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Chapter 6

Identification of peptides for tissue specific delivery

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Identification of peptides for tissue specific delivery

Abstract

Antisense-mediated exon skipping has shown to be a promising therapeutic approach and is in clinical trials for Duchenne muscular dystrophy. However, after systemic treatment the majority of the injected antisense oligoncucleotides (AONs) will not end up in the intended tissue. This mistargeting of AONs might have detrimental effects, especially with long term treatment and continuous accumulation of AONs. Further, even when no detrimental effects occur, mistargeted AONs are lost for exon skipping in the intended tissue. One way to reduce the amount of mistargeted AONs is by adding a peptide that specifically binds to and is taken up by the intended tissue. Such peptides can be found by screening phage display libraries. With in silico, in vitro and in vivo testing, the peptides that bind the intended tissue most efficiently and most specifically can be identified.

1. Introduction

In the last decade antisense oligonucleotides (AONs) have developed into a promising tool to interfere with splicing on the mRNA level. AON mediated exon skipping in Duchenne muscular dystrophy has even advanced into clinical trials (***1;2***). Further, AONs can also be used to switch protein isoforms or induce protein knockdown. This has various research and therapeutic applications (see Chapter 6 for an overview). However, for a robust therapeutic effect, it is essential that sufficient amounts are taken up by the intended tissue (usually the tissue affected by the disease). And even when AONs have the optimal design in sequence and chemistry, upon systemic delivery most of the injected dose will not end up in the intended organ, but in the organs with fenestrated endothelium, i.e. primarily in the liver and kidney (***3***). In some cases this can lead to detrimental effects, as the AONs change the expression/isoform of the target protein in an area that is not diseased. Further, accumulation of AONs in the kidney and the liver after long term treatment might lead to toxic effects, although this effect is not yet seen after short term treatment (***4***). Finally, even if mistargeted AONs do not induce adverse effects, they are lost for exon skipping in the intended tissue.

The delivery of the AONs to the intended tissue can be improved by the addition of homing peptides, and one way to identify such peptides is by phage display (***5;6***). Different cell types express different proteins on their surface, especially diseased tissues, and peptides binding to these proteins can increase the amount of AON delivered to the target tissue. An important obstacle in this approach is the endothelium. A normally functioning endothelium will prevent entry of most compounds, and targeting peptides will not be able to bind cells in the tissue. In this case peptides targeting the endothelium of the tissue of interest can be selected (***7***). Proteins expressed on endothelium also differ between tissues, again especially in a disease state, and a high local concentration of AON due to binding to the endothelium will lead to higher tissue uptake, for instance in the heart (***8***).

In diseases that induce an immune reaction, for instance Duchenne muscular dystrophy, the endothelium is more open, allowing tissue binding peptides to enter the tissue.

Bacteriophages (or briefly phages), are viruses that infect bacteria. Random peptide sequences can be added to their genome in such a way that they express these peptides on their surface **(*5;6*)** surface. This can be in a polyvalent or monovalent manner. Most libraries employing the M13 phage express the random peptides on all pIII proteins **(*6*)**. The attachment of the peptide to the phage can be on one side, or on two sides, giving the peptide a circular, more natural structure. All libraries contain different peptide sequences and it is important to realize that a given library will not give a complete list of all possible binding peptides. In fact, when two similar libraries with respectively 8 and 9mer peptides were compared, after enrichment in the same tissue, results did not even overlap **(*9*)**.

To identify peptides that ensure binding to (the endothelium of) the intended tissue, a phage display library can be added to a molecule, a cell line/culture or a tissue of interest and the bound or internalized phages can be isolated. After a first selection, phages can be amplified in bacteria to allow another round of selection. More rounds of selection will increase the portion of specifically binding peptides. Generally, 3-5 rounds of selection are performed, however a higher number of rounds introduces a bias, because phages encoding some peptides replicate faster than others.
Since not all peptides found with phage display will specifically bind the target sequence, in vitro and in vivo testing is needed to confirm specificity. Before testing, websites such as Pepbank **(*10*)** *(http://pepbank.mgh.harvard.edu/)* and SAROTUP **(*11*)** (*http://immunet.cn/sarotup/*), can help to make a first selection. For example, SAROTUP will tell you the phage expressing HAIYPRH in the Ph.D.-7

library (New England Biolabs) has a mutation that makes amplification of this phage faster **(*12*)**. These websites are only as good as the input they have, thus publishing complete lists of results will increase the usefulness of these tools. Possibly, massive parallel sequencing can reduce the amount of selection rounds needed. This way millions of phages can be sequenced, reducing the risk of missing a peptide that is only modestly increased after two rounds and reducing the risk of enriching for easily replicating phages **(*13;14*)** (‘t Hoen, manuscript in preparation). However, it is impossible to test all higher expressed peptides from such an experiment, since thousands of potentially interesting peptides will be identified. Therefore data analysis and filtering is required. The previously mentioned websites are a good starting point for this, but additional criteria should be added to rank peptides.

Phage display can be performed on specific proteins, cells or in vivo. If a protein is proven to be highly expressed in the target tissue or on the endothelium in that tissue, it is relatively easy to enrich for peptides efficiently binding to that protein. In cell culture, the amount of potential binding sites is enormous and less enrichment will be seen. The advantage of selection on cell culture is the possibility to isolate both membrane bound and internalized phages. Since AONs need to enter the cells in order to manipulate splicing, internalized phages might be more interesting. However, cells in culture are known to differ considerably from tissues in vivo and can express different proteins on their surface. Further, a lot of the proteins found on muscle cells in culture or in vivo might also be expressed by other tissues. Therefore, extensive in vivo testing of the peptides will be needed.

For in vivo selection, circulation time of the phages is important. Peptides should have enough time to bind their target, but if circulation time is too long they will be broken down. It takes about 30 minutes for a phage to be taken up by a cell

(***15***) and during this time phages are hardly broken down, since the amount of isolated phages is similar after 5 and 30 minutes circulation time (***16***). During a first round of in vivo selection, it is possible the most effective peptide is not present in the isolated tissue, especially in the case of skeletal muscle, where generally only one of the many muscles present in the body is isolated. To make sure none of the peptides is lost, a combination of in vitro selection followed by in vivo selection might be preferred. This way, the amount of muscle binding peptides will be increased in the in vitro selection, which increases the chance of finding them back in vivo.

Finally, it is important to keep in mind that, for a given target, several targeting peptides can be found and that some of these peptides may show better results compared to AON-only treatment, but they are not necessarily the most efficient peptides.

2. Materials

2.1 Phage experiments

2.1.1 In vivo phage panning

1. LB Medium: Per liter: 10 g Bacto-Tryptone, 5 g yeast extract, 5 g NaCl. Autoclave, store at room temperature.
2. Top Agar: Per liter: 10 g Bacto-Tryptone, 5 g yeast extract, 5 g NaCl, 7 g Bacto-Agar (or eletrophoresis grade agarose). Autoclave, dispense into 50 ml aliquots. Store solid at room temperature, melt in microwave as needed.
3. ER2738 culture on LB-agar plate.
4. Mice of a relevant strain and tools for injection of phages and organ isolation.
5. Electric pump for perfusion experiment.
6. TBS: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl. Autoclave, store at room temperature.
7. PEG/NaCl: 20% (w/v) polyethylene glycol–8000, 2.5 M NaCl. Autoclave, mix well to combine separated layers while still warm. Store at room temperature.
8. MagNa lyser green beads.
9. MagNa Lyser.

2.1.2 Phage amplification and isolation for sequencing

1.ER2738 culture on LB-agar plate.
2. LB Medium: see item 1 in section 2.1.1.
3. Top Agar: see item 2 in section 2.1.1.
4. Iodide Buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 4 M sodium iodide (NaI). Store at room temperature in the dark. Discard if color is evident.
5. PEG/NeCl: see item 7 section 2.1.1.
6. 70% ethanol.
8. TE buffer: 10 mM Tris-Cl, pH 7.5. 1 mM EDTA.

2.1.3 Phage titration

1.ER2738 culture on LB-agar plate.

2. LB Medium: see item 1 in section 2.1.1.
3. Top Agar: see item 2 in section 2.1.1.
4. IPTG/Xgal Stock: Mix 1.25 g IPTG (isopropyl-β-D-thiogalactoside) and 1 g Xgal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside) in 25 ml DMF (dimethyl formamide). Solution can be stored at –20°C.
5. LB/IPTG/Xgal Plates: 1 liter LB medium + 15 g/l agar. Autoclave, cool to < 70°C, add 1 ml IPTG/Xgal Stock per liter and pour. Store plates at 4°C in the dark.

2.2 Identification of most promising peptide

1. Human primary myoblast (or relevant primary cell culture).
2. Proliferation medium: Nut.Mix F-10 (HAM) medium supplemented with GlutaMax-I, 20% FBS (fetal bovine serum) and 1% P/S (penicillin/streptavidin).
3. Differentiation medium: Dulbecco's medium (without phenol red) with 2% FBS, 1% P/S, 2% Glutamax and 1% glucose.
4. Differentiation medium without serum.
5. PBS (phosphate buffered saline).
6. TBS (tris buffered saline).
7. Purified bovine dermal collagen for cell culture.
8. Distilled water.
9. Glass slides.
10. Fluorescently labeled peptide.
11. Methanol.
12. Mounting medium.
13. Fluorescence microscope.

2.3 Intramuscular test of fluorescently labeled peptides

1. Appropriate mouse model, in this case the *mdx* mouse.
2. Injection needle.
3. Fluorescently labeled peptide.

4. Dissection kit.
5. Isopentane.
6. Liquid nitrogen.
7. Cryotome.
8. Positive charged slides.
9. Acetone.
10. Mounting medium.
11. Fluorescence microscope.

2.4 Intramuscular test of peptides conjugated to an AON

1. Appropriate mouse model, in this case the *mdx* mouse.
2. Injection needle.
3. Peptide conjugated to an AON (peptide-AON) (*see* **Note 1**).
4. Dissection kit.
5. Isopentane.
6. Liquid nitrogen.
7. Cryotome.
8. Positive charged slides.
9. Fixation buffer: 4% formaldehyde and 5mM $MgCl_2$ in PBS.
10. Prehybridisation buffer: 40% deionized formamide, 2 x SSC (300 mM sodium chloride and 30 mM trisodium citrate (adjusted to pH 7.0 with HCl)).
11. Hybridisation buffer: 32% deionized formamide, 2 x SSC, 0.2 mg/ml BSA (bovine serum albumin), 10 mg/ml dextran sulphate, 1 mg/ml fish sperm .
12. sense AON.
13. Wash buffer: 40% deionized formamide, 2 x SSC.
13. Mounting medium.
14. Fluorescence microscope.

3. Methods

3.1 Phage experiment

Binding peptides can be identified through a phage display experiment for a specific protein, a specific cell type or in vivo after systemic injection. The method described below is for an in vivo experiment done with the Ph.D.-7 kit of New England BioLabs. Similar experiments can be done with other libraries.

3.1.1 In vivo phage panning

1. Inoculate 10 ml LB medium with ER2738 (plating culture for titration), and incubate 20 ml LB medium in a 250 ml Erlenmeyer flask (*see* **Note 2**) with ER2738. Incubate both cultures at 37°C with vigorous shaking.

2. Inject a mouse with the phage bank ($2 \cdot 10^{11}$pfu in 200 µl saline) through intravenous injection.

3. Let the phages circulate for 1 hour.

4. Perfuse mouse with PBS to remove phages that have not bound (*see* **Note 3**).

5. Remove the desired organs and wash them 3 times with TBS.

6. Homogenize the tissue in 2ml TBS with MagNa lyser green beads in the MagNa lyser according to the manufacturer's protocol.

The following steps are taken from the panning procedure described in the Ph.D.-7 manual *(www.neb.com/nebecomm/ManualFiles/manualE8100.pdf, step 11 onwards)*, and modified towards this in vivo experiment. When using a different library continue at a similar step in the protocol (i.e. the step after isolating the phages from targeted molecules, cells or tissues), and modify where necessary.

7. Titrate a small amount (~1 µl) of the eluate (see section 3.1.2 for phage tittering protocol). Plaques can be sequenced if desired. Or the whole eluate can be sequenced by mass sequencing.

8. Amplify the rest of the eluate by adding it to the 20 ml ER2738 culture from Step 4 (should be early-log at this point) and incubating with vigorous shaking for 4.5 hours at 37°C. (*see* **Note 4**)

9. Transfer the culture to a centrifuge tube and spin for 10 minutes at 12,000 g at 4°C. Transfer the supernatant to a fresh tube and re-spin (discard the pellet).

10. Transfer the upper 80% of the supernatant to a fresh tube and add to it 1/6 volume of 20% PEG/2.5 M NaCl. Allow the phage to precipitate at 4°C for at least 2 hours, preferably overnight.

Next day

11. Spin the PEG precipitation at 12,000 g for 15 minutes at 4°C. Decant and discard the supernatant, re-spin the tube briefly, and remove residual supernatant with a pipette. The phage pellet should be a white finger print sized smear on the side of the tube.

12. Suspend the pellet in 1 ml of TBS. Transfer the suspension to a microcentrifuge tube and spin at maximum (14,000 rpm) for 5 minutes at 4°C to pellet residual cells.

13. Transfer the supernatant to a fresh microcentrifuge tube and reprecipitate by adding 1/6 volume of 20% PEG/2.5 M NaCl. Incubate on ice for 15–60 minutes. Microcentrifuge at 14,000 rpm for 10 minutes at 4°C, discard the supernatant, re-spin briefly, and remove residual supernatant with a micropipet.

14. Suspend the pellet in 200 µl of TBS. Microcentrifuge for 1 minute to pellet any remaining insoluble material. Transfer the supernatant to a fresh tube. This is the amplified eluate, which can be used for another round of panning. The eluate can be stored for up to 3 weeks at 4°C. For long-term storage, add an equal volume of sterile glycerol and store at –20° C.

15. Titrate the amplified eluate on LB/IPTG/Xgal plates (see section 3.1.2 for phage tittering protocol), to calculate the amount of phages present and how much should be injected in the next round, to reach $2 \cdot 10^{11}$pfu.

With traditional sequencing methods, 3-5 rounds of panning should be performed for the best results (*see* **Note 5**).

3.1.2 Plaque Amplification and isolation for sequencing

Here we describe amplification and isolation of single colonies from titration plates. With massive parallel sequencing, the whole eluate can be sequenced; in this case the method for amplification and isolation will be different.

1. Dilute an overnight culture of ER2738 1:100 in LB. Dispense 1 ml of diluted culture into culture tubes, one for each clone to be characterized (*see* **Note 6**).

2. Use a sterile wooden stick or pipette tip to stab a blue plaque from a tittering plate and transfer to a tube containing the diluted culture (*see* **Note 7**). Pick well-separated plaques, this will ensure that each plaque contains a single DNA sequence.

3. Incubate the tubes at 37°C with shaking for 4.5–5 hours (no longer).

4. Transfer the cultures to microcentrifuge tubes, and microfuge at 14,000 rpm for 30 seconds.

5. Transfer 500 µl of the phage-containing supernatant to a fresh microfuge tube.

6. Add 200 µl of 20% PEG/2.5 M NaCl. Invert several times to mix, and let stand for 10–20 minutes at room temperature.

7. Microfuge at 14,000 rpm for 10 minutes at 4°C and discard the supernatant. Phage pellet may not be visible.

8. Re-spin briefly. Carefully pipet away and discard any remaining supernatant.

9. Suspend the pellet thoroughly in 100 µl of Iodide Buffer by vigorously tapping the tube. Add 250 µl of ethanol and incubate 10–20 minutes at room temperature. Short incubation at room temperature will preferentially precipitate single-stranded phage DNA, leaving most phage protein in solution.

10. Spin in a microfuge at 14,000 rpm for 10 minutes at 4°C, and discard the supernatant. Wash the pellet with 0.5 ml of 70% ethanol (stored at –20°C), re-spin, discard the supernatant, and briefly dry the pellet under vacuum.

11. Suspend the pellet in 30 µl of TE buffer. (*see* **Note 8**)

12. Measure concentration, for instance on a nanodrop.

13. Sequence with an appropriate primer. In this case the primer sequence is: CCCTCATAGTTAGCGTAACG.

3.1.3 Phage titration

1. Inoculate 5–10 ml of LB with ER2738 from a plate and incubate with shaking 4–8 hrs (mid-log phase, OD600 ~ 0.5). (*see* **Note 9**)

2. While cells are growing, melt Top Agar in a microwave and dispense 3 ml into sterile culture tubes, one per expected phage dilution. Maintain tubes at 45°C.

3. Pre-warm, for at least one hour, one LB/IPTG/Xgal plate per expected dilution at 37°C until ready for use.

4. Prepare 10 to 10^3-fold serial dilutions of phage in LB; 1 ml final volumes are convenient. Suggested dilution ranges: for amplified phage culture supernatants, 10^8–10^{11}; for unamplified panning eluates, 10^1–10^4. (*see* **Note 10**)

5. When the culture in Step 1 reaches mid-log phase, dispense 200 µl into microfuge tubes, one for each phage dilution.

6. To carry out infection, add 10 µl of each phage dilution to each tube, vortex quickly, and incubate at room temperature for 1–5 minutes.

7. Transfer the infected cells one infection at a time to culture tubes containing 45°C Top Agar. Vortex briefly and IMMEDIATELY and pour culture onto a prewarmed LB/IPTG/Xgal plate. Gently tilt and rotate plate to spread top agar evenly.

8. Allow the plates to cool for 5 minutes, invert, and incubate overnight at 37°C.

9. Count plaques on plates that have approximately 100 plaques. Multiply each number by the dilution factor for that plate to get phage titer in plaque forming units (pfu) per 10 µl.

3.2 Identification of most promising peptide

3.2.1. In silico analysis

1. Perform a BLAST analysis (Basic local alignment search tool) to see whether the sequenced peptides are homologous to known proteins

(*http://blast.ncbi.nlm.nih.gov*). Peptides could, for instance, have similarities with proteins that are known to be taken up by the target cells.

2. Check the available information about the peptides (Pepbank (*http://pepbank.mgh.harvard.edu/*); SAROTUP (*http://immunet.cn/sarotup/*); Pubmed (*http://www.ncbi.nlm.nih.gov/pubmed*); Google (*http://www.google.com*)).

3.2.2. In vitro analysis

To verify whether the selected peptides really bind to the targeted cell type (*see* **Note 11**), fluorescently labeled peptides can be tested in vitro in the desired cell type (*see* **Note 12**). In this example we will test binding to muscle cells.

1. Coat glass slides in a six well plate with collagen (diluted 1 in 10 in distilled water) for 30 minutes.

2. Wash with TBS and let dry for 1 hour.

3. Seed human primary myoblasts in 3 ml proliferation medium.

4. When cells are confluent, remove the proliferation medium, wash them twice with PBS and add 3 ml differentiation medium.

5. When cells have fused with each other, to form myotubes, remove medium and wash twice with PBS.

6. Add 1 ml medium without serum (to reduce background staining) plus 2.5 nmol of fluorescently labeled peptide.

7. After 3 hours add 2 ml of differentiation medium with 2% serum.

8. After 48 hours remove the medium and wash three times with PBS.

9. Fix the cells with -20ºC methanol for 5 minutes and let them dry for 30 minutes.

10. Mount the cells in mounting medium.

11. Assess cell binding with a fluorescence microscope (*see* **Note 13**).

3.3 Intramuscular test of fluorescently labeled peptides

To verify whether the selected peptides efficiently bind the targeted cell type and not another cell type in the same tissue they can be tested by local injection. In this example we test intramuscular injection.

1. Inject 2.5 nmol fluorescently labeled peptide in 40 µl saline in a gastrocnemius muscle of a mouse.
2. Sacrifice the mouse at the desired time point (for instance after 1 hour).
3. Isolate the gastrocnemius and snap freeze the muscle in liquid nitrogen cooled isopentane (*see* **Note 14**).
4. Cut 8 µm sections of the muscle with a cryotome (perpendicular to the muscle fibers), and collect them on a positively charged glass slide (the charged slides will automatically attract the sections through electrostatic force).
5. Fix the sections with acetone of -20ºC and mount in mounting medium.
6. Analyze for cell binding with fluorescence microscope (*see* **Note 13**).

3.4 Intramuscular test of peptides conjugated to an AON

To test whether the selected peptides still efficiently bind their target when they are conjugated to an AON, the peptide-AON conjugates can be injected intramuscular and localized with a so-called 'sense-staining' of the AON.

1. Inject 2.5 nmol of the peptide-AON in 40 µl saline into the gastrocnemius of a mouse.
2. Sacrifice the mouse at the desired time point (for instance after 1 hour).
3. Isolate the gastrocnemius and snap freeze the muscle in liquid nitrogen cooled isopentane (*see* **Note 14**).
4. Cut the muscle in 8 µm cross sections with a cryotome, perpendicular to the muscle fibers, and collect the sections on a positively charged glass slide (the charged slides will automatically attract the sections).
5. Fix sections for 30 minutes in fixation buffer.
6. Wash slides in 1 x PBS/5mM $MgCl_2$ for 30 minutes.

7. Prehybridize slides for 30 minutes in 125 µl prehibridization buffer.

8. Remove prehybridization buffer and incubate overnight in 250 µl hybridization mix in a water bath at 37ºC (keep dark and humid).

9. Wash the slides in wash buffer for 2 x 20 minutes in a water bath at 37ºC (keep dark).

10. Wash the slides in 1 x SSC for 20 minutes at room temperature (keep dark).

11. Quickly wash in 1 x PBS + 1:5000 DAPI to stain nuclei.

12. Mount in mounting medium.

13. Determine the fate of the conjugate with a fluorescence microscope (an example picture is seen in figure 2).

Exon skip levels can also be determined in these muscles. Results will not be very informative about the targeting efficiency, since the injection is local, but it can be used to determine whether the AON it is still capable of inducing exon skipping, after being conjugated to an AON (*see* **Note 15**).

4. Notes

1. For optimal results, the AONs should be bound to the same side of the peptide as the phage, for the phages used here this is the N-terminal site.

2. Do not use a 50 ml conical tube, bacteria should be grown in an erlenmyer flask for optimal growth.

3. To perfuse the mouse, it is anesthetized and immobilized, lying on its back. The chest cavity is cut open, exposing the heart and a butterflyneedle attached to a PBS supply is inserted in the lower tip of the heart, into the left ventricle. The needle should be immobilized. Then make a cut in the right atrium and, when blood comes out, immediately allow PBS to flow in (either by a pump or by gravity).

4. The remaining eluate can be stored overnight at 4°C at this point, if preferred, and amplified the next day. In this case, inoculate 10 ml of LB+Tet with ER2738 and incubate with shaking overnight at 37°C. The next day, dilute the overnight culture 1:100 in 20 ml of LB in a 250-ml Erlenmeyer flask (see **Note 2**) and add the unamplified eluate. Incubate with vigorous shaking for 4.5 hours at 37°C and proceed to Step 9.

5. More rounds of panning will increase the presence of specifically binding peptides, however it will also increase the presence of peptides which are easy to amplify, because of their sequence.

6. Because of the many potential binding sites, it will be difficult to find a consensus binding sequence. This problem can be solved by more elaborate sequencing (e.g. massive parallel sequencing) or elaborate testing of candidate peptides.

6. Plates should be <1–3 days old, stored at 4°C and have <100 plaques.

7. The template can be suspended in H_2O instead of TE if desired, but this is not recommended for long-term storage. In TE buffer, the phage DNA should be stable indefinitely at –20°C.

8. The number of plaques will increase linearly with added phages only when the multiplicity of infection (MOI) is much less than 1 (i.e., cells are in considerable excess). For this reason, it is recommended that phage stocks be titrated by diluting prior to infection, rather than by diluting cells infected at a high MOI. Plating at low MOI will also ensure that each plaque contains only one DNA sequence.

9. Use aerosol resistant pipette tips to prevent cross-contamination, and use a fresh pipette tip for each dilution.

10. Keep in mind that the peptide might have different characteristics when it is eventually bound to an AON.

11. You can also test for **not** binding to an undesired cell type.

12. To be able to compare the results for different peptides, pictures should be taken with the same exposure time and intensity.

13. Tissues should be snap frozen to be able to cut good quality slides. This can be achieved with isopentane, which reduces surface charge of the tissue, preventing the formation of gas bubbles around the tissue (as is the case when tissues are directly immersed in liquid nitrogen). A tube of isopentane should be placed in liquid nitrogen, and when the isopentane stops fuming and becomes a bit viscous, it is cold enough for snap freezing a tissue. The time needed for snap freezing depends on the size of the tissue, a gastrocnemius will be snap frozen after 20 seconds. The isopentane will freeze solid after a while. Therefore, if a lot of tissues need to be snap frozen, it will be more practical to use two tubes.

14. Checking whether an AON can still induce skipping after conjugation to a peptide can also be done in vitro. When the peptide-AON is transfected into cells, is should result in skipping levels comparable to levels in cells transfected with AON.

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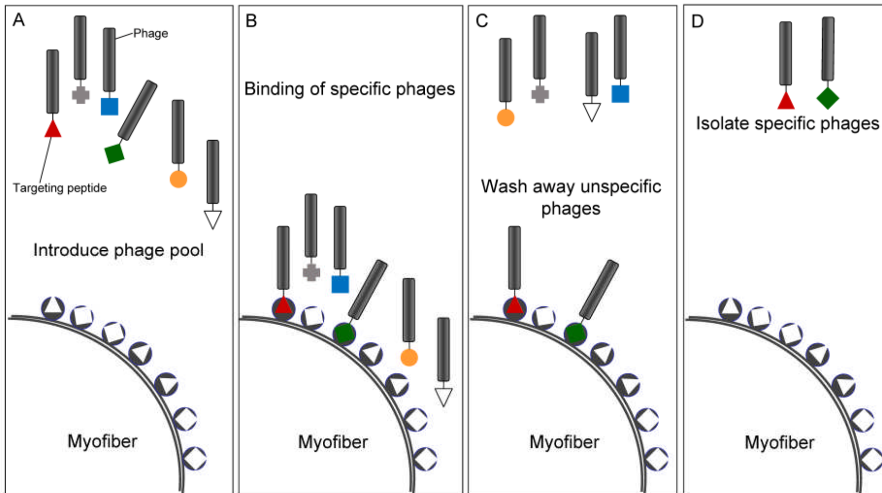
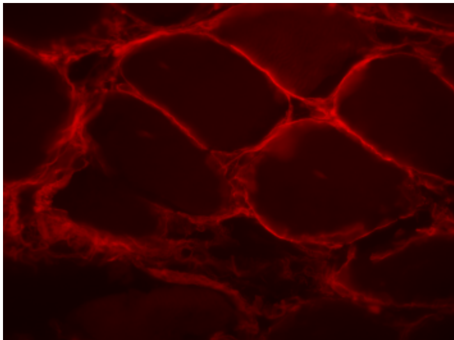
Figures



1. Schematic overview of phage display on cells. A. A pool of phages expressing random peptides on the surface is added. B. Some peptides will be able to bind, others will not. C. Unbound phages are washed away. D. Binding phages are isolated and can be amplified for a second selection round.



2. Example of a 'sense staining' of a section injected with a peptide-AON