REIZENDE DNA LABS

Student manual

Level: **expert**

Healthy or ill: Just a single wrong fold

Universiteit
Leiden
Wiskunde en Natuurwetenschappen

Amgen Biotech Experience
Scientific Discovery for the Classroom

Developed by the University of Leiden in cooperation with the Centre for Medical Systems Biology

With questions and/or comments please contact the DNAlabs on the Road (leiden@dnalabs.nl).

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DNA-labs on the road

DNA-labs on the road forms a link between biology and chemistry at school as well as the latest developments in genomics, by letting students work with advanced techniques and current developments and subjects from modern science.

The DNA-labs are designed by Dutch universities and the Genomics Centres of Excellence. Part of the project includes the website www.allesoverdna.nl, a source of information on genomics, especially written for high school students.

There are six different DNA-labs, each containing different aspects of modern DNA research. All DNA-labs show that knowledge of genes and molecules in cells play a great role in everyday topics: nutrition, health and the environment. In addition, the experiments clearly show that scientific progress often raises social questions.

Method of teaching

The module consists of four class hours. The preparatory lesson (lesson 1) and the concluding lesson (lesson 4) will be given by the school teacher. The practical course (lesson 2 and 3) will be completely given by students of the university. Both the preparatory and the concluding lesson are important for proper implementation and understanding of the practical work.

Lesson 1: Preparatory lesson

Let's refresh: Cells, DNA and proteins

The human body is constructed out of approximately onehundredthousand billion cells. You can see this enormous amount of cells as tiny individual factories in which all processes take place that make life possible. DNA and proteins play an important role in these processes. The DNA specifies how proteins will look like and which function they will have. A mistake in a gene can lead to a change in a protein with possible catastrophic consequences like diseases. Proteins are the end products that arise when genes are transcribed into RNA in the cell. The RNA will become translated in proteins. (Figure 1)

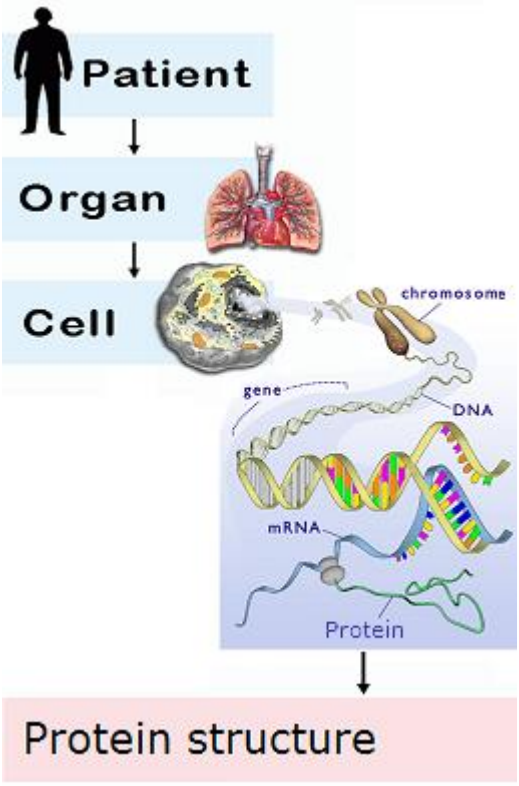

Figure 1: At the top is a patient who noticed from symptoms that certain organs in his body do not function properly. Causes here fore are retraceable in the cells from which the organ is made of. Cells can communicate with each other via proteins and cooperatively form functioning organs. The DNA specifies which proteins are made. Only proteins with the correct structure can function in the way it was meant to perform.

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Diseases and proteins

Because proteins are vital to all body processes, it is essential that all proteins fulfill the correct function. These functions are largely determined by their folding causing a specific structure. Subsequently, the structure of a protein is determined by the sequence in which different amino acids are coupled.
When a mistake in the DNA arises, the protein cannot function correctly any more and causes a certain body process to dysfunction. This can give rise to several diseases like cystic fibrosis, Alzheimer's disease and cancer. These diseases are caused by a protein having a different structure than it should have. Proteins with wrong structures cannot fulfill the right function, which causes difficulties in vital body processes.

Cystic fibrosis: an instable protein

Paul is 16 years old. He was born with a serious genetic disorder, which is not yet curable. Paul always has problems with his lungs, which makes sports almost impossible. Besides that, Paul has growth retardation and visiting the physiotherapist is a daily ritual to him. Paul's life expectancy is around 30 years, just like his approximately 1200 Dutch fellow sufferers with cystic fibrosis (CF).
A special protein related to this disease, playing an important role in mucus production in the lungs, is instable. The unstable protein cannot fulfill its role and is broken down by the body. This causes the mucus in lungs to grow out of proportion. It is getting so tough, the body cannot cope with it any more. Mucus build-up and infections are the bad outcome (Figure 2).

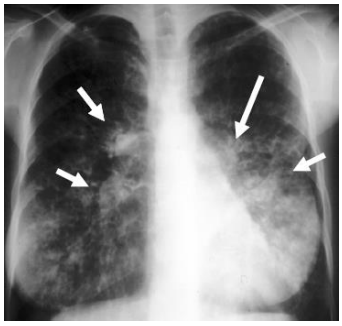

Figure 2: An X-ray photograph of a patient with cystic fibrosis. The mucus is not visible on a photo of a healthy person, however, the tough mucus of a CF-patient is visible as white spots in the picture (see arrows).

Alzheimer's disease: a too stable protein

Mr. Den Oudsten is 72 years old. Since several years he noticed to regularly forget what he is doing and he doesn't recognize his children as good as before. As well as 250.000 other Dutch people, Mr. Den Oudsten suffers from Alzheimer's disease. The disease alters the brain structure so that nerve cells (neurons) do not perform their tasks anymore. The changes in the brain occur by a specific protein being too stable. The body cannot break down this protein anymore. The result is a protein build-up in the brain of Alzheimer patients (Figure 3). Brain cells are damaged and finally killed by these protein aggregates.

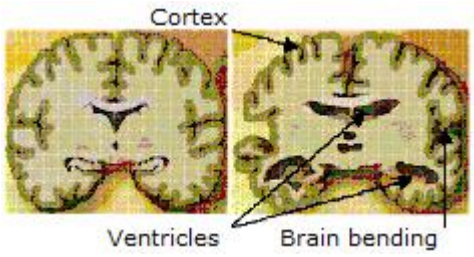

Figure 3: On the left an intersection of a healthy brain; on the right an intersection of the brain of an Alzheimer's disease patient. The exterior parts of the brain (cortex) are diminished; there is more space between the brain folds and the ventricles are enlarged.

Now make question 1.1 to 1.6

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Stable and instable proteins

A protein that cannot properly perform is function can have unpleasant consequences, as stated in the case of cystic fibrosis and Alzheimer's disease. The reason why a protein can loose its function is not always the same. You have read that with cystic fibrosis patients an important protein in the lungs is unstable. This protein causes mucus breakdown to normal levels in healthy people but with cystic fibrosis patients, the protein is broken down too fast and mucus build-up occurs. In Alzheimer's disease the opposite is happening. A too stable protein cannot be broken down anymore and protein build-up in the brain causes the problems.

Structural folding of proteins

Proteins have a structure or a certain folding. The structure of a protein is determined by the amino acid sequence and their interaction. Depending on the order in which the amino acids are linked to each other in the protein, amino acids can or can't come close to each other. Amino acids that are attracted to each other will go near each other, while amino acids that are repulsive to each other will try to get out of their way. Several amino acids are acetic others are basic. Some are hydrophilic and will orient themselves towards the outer parts of the protein, while hydrophobic amino acids want to be in the core of the protein.
So the protein takes shape depending on the properties of the different amino acids. This shape is build up on four different levels in which you can consider protein structure. Figure 4 gives a summary of these four levels of protein structure.

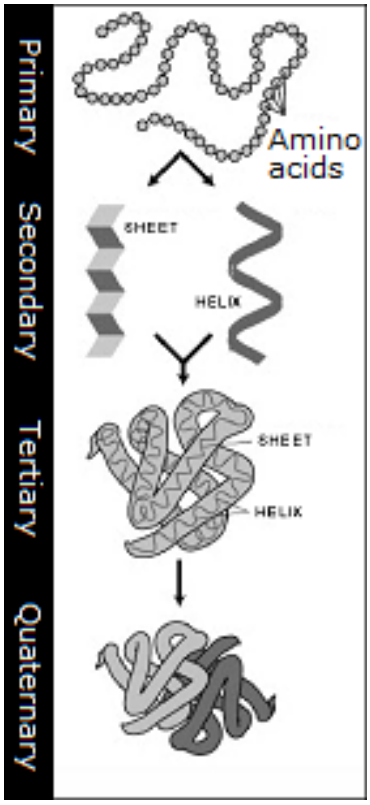

Figure 4: the four different levels of protein folding

The primary (first) level is the enumeration of the amino acid chain from which the protein is build.

The secondary (second) level arises when the chain of amino acids is attracted to each other due to hydrogen bonds. The two most common configurations (sheet and helix) are depicted on the left.

The tertiary (third) level describes how the secondary structures attract or repulse each other. This gives the protein the form of a tangle.

The quaternary (fourth) level is constructed out of more then one chain of amino acids. The way in which different chains are coupled determines the final structure of complex proteins and thereby also their function.

Now make questions 1.7 to 1.9

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Three-dimensional representation of a protein

Protein structures can be depicted in several ways (figure 5). The different ways of depicting are often used interchangeable in literature. Depending on their relevance in the context a certain model is suitable for representing what the three-dimensional structure and folding of a protein is.

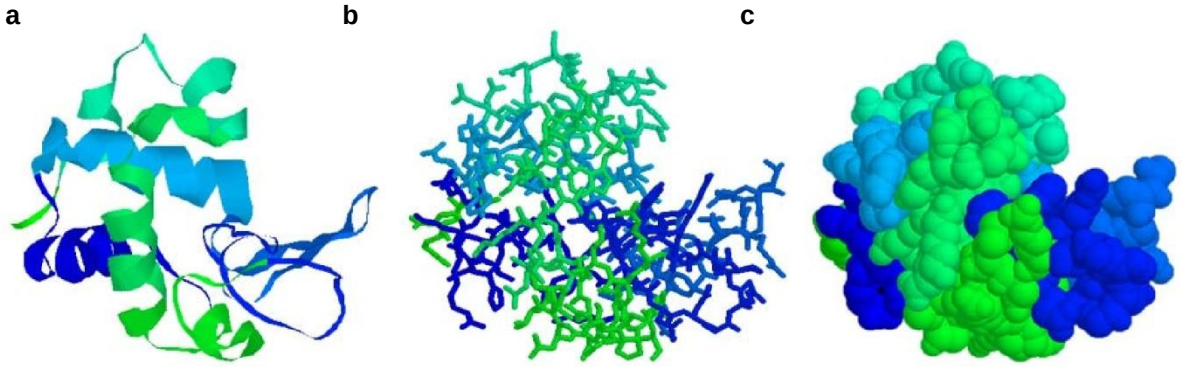

Figure 5: Protein folding can be depicted in several ways. In this figure three different models are shown of the lysozyme protein. The lysozyme has a role in the demolition of proteins in bacteria. In the ribbon model (a) you can distinguish nicely how the different tertiary structures look like, while in the stick model (b) the interconnecting bonds between amino acids are shown. The space-filling model (c) gives an image of the volume a protein occupies and thereby represents the most native form of a protein.

Protein functions

Proteins come in different sizes and shapes. Every process in the human body is taken care of by one or more proteins that facilitate that process. For instance the amylase protein is a protein that is involved in the breakdown of starch in saliva. It thereby fulfills an important role in the start of the digestion.

The folding of a protein determines what function a protein has. You can imagine it as a key and a lock. The protein is the lock and the substrate is the key. Substrates are very specific for the protein with whom they interact. If a protein structure changes a little it might cause it to lose its functionality and it might not be able to fulfill its task anymore. This is shown in figure 6.

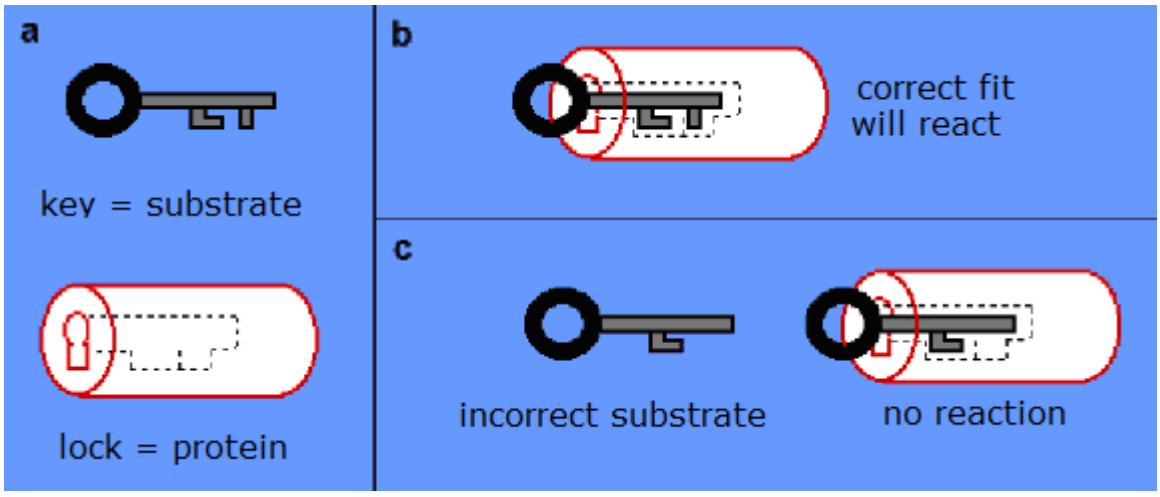

Figure 6: The shape of the protein determines its function. The principle is comparable with a lock and a fitting key (a). When the right substrate comes to a specific protein, the protein will react (b). When a faulty substrate comes, there will be no reaction from the protein (c).

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A single wrong fold

As you might know by now, proteins play a very important role in the lungs and in the brain. This doesn't only count for these two organs but for all the organs in the human body. All organs are built up from specialized cells. Without proteins there will be no cell in the body that can perform its specific task. Because proteins are so important for all body processes, it is of the utmost importance that all proteins fulfill their specific tasks.
The function of a protein is predominantly determined by its shape and folding: the protein structure. This is shown in figure 4. The structure of a protein can change when there are abnormalities in the DNA. A protein can change so much that is might get instable and cannot fulfill its function anymore, like in cystic fibrosis. It might also occur that a protein will get too stable and cannot be broken down anymore, like with Alzheimer's disease. Multiple diseases with totally different characteristics can be caused by the same, a incorrectly folded protein.

Now make question 1.10

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Research

In order to understand how diseases originate from incorrect protein folding it is necessary to do a lot of scientific research. To know what is going wrong in these diseases is giving insights in how medicines should invade and might give chances to new treatments. In protein folding diseases you first need to know how to distinguish correctly folded proteins from the incorrect ones. Cells from healthy people can then be compared with patients. When you know that certain proteins differ in the healthy and diseased people, it is interesting to investigate how these proteins are different. This brings up the need of the protein structure. It is of great importance to check whether or not a difference in protein structure leads to disruption in protein function, causing the disease.
For a good view on what exactly is going wrong in protein folding diseases like cystic fibrosis and Alzheimer's disease scientists can conduct the following experiments.

Read the text carefully and answer the corresponding questions.
During the performance of the practical course there will be supplementary information via a presentation.

The differences between proteins of healthy and diseased people

The differences between healthy and diseased people are retraceable in their cells, in which the DNA and proteins are located. In order to check for differences, proteins in the cells of healthy and diseased people can be compared with the help of scientific research.

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Immunofluorescence
Lung cells of healthy humans have a good working CFTR protein. By making this protein visible in some sort of way, you can see that it is actually in the lung cells of healthy people. However, in lung cells of cystic fibrosis patients, the CFTR protein cannot work properly. Because the CFTR protein is folded different in cystic fibrosis patients in comparison to healthy humans, it becomes unstable and will not be visible like the correctly folded CFTR.
It is possible to compare specific proteins in cells of healthy and diseased people with immunofluorescence. In this technique one uses antibodies. This is a protein that specifically binds to another distinct protein. A fluorescent probe is attached to the antibody in order to make it possible to see it with a special microscope (fluorescence microscope). You can then see where the antibody has bound and so where the protein is to which the antibody binds. Depending on the fluorescent molecule, its color can differ (figure 7).

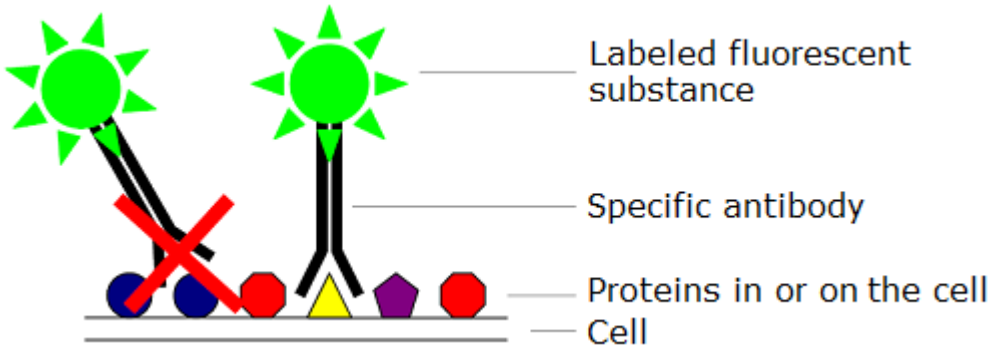

Figure 7: The principles of immunofluorescence staining. An antibody that is attached to a specific protein contains a linked fluorescent molecule that makes visible where the specific protein is located. The antibody will not bind to other proteins than the protein of interest.

Because there is no commercial available antibody that specifically binds to the CFTR protein, you shall be working with an antibody specific for the protein tubulin in this experiment. Tubulin is a protein that plays a major role in rigidity of some cells and is part of the skeleton of cells – the cytoskeleton. A part of the cells that come in contact with the antibody do not have tubulin, while the other part does have the protein. The antibody is coupled to a green fluorescent molecule called fluorescein isothiocyanate.

After the experiment, a green color can be observed where the antibody has bound and thus where tubulin is present (figure 8). In cells that do not contain tubulin, no green color will be detected. In this way you can discriminate which cells have and which do not have the tubulin protein.

When it is known that a good functioning protein is present in lung cells of healthy people, but the same protein is not functioning in diseased people, the next step is to discover what the difference is between the protein of the healthy and diseased people. However, a problem is how to get these proteins. You cannot just take out a lung or brain out of a patient.

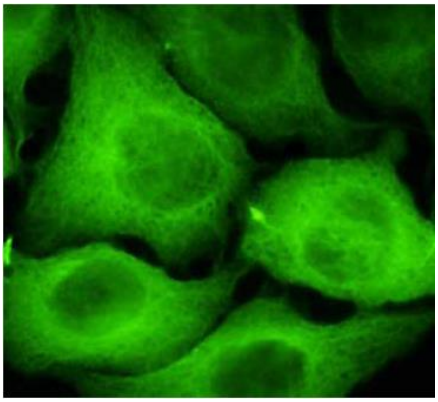

Figure 8: Cells containing tubulin that have been made visible by binding to a green fluorescent labeled antibody.

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X-ray diffraction
When the relevant proteins from healthy and diseased humans are finally available, there are different ways to determine the differences between them. One of the ways to do it is via a technique called X-ray diffraction. With the help of this technique, the three-dimensional molecular structure of the protein can be determined. The protein folding can be made visible in this manner.

In X-ray diffraction the protein is crystallized. In a crystal all molecules are aligned properly, like in frozen water (ice crystals) of in salt (NaCl, figure 9). Subsequently, the crystals are irradiated with X-radiation. The molecules in the crystal bend this radiation and the deflected rays are collected. You can use the pattern information from the collected rays to calculate where the different atoms are with respect to each other in the protein. Eventually it is possible to calculate the three-dimensional structure of the protein (figure 10).

When you determine the three-dimensional structure of both the correctly and incorrectly folded protein you can observe differences in protein folding by comparing the two images. One can make crystals out of protein solutions. The crystallization, or the process in which the molecules or atoms align is generally in two phases. First a growth point or nucleus needs to be formed (nucleation). When the nucleus is formed more and more particles can align to the nucleus. This causes the crystal to grow (growth). Once the crystal is big enough it can be analyzed with the X-radiation to determine the protein structure.

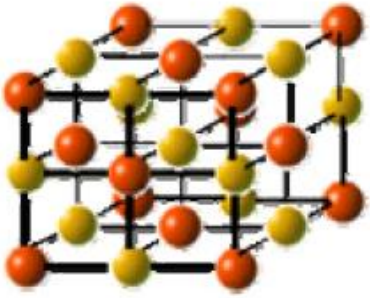

Figure 9: The crystal structure of salt (NaCl).

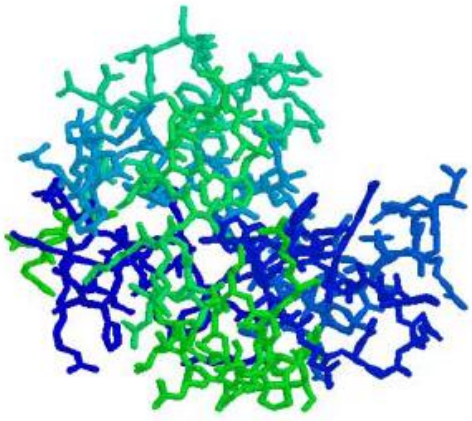

Figure 10: 3D-structure (stick model) of the lysozyme protein.

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The consequences of a structural difference

The structure of a protein is closely related to its function. When a protein is folded different than it should have been, it has consequences for the functionality of the protein. In cystic fibrosis the folding of the protein is so that the protein cannot function any more: there is no transporting of chloride out of the cell anymore.
However, it is not clear if a change in folding always causes a protein to become inactive and if this really causes the disease. For this reason it is worthwhile to check this, because it could happen that a protein just occurs in different forms or that the protein under investigation is not specific at all for the investigated disease.

Now make question 1.11 and 1.12

Practical course

In the practical course given after this lesson you will be the researcher and do the examinations stated earlier yourselves. You will work with immunofluorescence and X-ray diffraction in order to experience how these experiments work.

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In depth

Cystic fibrosis: the wrongly folded protein

People who suffer from cystic fibrosis have a certain protein that is instable. The human body is breaking down this protein too fast. The full name of the protein is the cystic fibrosis transmembrane conductance protein, in short CFTR. This protein is located in the membrane of lung cells that produce mucus. Its function is to regulate the composition of the mucus. In particular, the protein regulates the amount of chloride in the mucus by exporting chloride from the cell. When chloride leaves the cell it also causes the efflux of water, making the mucus more fluid so it can flow trough the lungs in order to help removing waste products and thereby cleaning the lungs.

Patients with cystic fibrosis have a wrongly folded CFTR protein. The cause of the wrong fold is retraceable in the primary structure of the protein, in the amino acid sequence from which the protein is built. The normal CFTR protein consists of 1480 amino acids with on position 508 the amino acid phenylalanine. In 70% of the cystic fibrosis patients the phenylalanine amino acid isn't present at this position. A schematic representation of the CFTR gene and the location of the mutation are shown in figure 11. The mutation in the CFTR gene on position 508 has such an impact that is changes the secondary and tertiary folding of the protein, causing it to dysfunction. This shows that a tiny change in the build-up of the protein can have a major influence on its functionality.

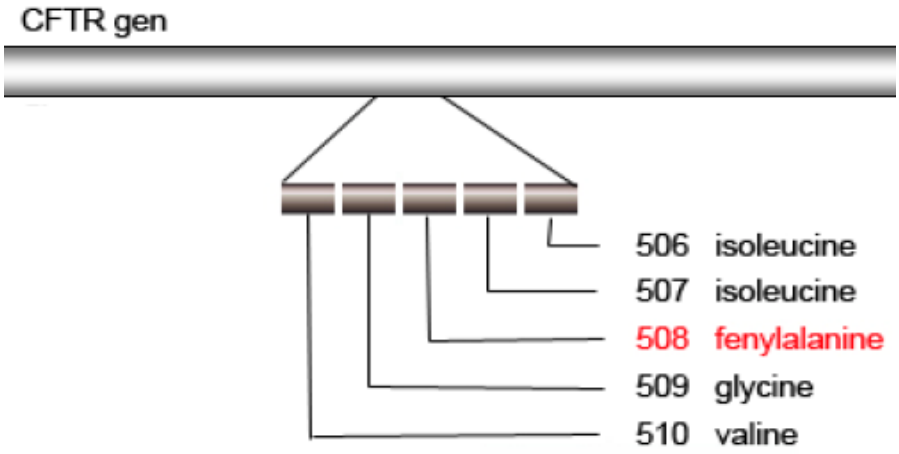

Figure 11: Schematic representation of the CFTR gene and the location of the most common mutation in cystic fibrosis: the phenylalanine on position 508 is not present.

Due to the malfunctioning of the CFTR protein in cystic fibrosis patients, the mucus in the lungs gets too thick. This makes the airways tighter and causing breathing complications. The thick mucus also serves as a good host for bacteria to live in. Serious bacterial infections can take place and this is the foremost reason of death in cystic fibrosis.

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Alzheimer's disease: the wronly folded protein
Patients with Alzheimer's disease have a protein in their brain that causes problems due to the fact that it is too stable and cannot be broken down by the body anymore. The protein causes build-ups that are clearly visible when comparing the brain of a healthy older person with a person with Alzheimer's disease.
In Alzheimer's disease infected people you can find so called plaques and tangles (figures 3 and 12).

Plaques are build-ups from a certain protein between the brain cells. This protein build-up is called amyloid. Older people and in particular people with Alzheimer's disease do not break down this protein efficient anymore. This causes the formation of a kind of protein aggregate between the brain cells that probably cause interference between communications in the brain. In due time the nerve cells themselves are also affected. This is for instance visible due to the presence of tangles. A tangle is a pile of threadlike protein in a nerve cell that make normal nerve cell functioning impossible.

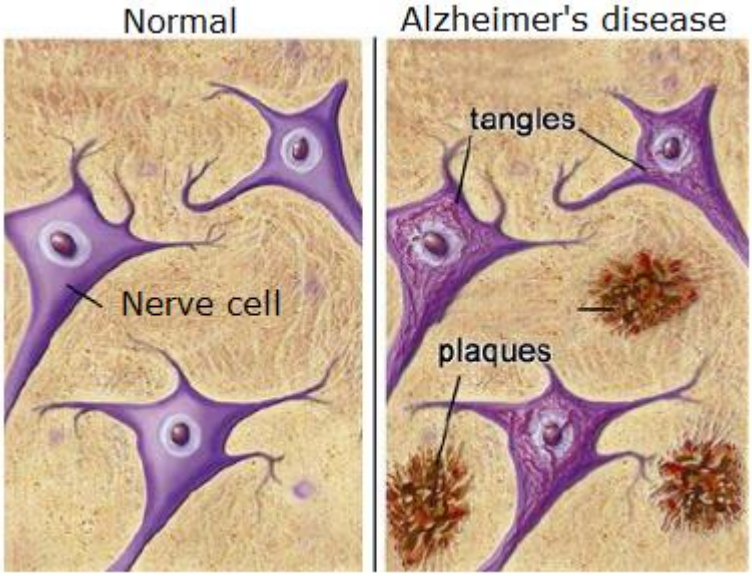
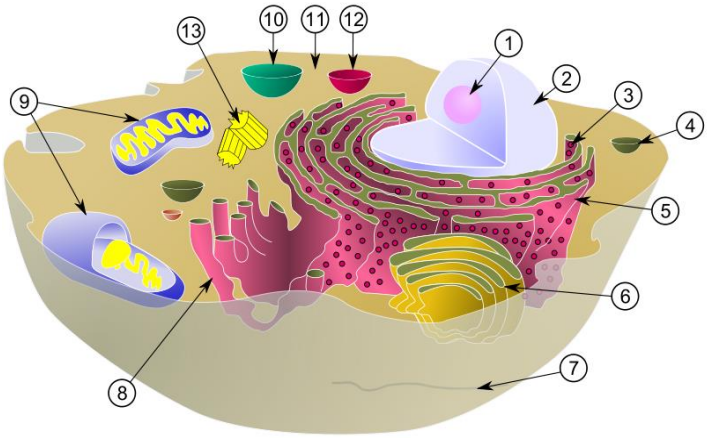


Figure 12: The difference between nerve cells in healthy elderly people (left) and those of patiens with Alzheimer's disease (right). In Alzheimer's disease plaques arise due to the build-up of the amyloid protein. Inside the cells tangles are present, consisting of insoluble protein threads.

Now make questions 2.1 to 2.6

Questions lesson 1

Question 1.1
State the names of the different cellular organelles in the figure below.



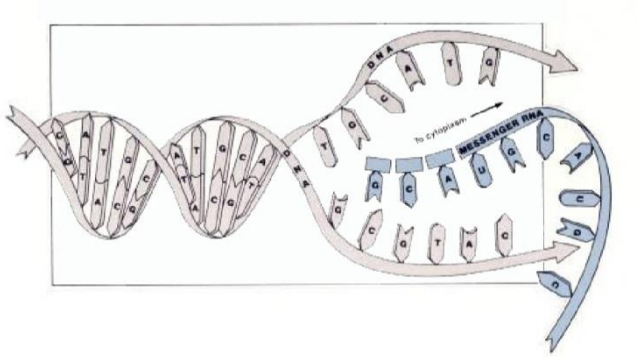
Question 1.2
What is DNA and what are genes?

Question 1.3
What is the important role of DNA and proteins in cellular processes?

Question 1.4
What are amino acids and how many different amino acids exist?

Question 1.5
Name the similarities and differences between cystic fibrosis and Alzheimer's disease based on the given information.

Queston 1.6
Briefly describe how a cell makes proteins from the information that is located in the genes on the DNA. Make use of the figure below.



Question 1.7
Why do hydrophilic amino acids tend to go towards the outer part of the protein?

Question 1.8
When you change one amino acid in the chain of a protein, on which level of protein folding you could indicate this best.

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Question 1.9
Do you think that changing one amino acid could also influence the rest of the protein folding levels? If so, how?

Question 1.10
How would you do scientific research on protein folding diseases?

Question 1.11
How do you think that proteins of healthy and diseased people can differ?

Question 1.12
How do you think that you can do research on protein from cells in lungs and brains without removing them from a patient?

Question 2.1
Why is it useful to depict protein folding in different ways?

Question 2.2
Name three body processes in which proteins are involved and state the protein names if you know them.

Question 2.3
Imagine a non-fitting substrate reaches a protein. Will the protein react on the substrate and if so, how?

Question 2.4
Cells from patients with cystic fibrosis cannot excrete chloride anymore. Why does this lead to ticker mucus?

Question 2.5
How can the lack of only one amino acid in the CFTR protein cause such harsh consequences?

Question 2.6
How do plaques in the brain of an Alzheimer's disease patient cause mental health problems like confusion, one of the signs of this disease?

Lesson 2 and 3: Practical course

Experiment 1a: Immunofluorescence staining

The differences between healthy and diseased cells

Goal of the experiment

The cells of healthy and diseased people differ. These differences can occur in several ways, for instance in the shape or the size of the cells or in the cooperation between cells. In cystic fibrosis the difference lies in the stability of the CFTR protein. At first sight the cells look the same, but the difference between the stable correctly working and instable faulty working protein can be made visible with fluorescent staining. Apart from CF-cells, human cancer cells can also serve as an example for proteins that cannot be made anymore. CF-cells are not (commercially) available but fixated tumor cells are. This is why this experiment will be done with fixated, dead, HeLa cells.

The green fluorescent substance FITC is coupled to an antibody that specifically binds to the tubulin protein. When this protein is present in a cell, you will see a green color appear under a fluorescence microscope after staining. In cells that don't contain / have fewer tubulin, the green color will be absent or strongly reduced.

Now first make question 1.1

Requirements

- A cover slip with fixated tumor cells (HeLa)
- PBS/tween buffer
- Pasteur pipette
- Anti-tubulin labeled with FITC (AB)
- Propidium-jodide (PI)
- Fluorescence microscope

Instructions

Always follow the instructions from the assistants to the letter and pay great attention to the presentations!

- Wash the cover slip with fixated tumor cells by pipetting 1 or 2 mL PBS/tween-buffer in the dish with the Pasteur pipette. Swivel for 10 seconds and remove the fluid with the same pipette. You can tilt the petridish slightly while doing this.
- Spread 150 µl antibody (AB; FITC-conjugated anti-tubulin) over the cells on the cover slip.
- Cover the petridish with aluminum foil and incubate for at least 15 minutes in the dark at room temperature.

During the incubation you will do experiment 2. Note that you should keep these two experiments separated.

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Experiment 2: X-ray diffraction

Determining the three-dimensional structure of the protein

Goal of the experiment

In order to determine the exact difference between an active (correctly folded) and inactive (faulty folded) protein, one can use X-ray diffraction. The proteins have to be in crystal form for this technique. The whole experiment depends on the quality of the crystals. All the molecules in the crystal need to be aligned properly in order to recover the right three-dimensional structure.

Now first make questions 2.1 and 2.2

Requirements

- Lysozyme: 100 mg/ml stock solution
- Precipitation mix: 30% mono-methyl-PEG 5000 in 1M NaCl, 50 mM Na acetate, pH 4.7
- Slide
- Pipette with a volume of 15 µl
- Cover slip

Instructions

Always follow the instructions from the assistants to the letter and pay great attention to the presentations!

- Use the letters on the slide to focus the microscope.
- Place the cover slip on the desk and pipette 15 µl lysozyme solution on it..
- Pipette 15 µl precipitation mix about 1 cm next to the lysozyme solution.
- Mix the fluids by dragging the pipette point between the two solutions. (move the point 3 times to and tro)
- Put the cover slip under the microscope and check what is happening. (check every few minutes).

Now make questions 2.3 and 2.4

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Experiment 1b: Immunofluorescence staining

The differences between cells of healthy and diseased people

The continuation of experiment 1a

- Remove the cover slip from the dark area and wash it 3x by pipetting 1 to 2 mL PBS/tween-buffer in the dish with a Pasteur pipette. Swivel for 10 seconds and then remove the fluid with the same pipette. You can tilt the petridish slightly while doing this. Make sure that the cover slip cant dry up.

Pay great attention to the assistant's instructions for the next step!

- Spread out 150 µl propidium iodide (PI) over the cells on the cover slip and let this incubate in the dark for a maximum (!) of 30 seconds.
- Again remove the cover slip from the dark area and now wash 3x with PBS/tween-buffer.
- Examine the cover slip with the fixated cancer cells on it together with the assistant under the fluorescence microscope at a magnification of 1000x. Draw how the cells look like. Preferably use crayons.

After doing this, make sure the cell are stored in the dark until the assistant arrives to help you prepare your sample for the fluorescence microscope.

Now make questions 1.2 and 1.3

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Questions lesson 2 and 3

Experiment 1

Question 1.1
Formulate the research question for experiment 1.

Question 1.2
Can you see stained tubulin in HeLa cells? State the difference with the stained fibronectin and explain what this means.

Question 1.3
Conclusion: You have just examined the difference between the cells of a healthy and a diseased person. What can you conclude from this experiment with respect to the research question formulated in question 1.1?

Experiment 2

Question 2.1
State the different steps that are necessary to determine the three-dimensional structure of proteins via X-ray diffraction. (You can use the information in the presentation)

Question 2.2
Formulate the research question for experiment 2.

Question 2.3
Draw what you see through the light microscope after 1, 3, 5 and 7 minutes.

Question 2.4
Conclusion: You have just examined how to make a protein crystal for the use in three-dimensional protein structure determination via X-ray diffraction. What can you conclude from this experiment with respect to the research question formulated in question 2.1?

Lesson 4: Concluding lesson

After finishing the preperatory lesson and the practicle course there is a concluding lesson. The teacher has several options for this lesson, for which different manuals are available. For more information on the topics discussed in this practical course you can go to www.allesoverdna.nl.