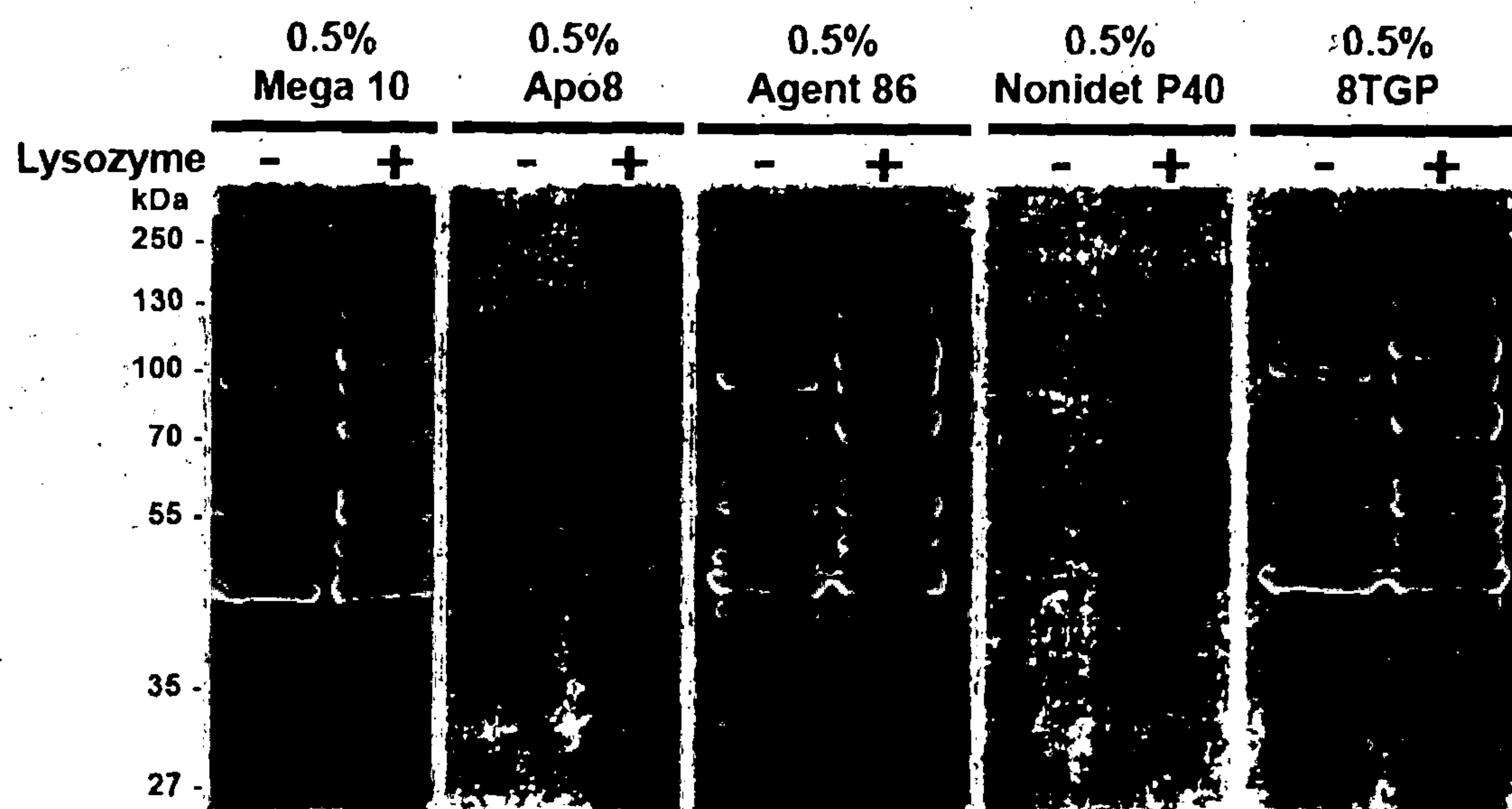


Figure 2

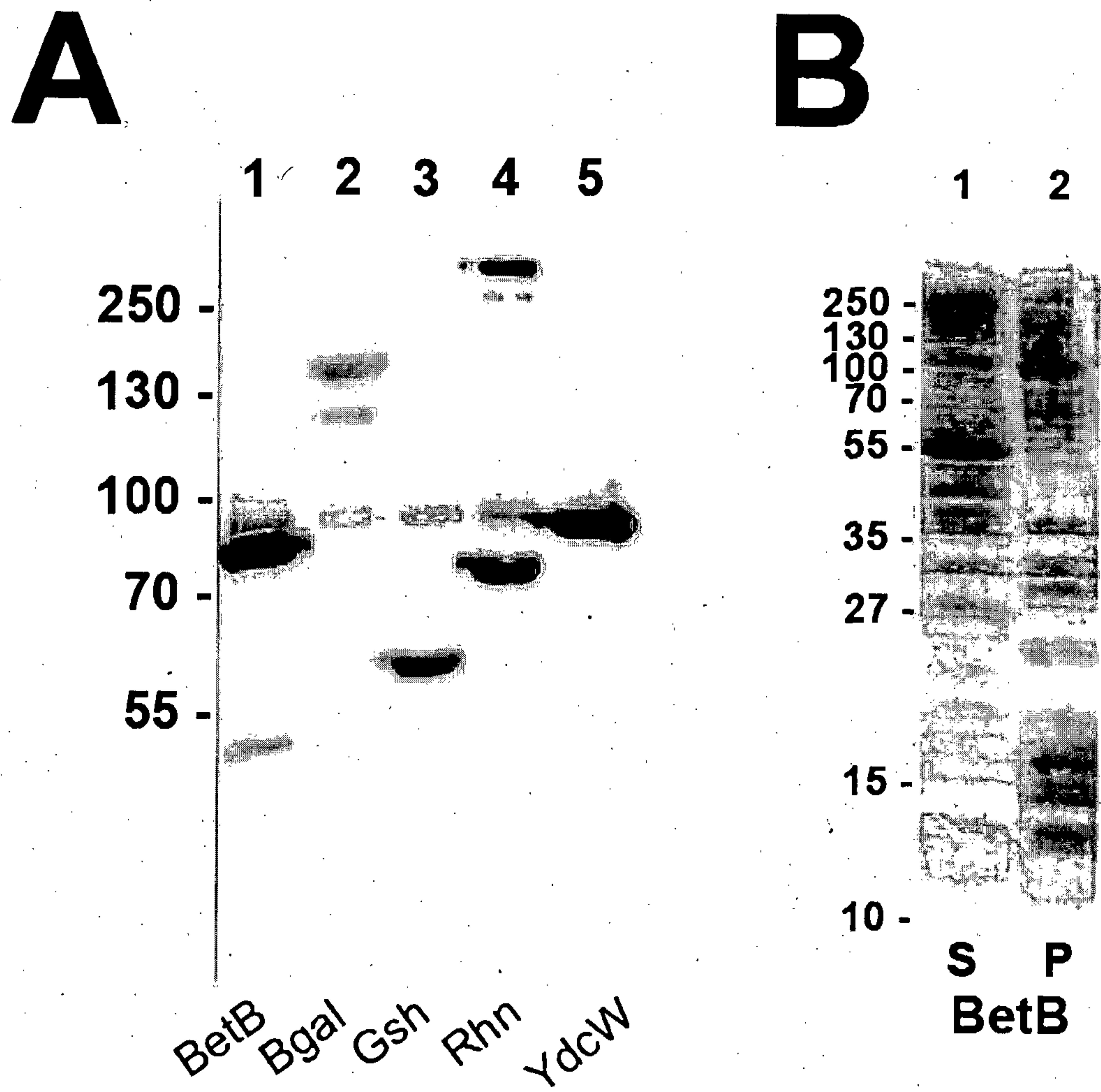


Figure 3

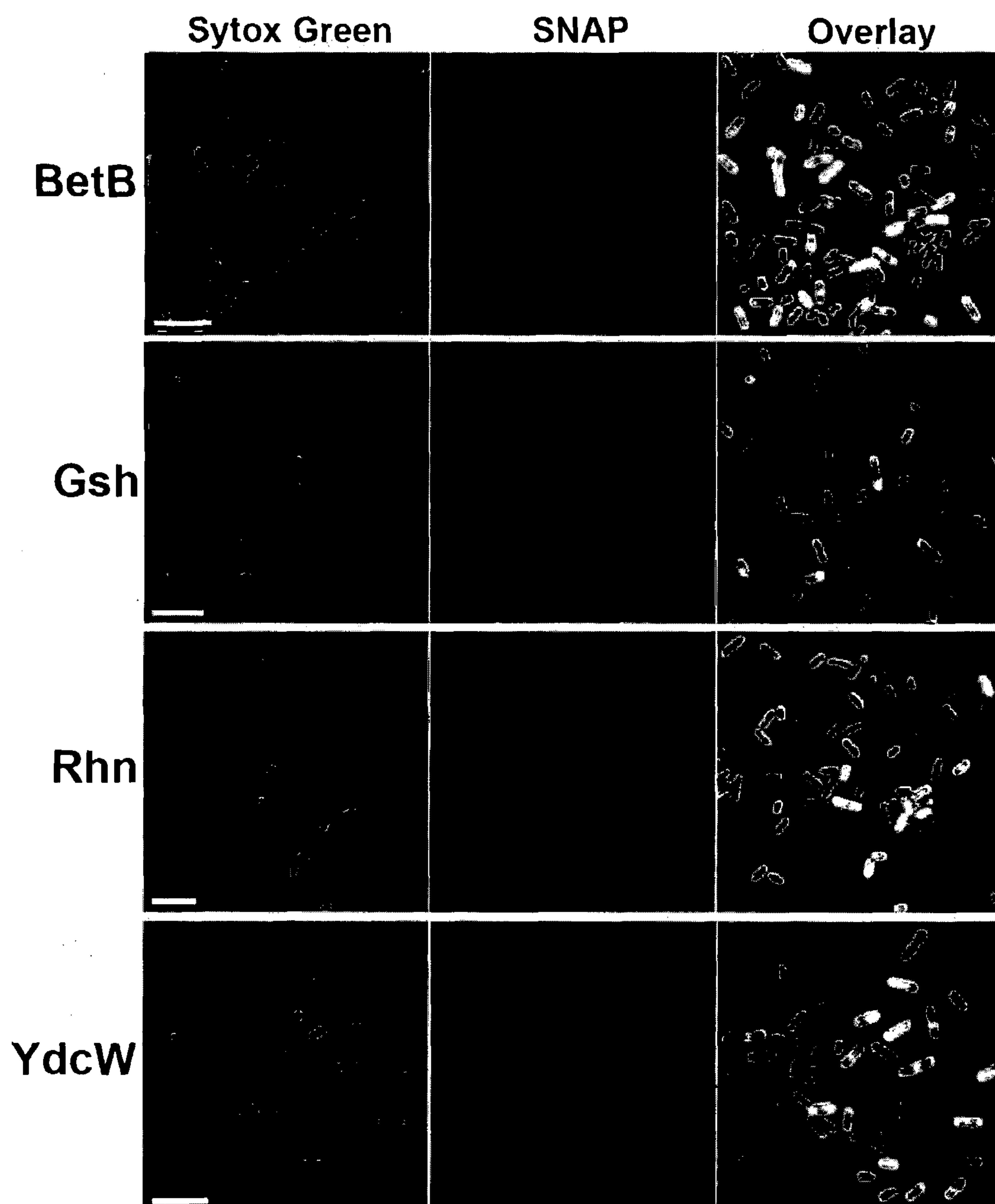
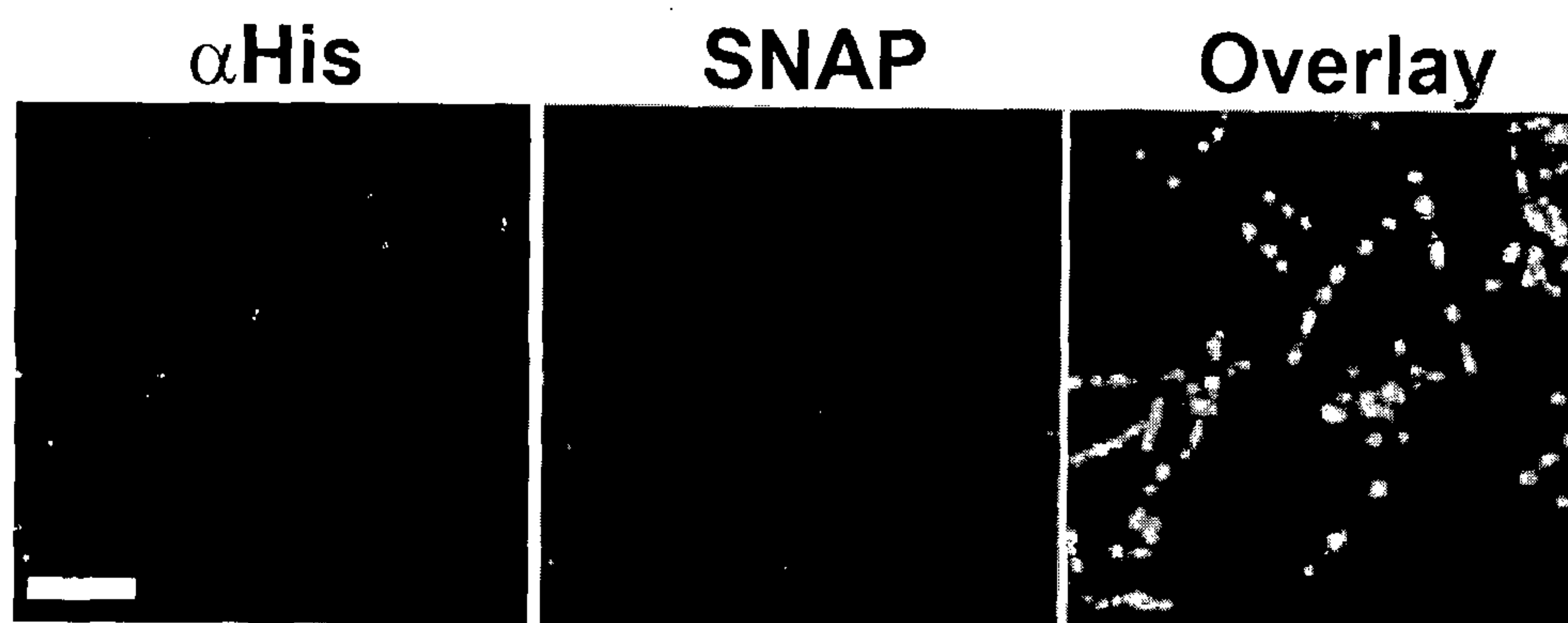


Figure 4

**Figure 5**

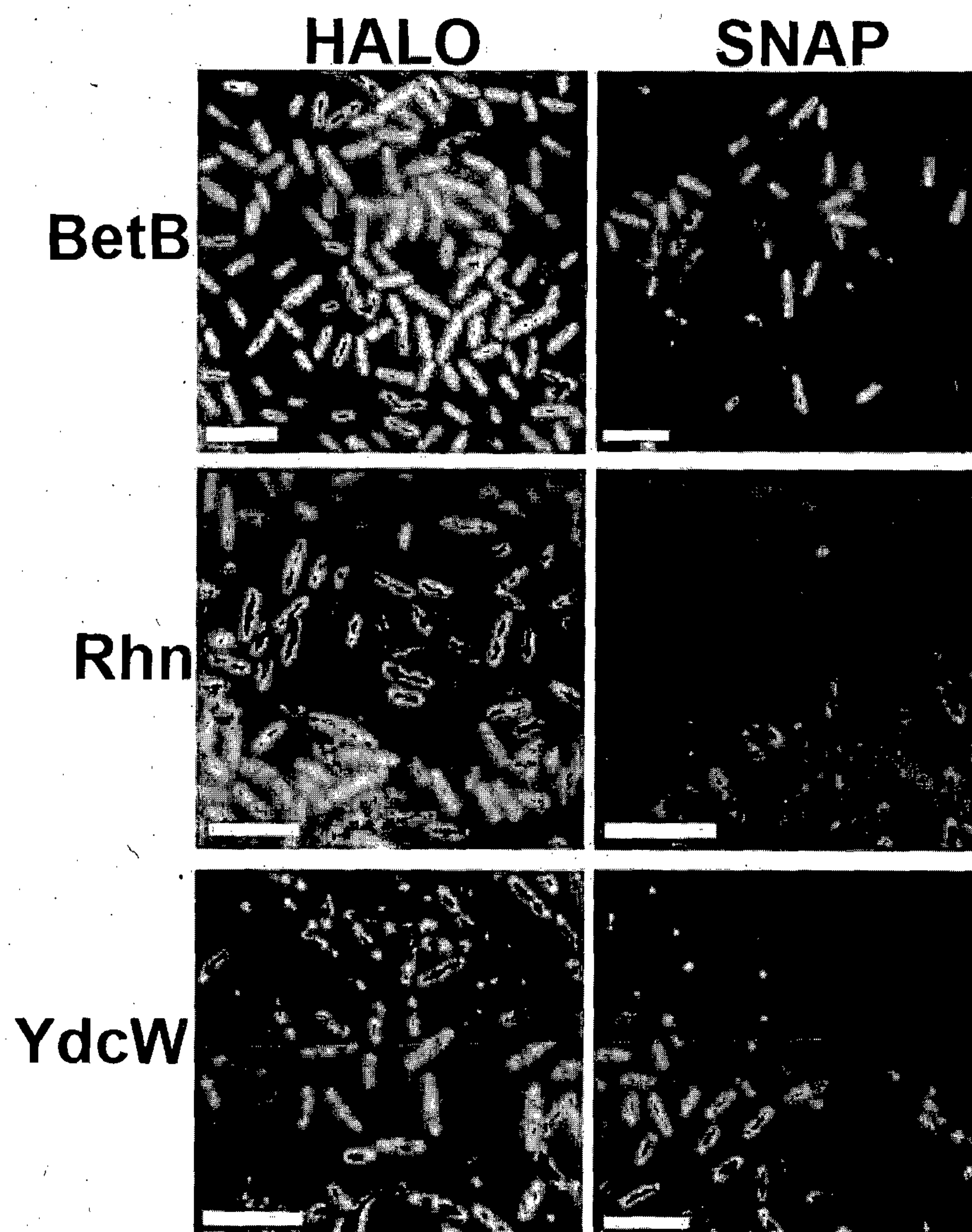


Figure 6

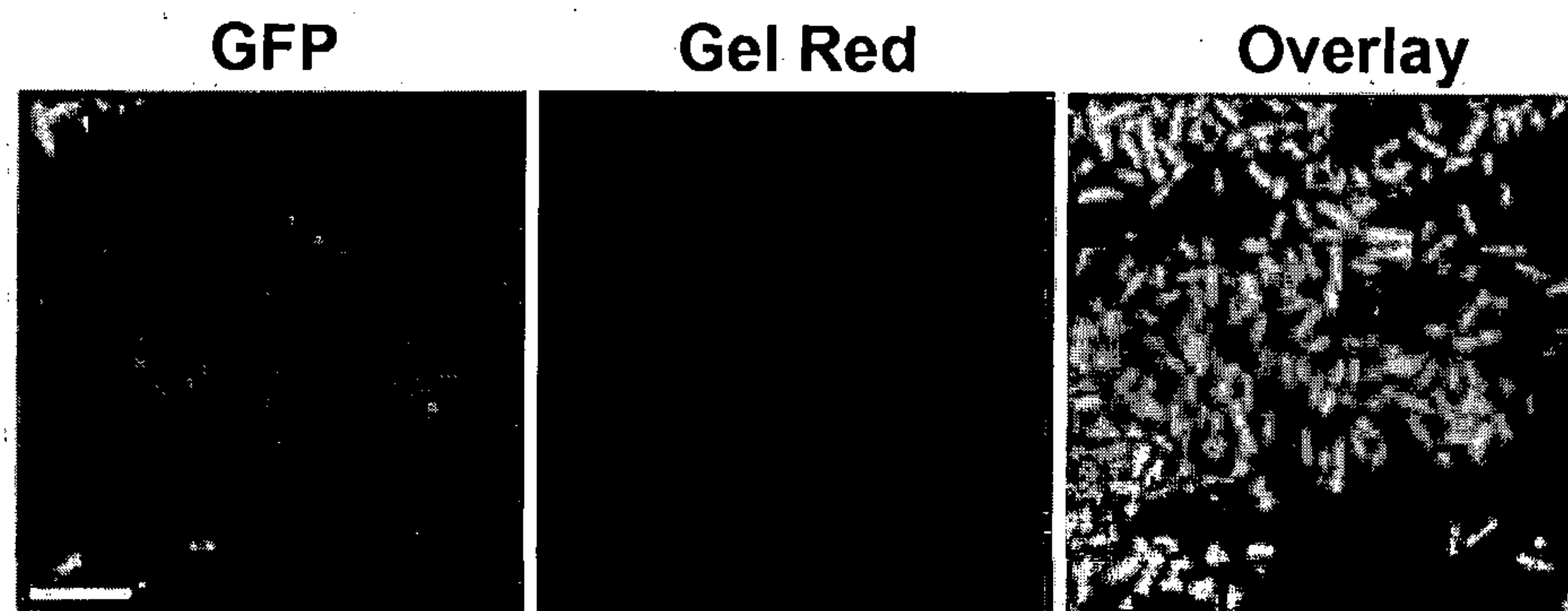


Figure 7

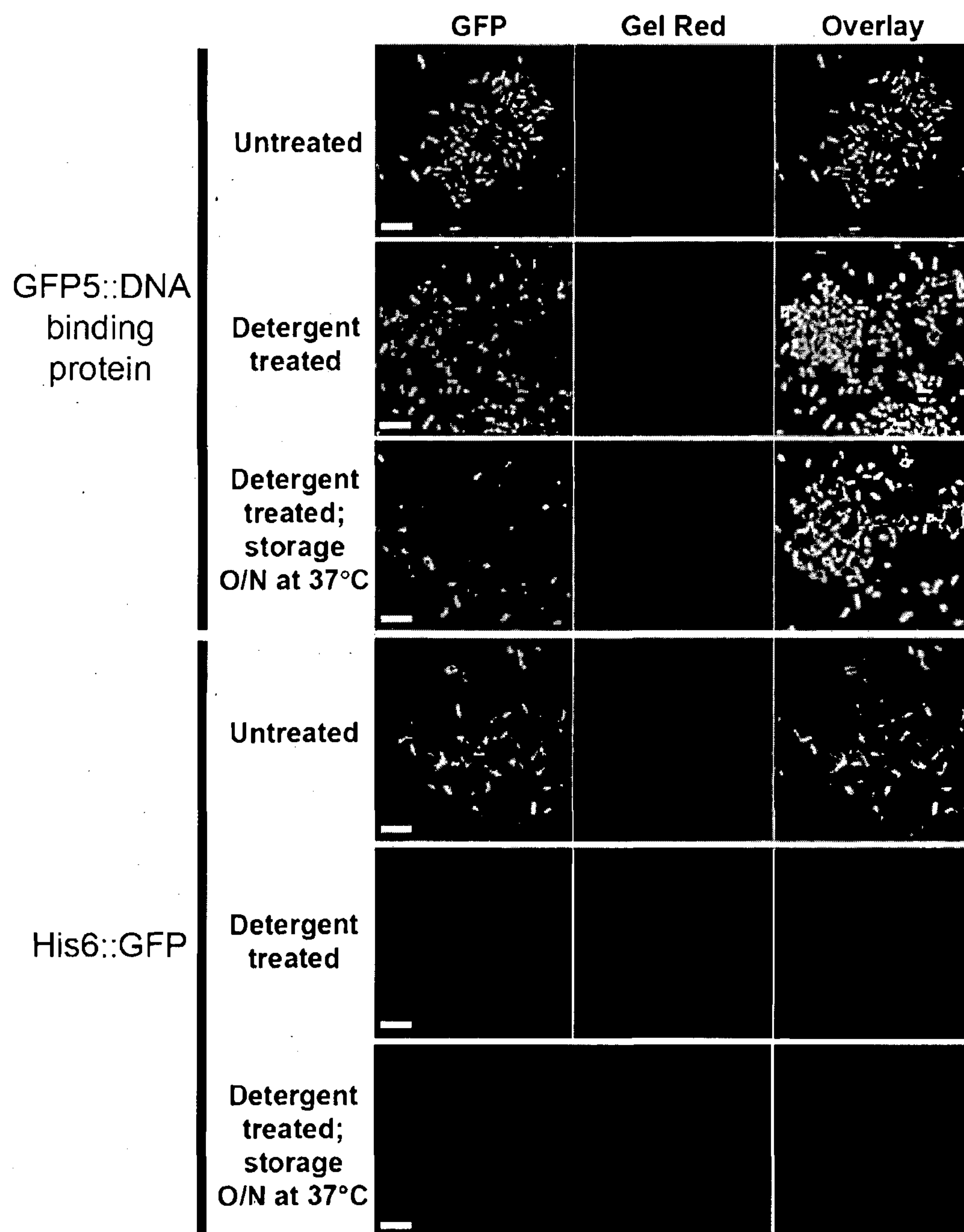


Figure 8

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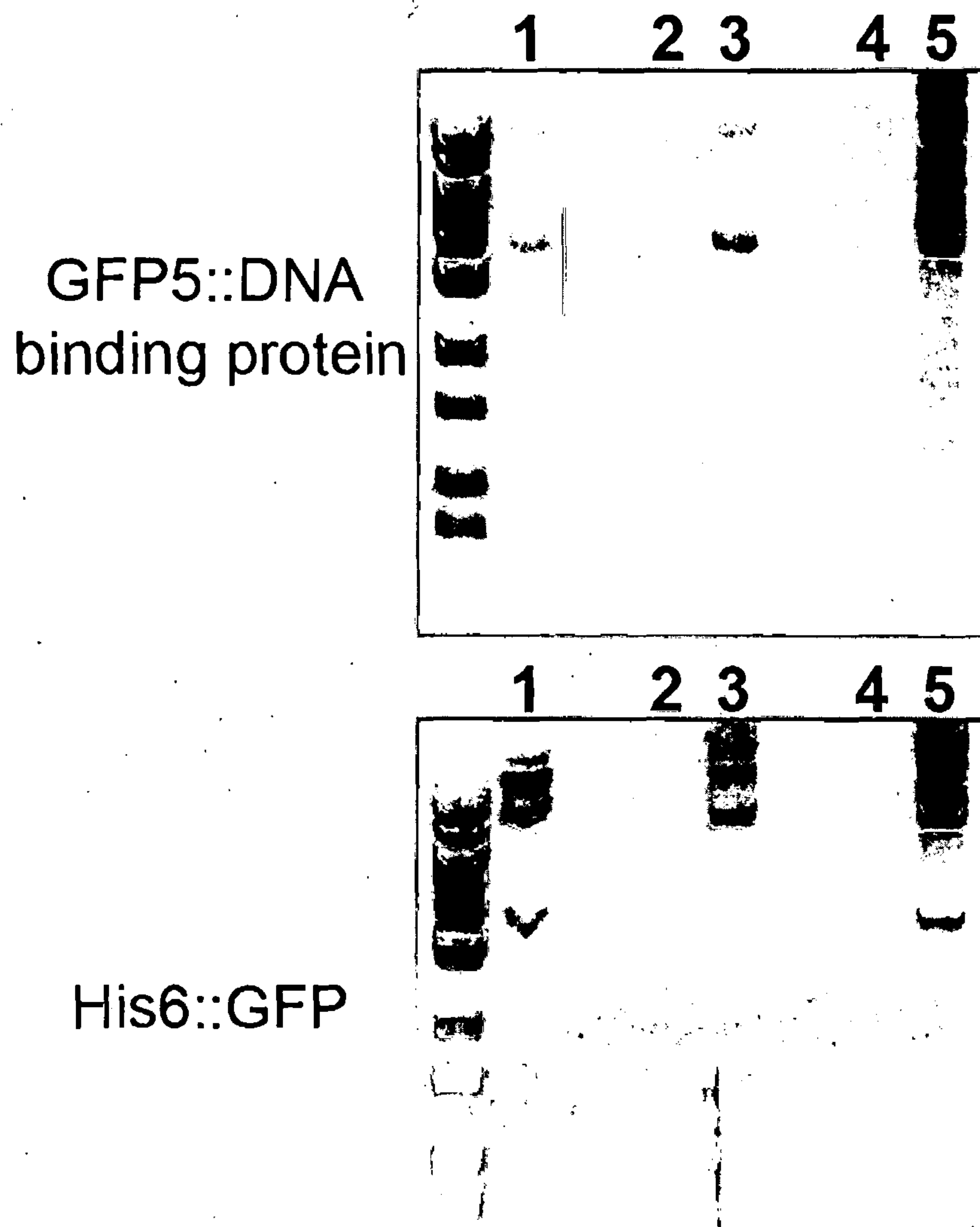


Figure 9

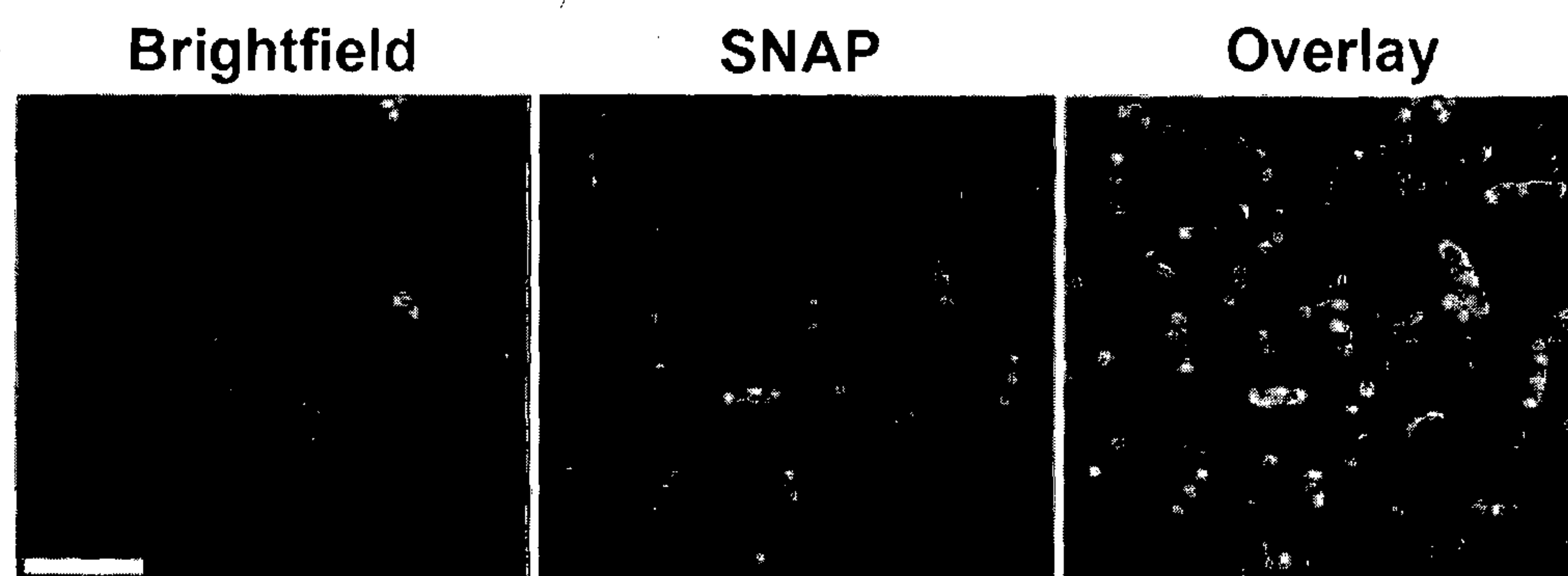
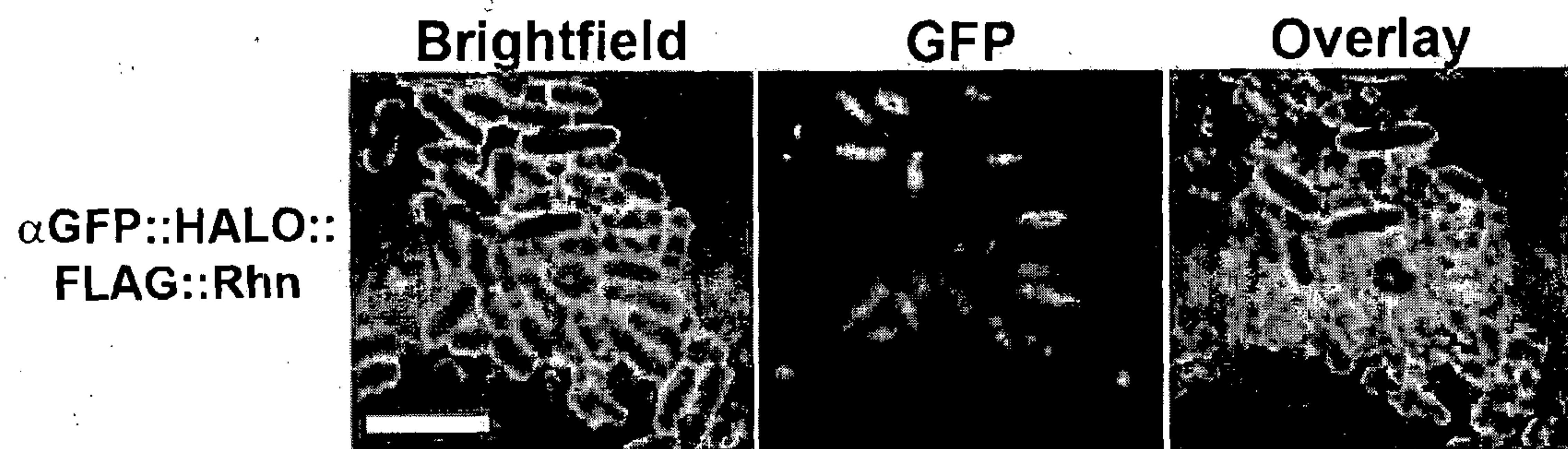
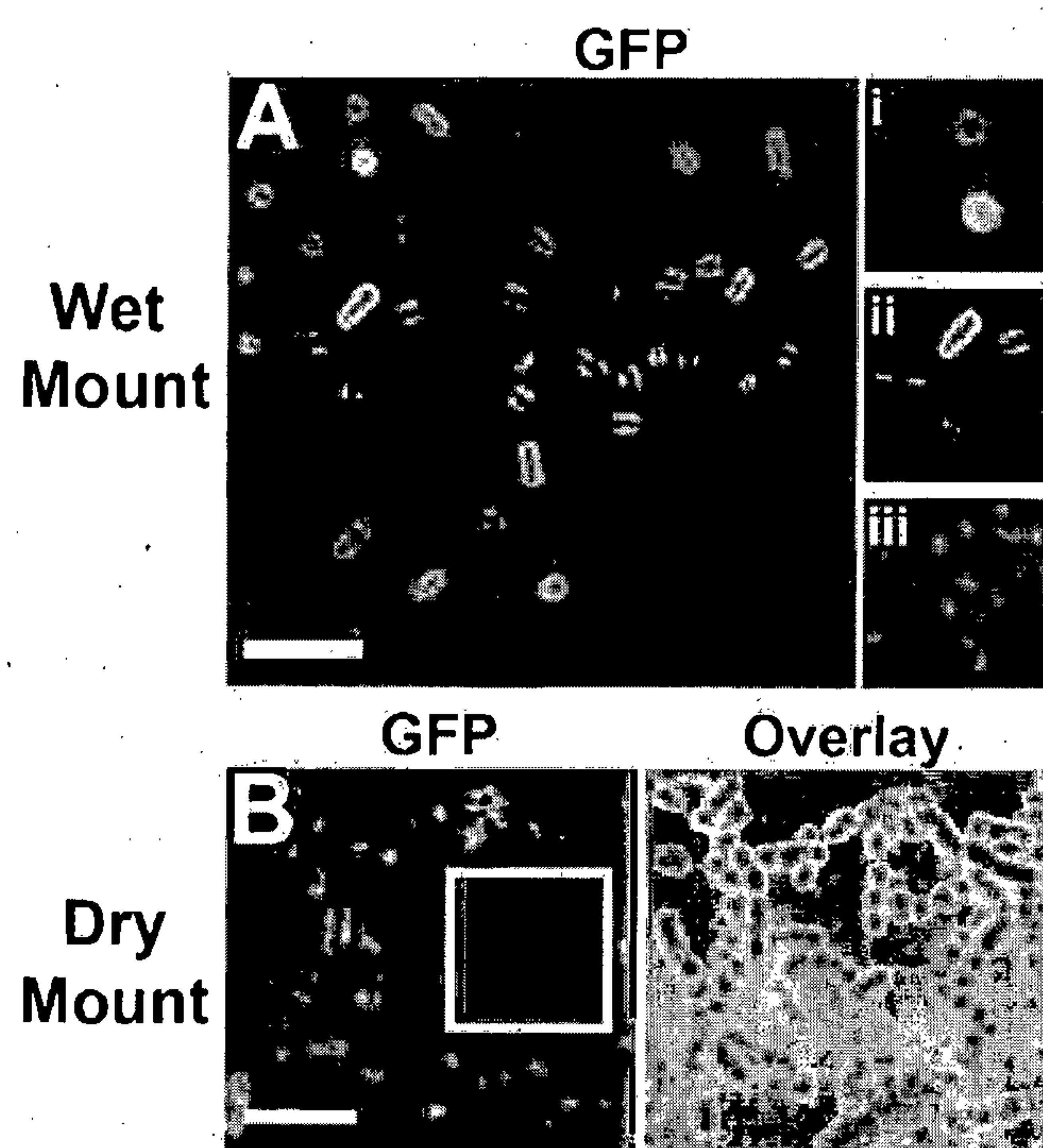


Figure 10

**Figure 11**

12/25

**Figure 12**

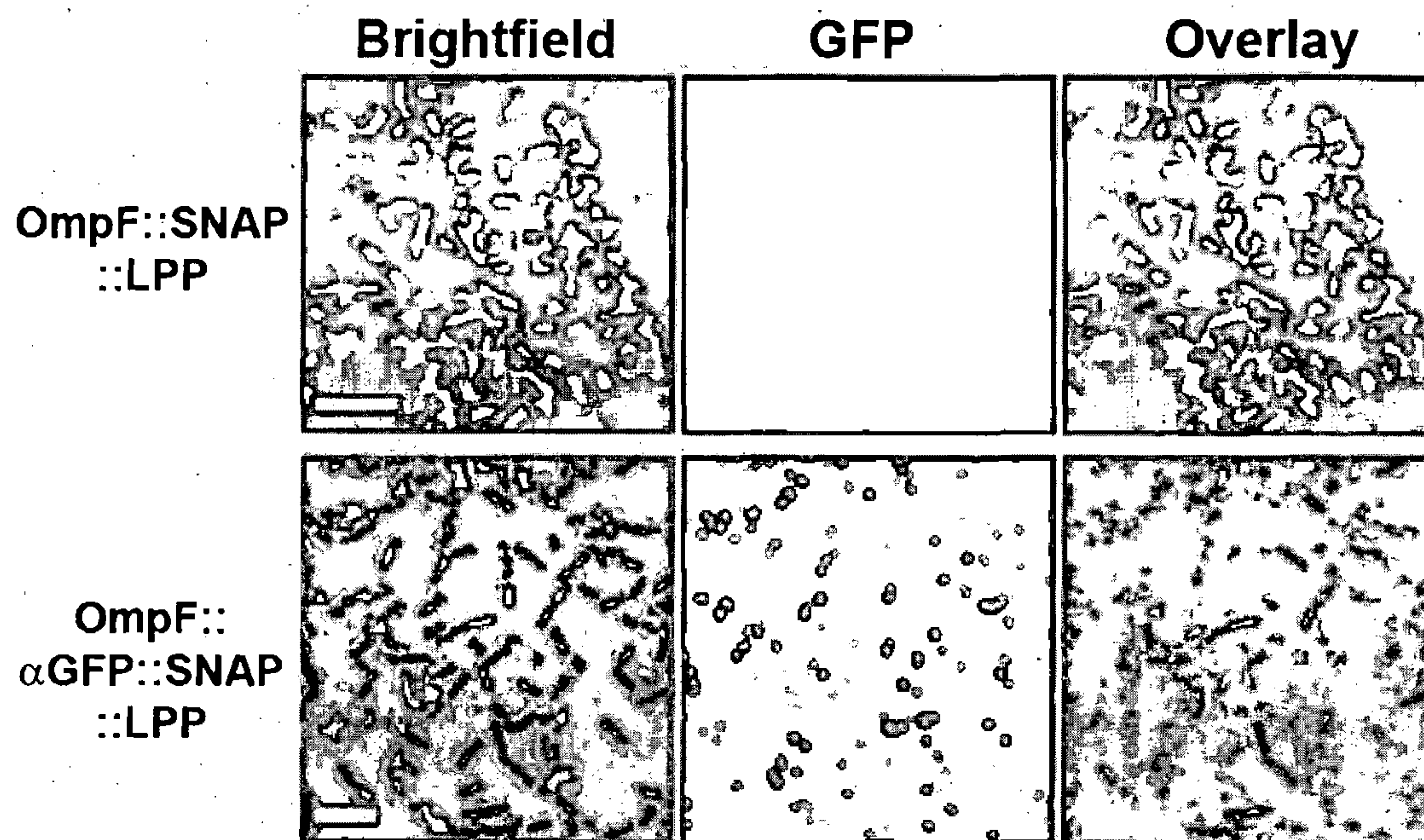
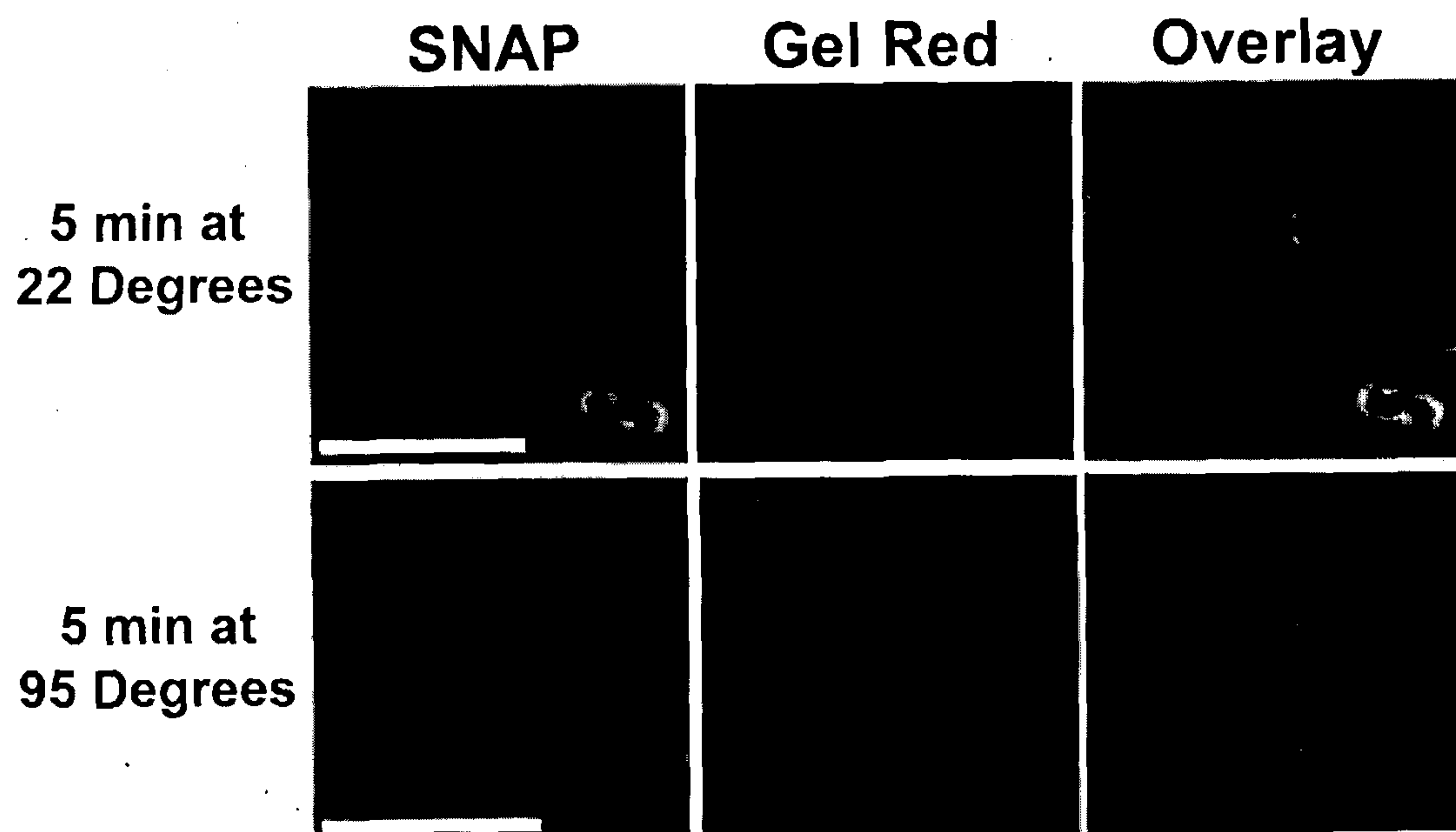


Figure 13

**Figure 14**

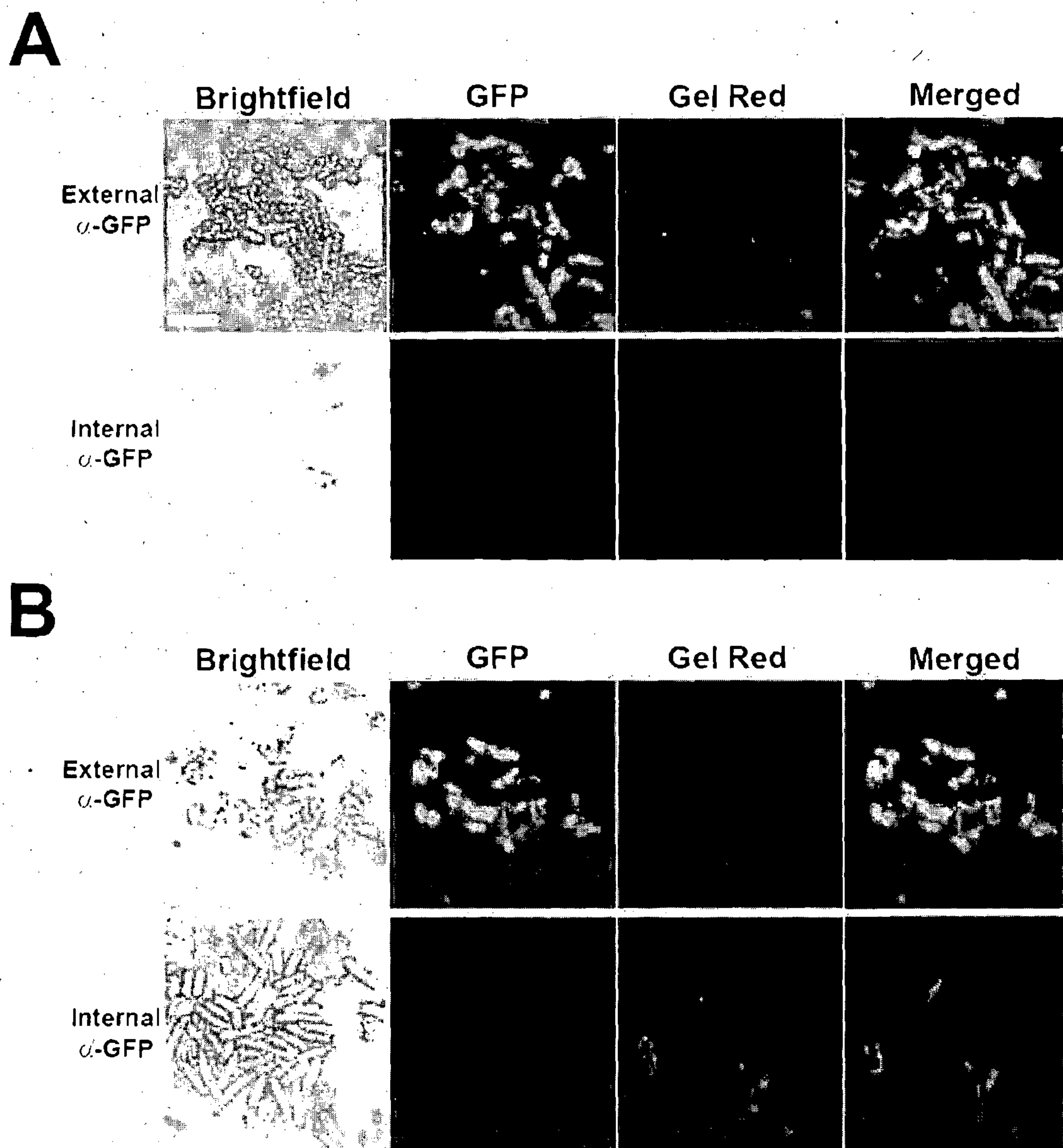


Figure 15

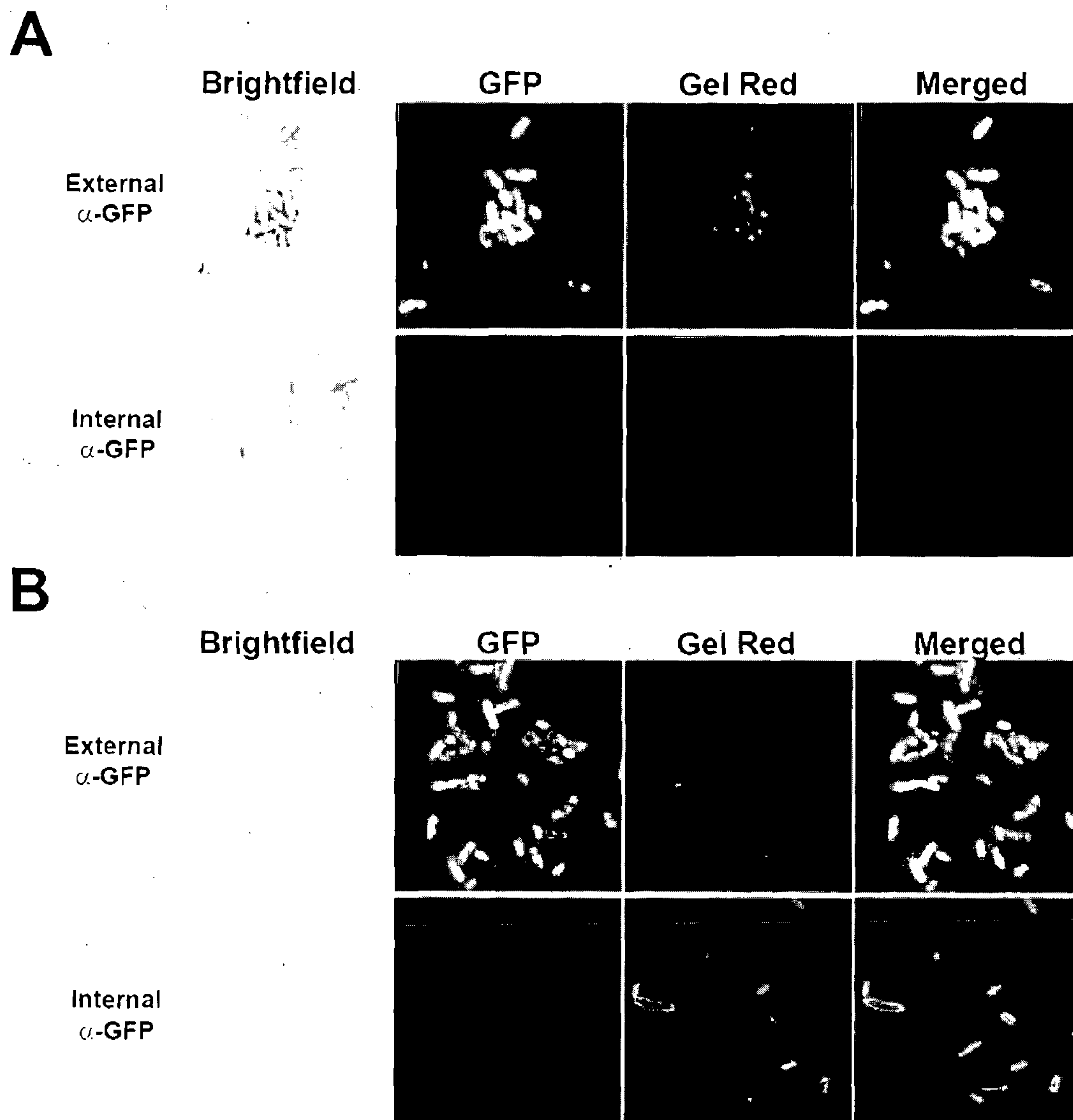


Figure 16

FACs cell sorting using eGFP, SNAP green and BetB Bacteria

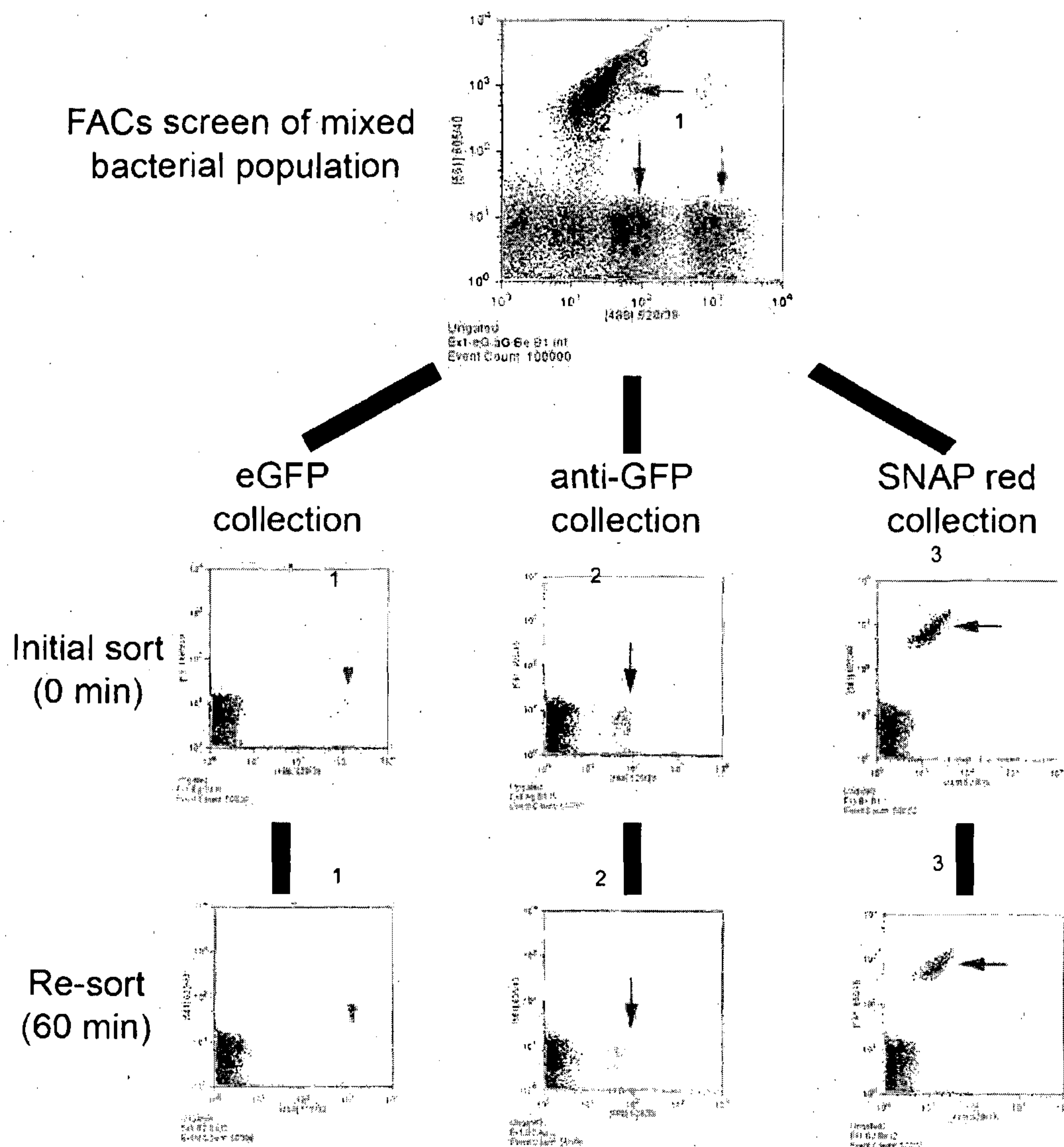


Figure 17

RL6 binding to His-Pur Cobalt resin

GFP

SNAP red

Overlay

**Figure 18**

Magnetic bead binding

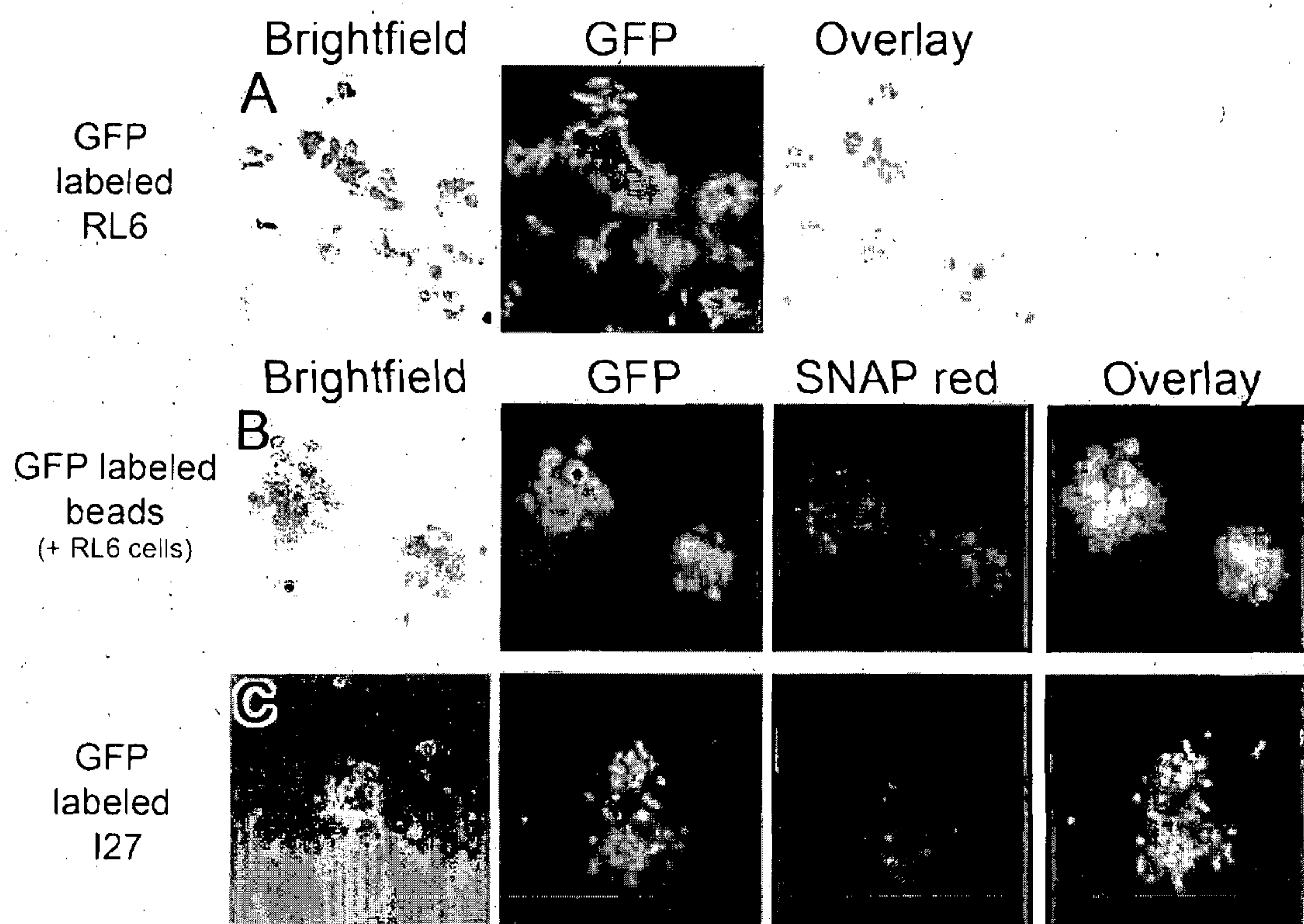


Figure 19

Location of expressed protein

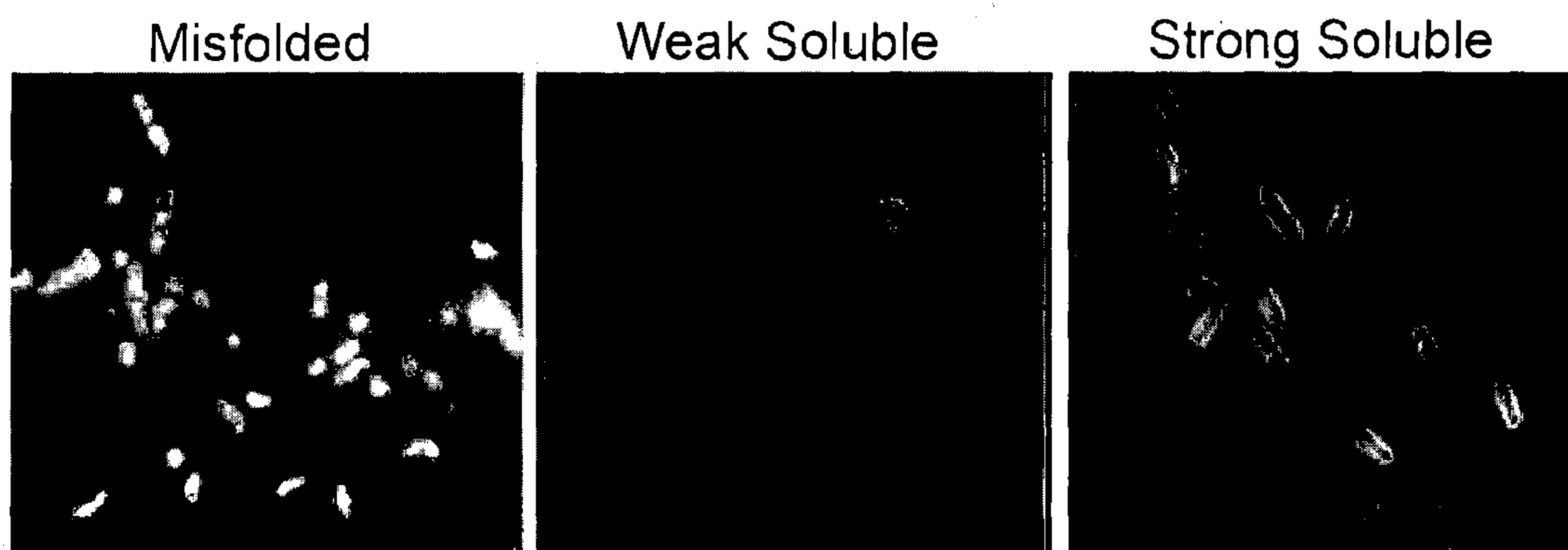


Figure 20

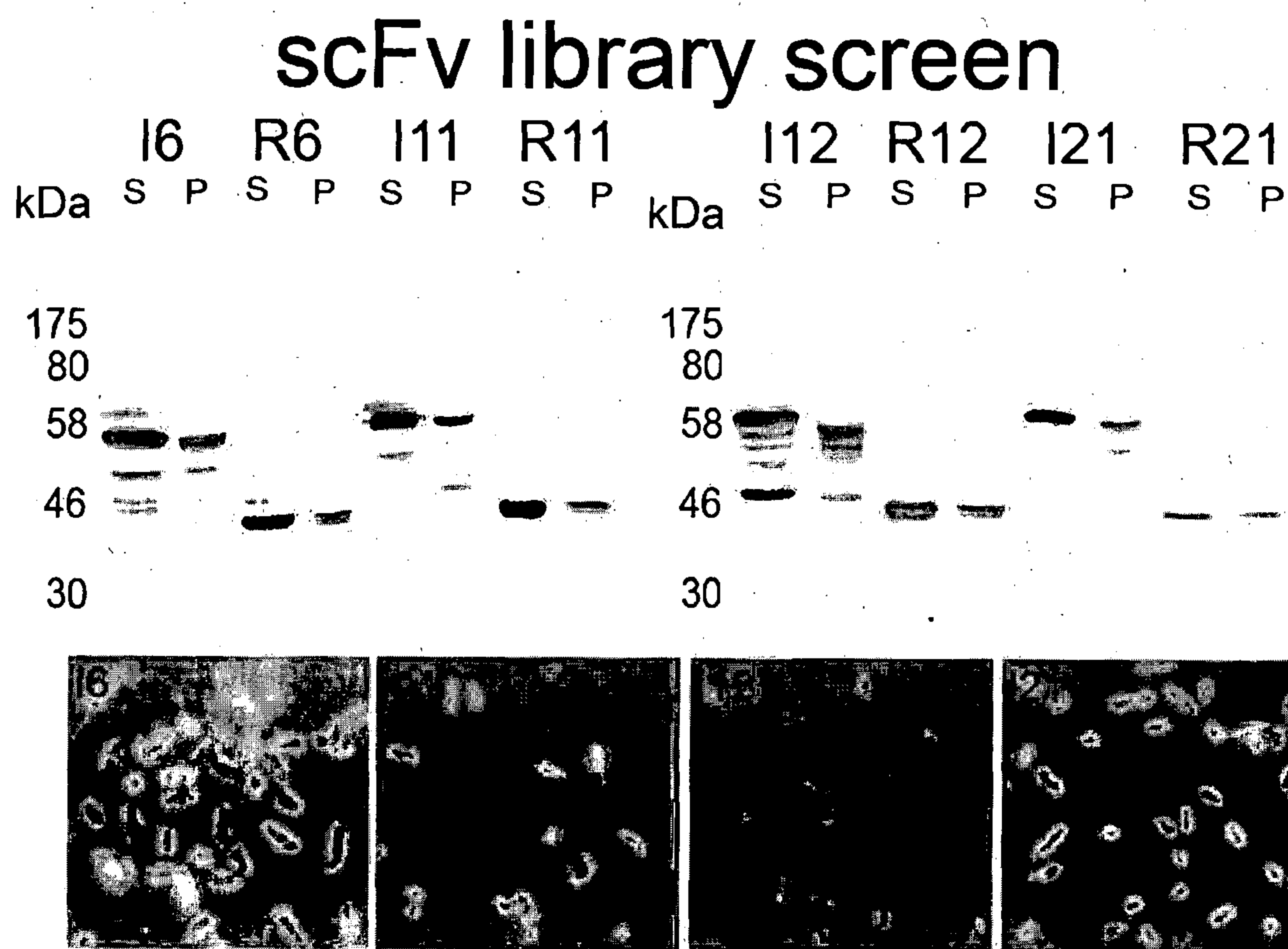


Figure 21

A**Gel Red permeabilisation****-ve****+ve**

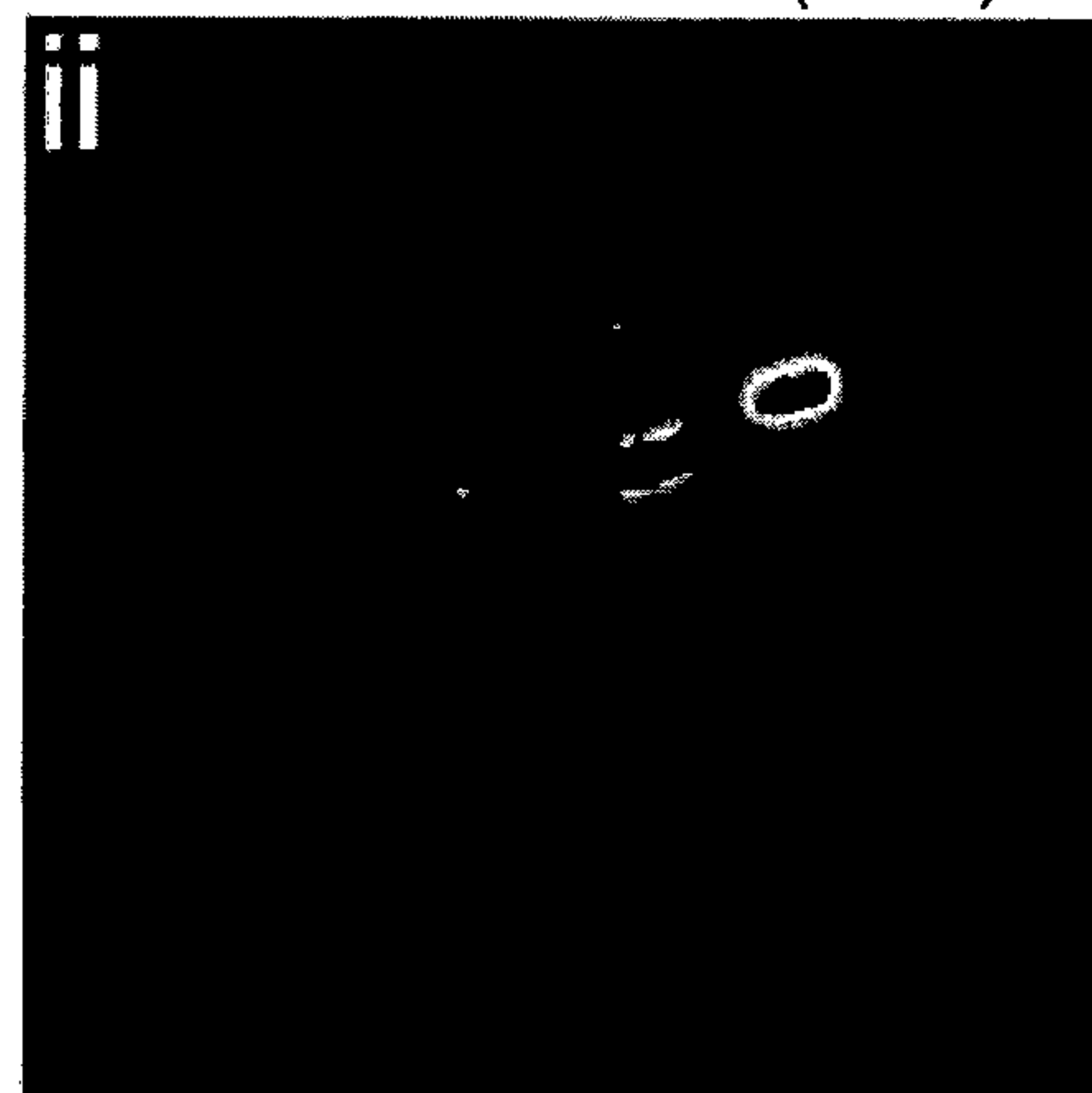
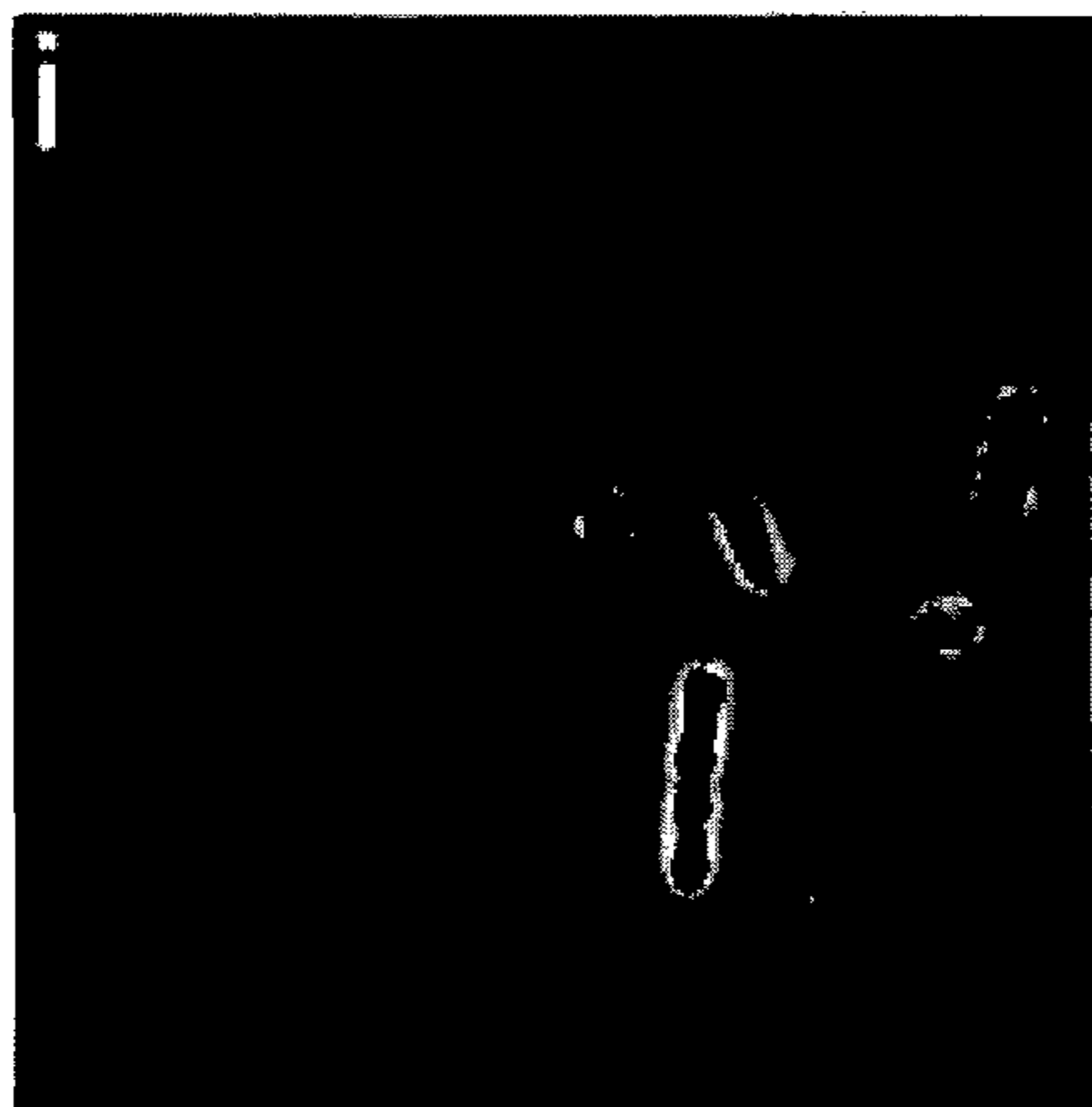
10-30% Ethanol
10-30% Methanol
10-30% Isopropanol
10-30% DMSO
10-30% Acetone
1M Tris
50mM Tris

Butanol (1:5)
Chloroform (1:5)

B**GFP permeabilisation**

0.5% 8TGP

Chloroform (1:5)

**Figure 22**

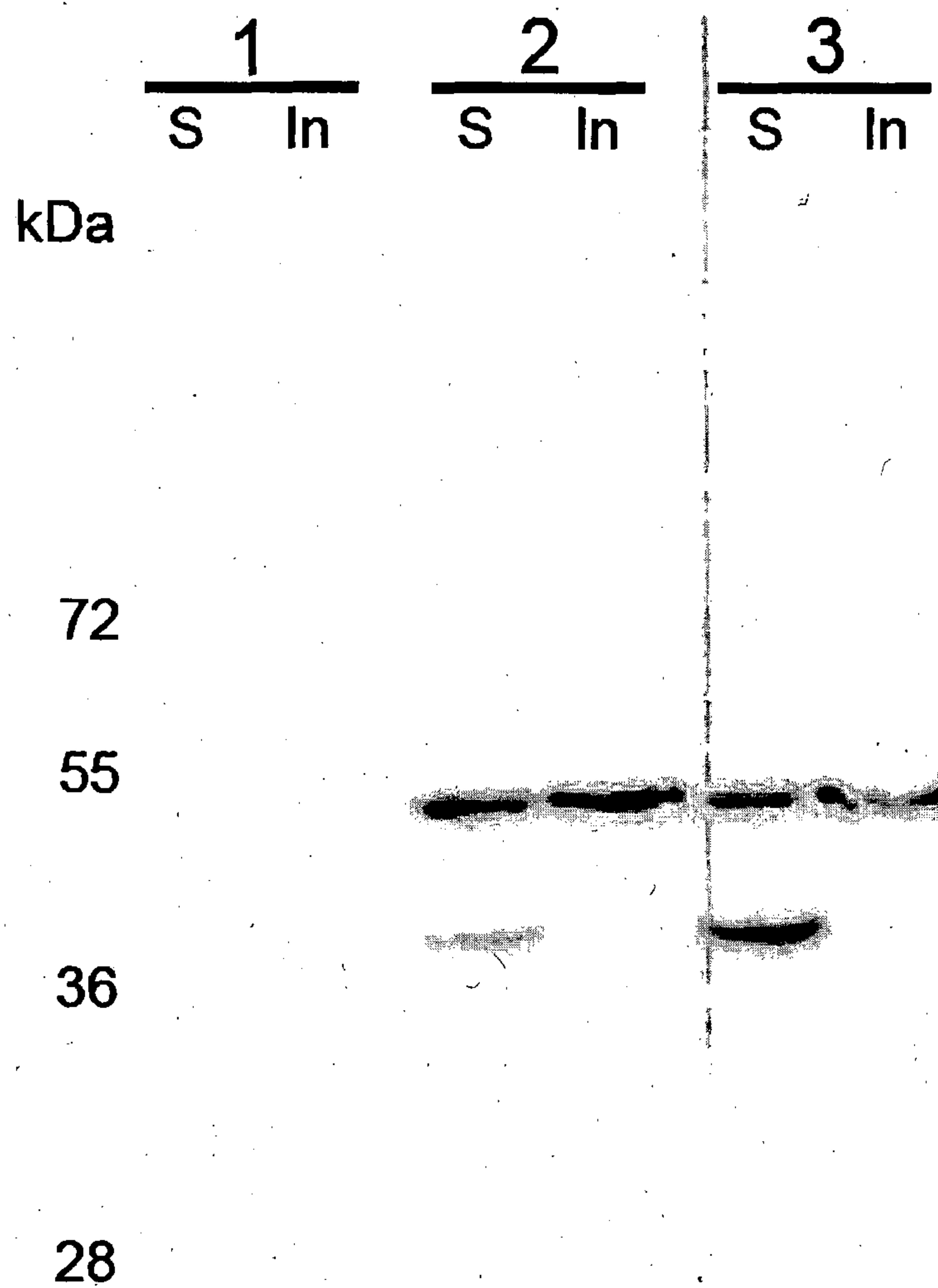


Figure 23

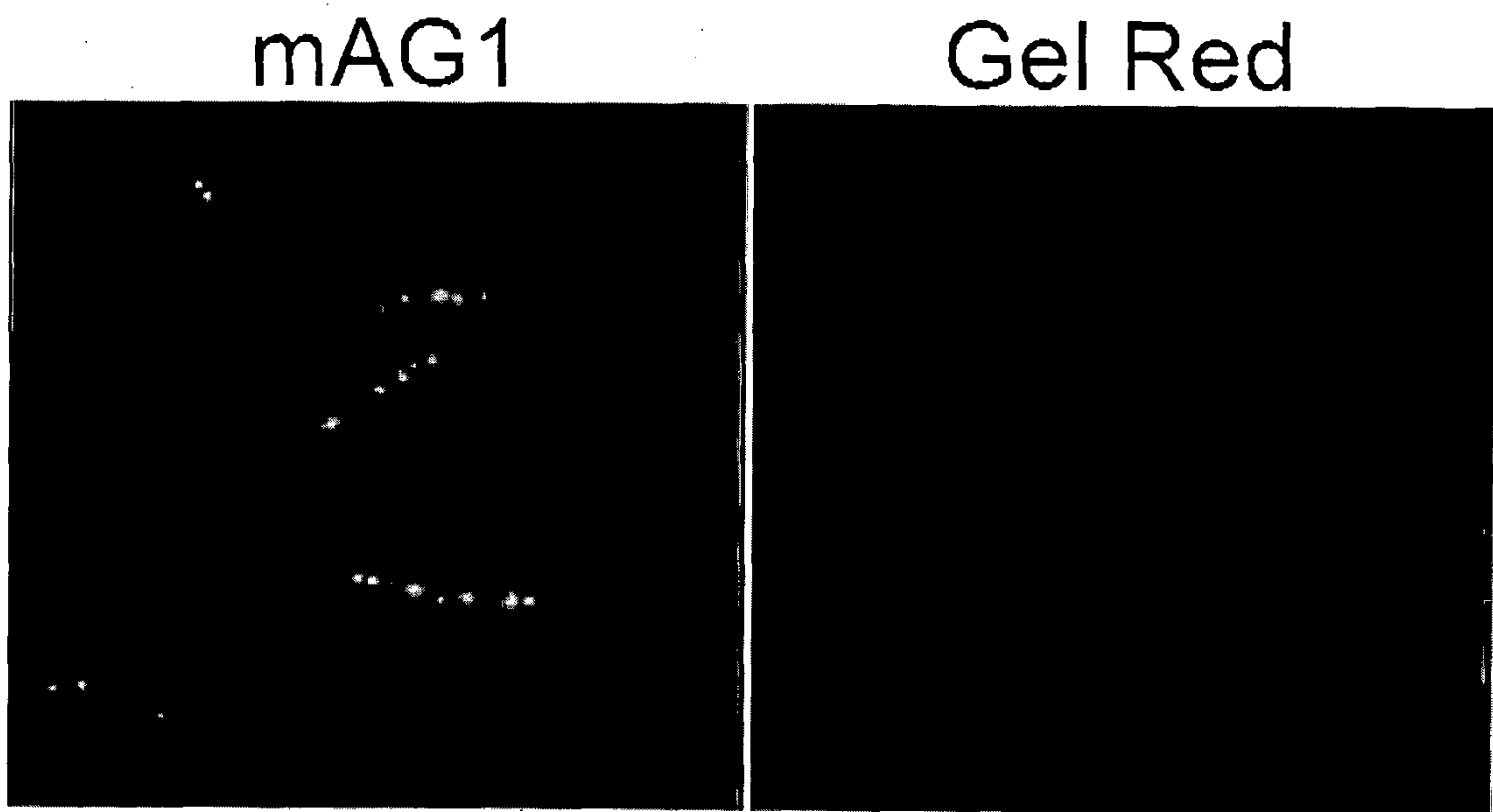


Figure 24

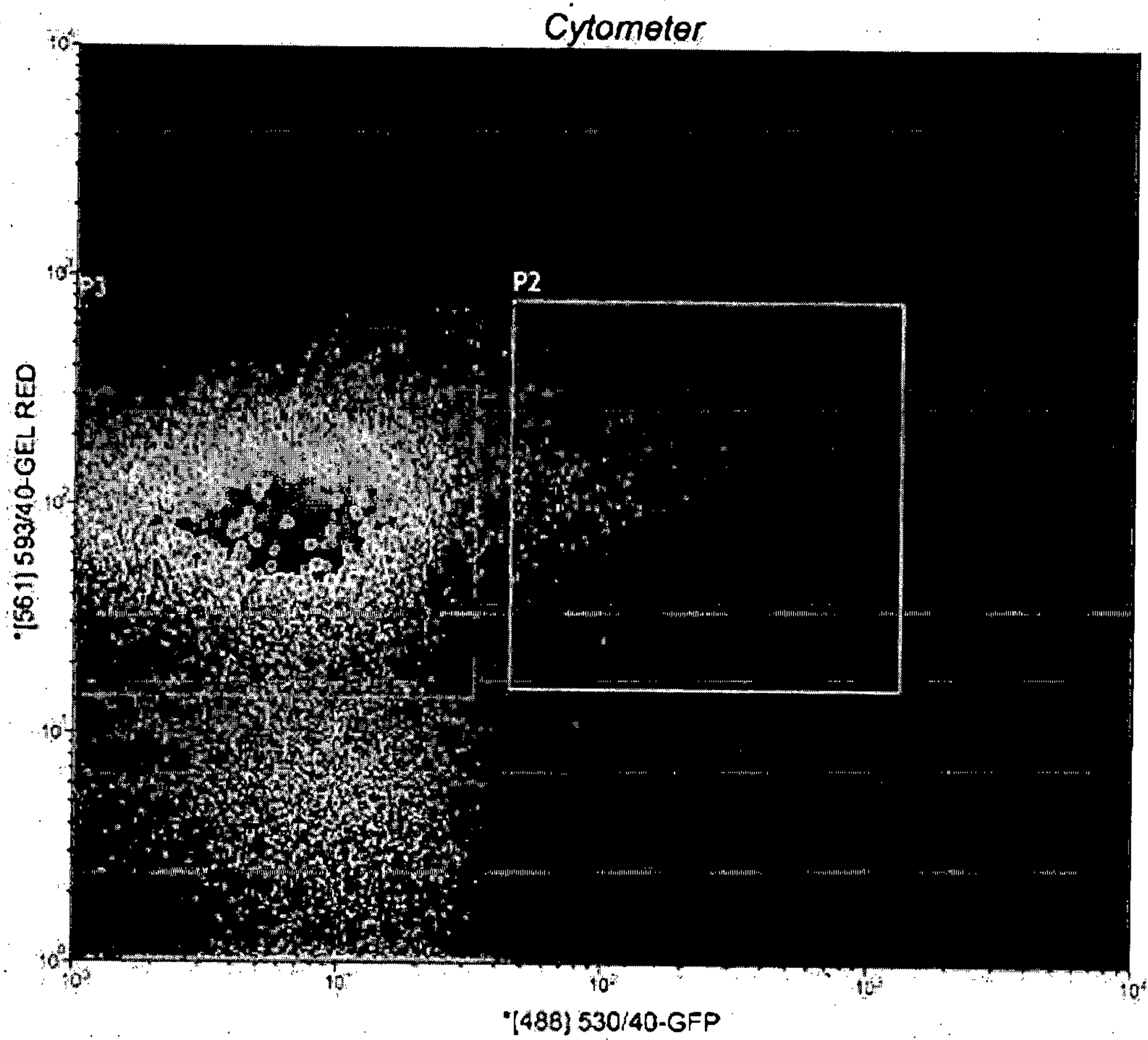


Figure 25