

The Pigments of Life

How to illustrate the evolution and the variation of biological pigments among different photosynthetic organisms

OBJECTIVES

This protocol has been developed to support the teaching of a lesson on photosynthesis and evolution, with a specific focus on the *chloroplasts' endosymbiosis theory*. The purpose of the experience is to provide an activity that triggers an interactive lesson on the evolution of different photosynthetic machineries in different photosynthetic organisms. Thanks to a simple and visual (coloured pigments) protocol, this activity allows teachers to address the complex topic of evolution focusing on organisms that are phylogenetically separated (*i.e.* cyanobacteria, unicellular algae and higher plants).

INTRODUCTION

Very diverse organisms such as cyanobacteria (prokaryote, bacteria), unicellular green algae (eukaryote, chlorophyta) and higher plants (eukaryote, tracheophyta) are all defined as *autotroph organisms*, thus able to produce for themselves the organic compounds that they need to survive. The chemical reaction that allows them to do so is called photosynthesis and it's directly fuelled by sunlight (see also BiOutils experience 17: photosynthesis). In different organisms such as prokaryotes and eukaryotes, the photosynthetic process shows some slightly different characteristics. For example, in cyanobacteria photosynthesis takes place within intracellular lamellae (*i.e.* in the cytosol), whereas in algae and higher plants, it takes place within the chloroplast, a highly specialized cellular organelle. In both cases, however, photosynthetic machineries use light photons and CO₂ to synthesize energy-storing molecules such as ATP and NADPH, while freeing O₂ from H₂O.

The ability of organisms that are coming from two separate taxonomic kingdoms, such as *Bacteria* and *Plantae*, of similarly performing photosynthesis, has been explained by the *endosymbiosis theory*. **According to this theory, chloroplasts are the result of an endosymbiosis between an ancient eukaryotic cell and an ancestor of the modern cyanobacteria.** This early cyanobacterium would have then be assimilated by the eukaryotic cell, evolving with time to be the actual chloroplast. Some clues of this ancient endosymbiosis may be directly observed by comparing the components of the photosynthetic machineries that have been conserved through algae, higher plants' and modern cyanobacteria (Fig. 1). A good example of this comparison can be done through the visual observation of the light-harvesting pigments family.

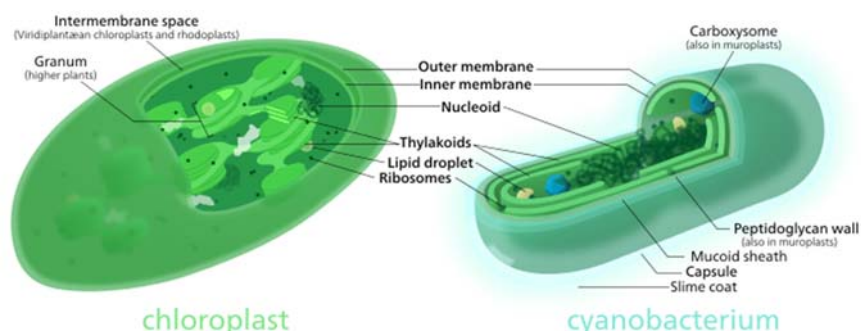


Fig. 1: comparison of the morphological structures of a chloroplast with a modern cyanobacterium. Wikipedia, CC BY-SA

Biological pigments are widely present in nature and, due to their property of absorbing specific wavelengths of the visible light spectrum (while reflecting others), they are responsible for the coloration of most of the living tissues. The absorption spectrum of each pigment (and therefore its visible color) varies according to the type of chromophore that it is embedded within its chemical structure. In photoautotroph

organisms, biological pigments cover a crucial role for light-harvesting. Photosynthetic machineries take advantage of their absorption properties in order to capture light photons, transform their energy in chemical energy and eventually channel it to the photosynthesis reaction centers (*i.e.* photosystems). The main biological pigments that are present in photoautotroph organisms can be divided in three main families *i.e.* chlorophylls, carotenoids and phycobilins, each characterized by a different chromophore and therefore by a different absorption spectrum.

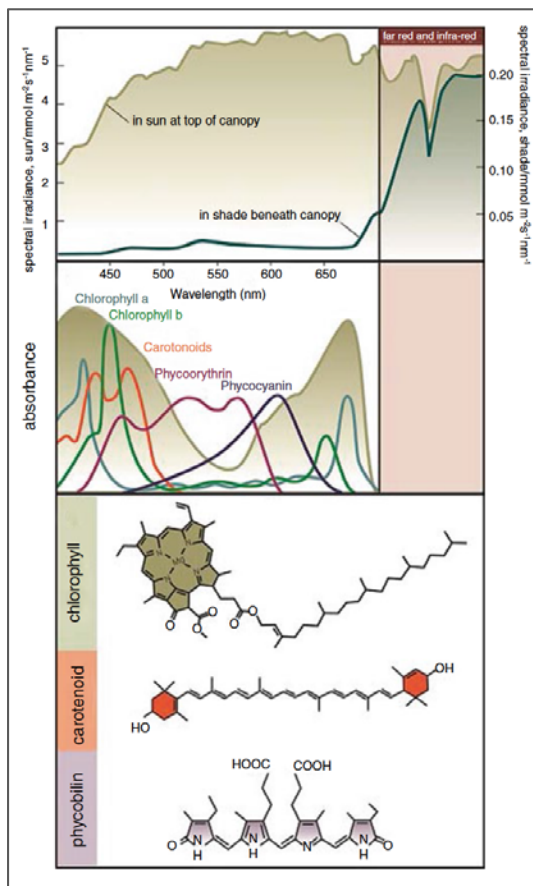


Fig. 2: A) wavelength spectra in the top of the canopy, dark green line, compared to the wavelength spectra in the canopy shade, light green line; B) absorbance spectrum of the different pigments' species. Each peak corresponds to the wavelength maximally absorbed by the pigment; C) chemical structures of the main classes of pigments. Mikovic and Scholes, *Photobiology: The Science of Light and Life*. 2015

• Chlorophylls

Chlorophylls are green pigments, present in all photoautotroph organisms. They are the most important pigment during the photosynthetic process being also called *photosynthetic pigments*. Their chemical structure is made up of a porphyrin composed by four pyrrolic groups. In the center of the porphyrin, an atom of Magnesium (Mg) is kept in position by four atoms of Nitrogen (N) (Fig. 2). Their green coloration is due to the fact that they absorb in the red and blue wavelengths, while reflecting the green. In higher plants we find two different chlorophylls: a) chlorophyll a - blue/green coloration - with a methyl group bound to the 2nd pyrrol; b) chlorophyll b - green/yellow coloration - with an aldehyde group bound to the 2nd pyrrol.

• Carotenoids

Carotenoids are non-nitrogenous pigments that in solution display a characteristic orange/yellow coloration. They are called *accessory pigments* since they are able to absorb light in spectral regions where chlorophyll absorbs weakly and channel it towards chlorophyll pigments. Furthermore they exert a *photo-protective function*, dissipating the excess of light. Their chemical structure is made by a polymer of isoprene units up to an average of 8 units for each carotenoid molecule (Fig. 2). Carotenoids can be divided in two big groups according to the presence or not of oxygen within their structure: a) Carotenes, oxygenated compounds and b) Xanthophylles, non-oxygenated compounds.

• Phycobilins

Phycobilins are a group of pigments mostly found in red algae (*i.e.* Rhodophyta) and Cyanobacteria. **Their universal presence among these primitive species, suggests that phycobilins may represent an example of ancestral pigments that have been eventually lost through the evolution of higher plants and other algae** (Ó Carra and Ó hEocha, *Chemistry and Biochemistry of Plant Pigments*, 1976). As it is for carotenoids, also phycobilins act as accessory pigments, channeling energy quanta towards chlorophylls. Their chemical structure is made up of a chain of four pyrrole rings similarly to chlorophylls. However, phycobilins display an open chain of pyrroles whereas in chlorophylls the pyrroles are arranged in a ring with a Mg atom in the center (Fig. 2). Phycobilins are divided in two major groups *i.e.* a) Phycoerythrins, characterized by a clear red color and b) Phycocyanins, characterized by a blue color, with a strong emitted red fluorescence.

The experience hereby proposed, will allow the physical separation and the visual observation of the biological pigments present in: 1) a higher plant (*Petroselinum crispum*, Parsil); 2) a unicellular green algae (*Chlorella regularis*) and 3) a cyanobacteria (*Arthrospira platensis*, Spirulina). Importantly, parsil and chlorella are both two eukaryotic organisms (*i.e.* photosynthesis takes place within the chloroplast),

whereas spirulina is a prokaryotic organism (*i.e.* photosynthesis takes place in the cytosol within the intracellular lamellae). The observed pattern of pigments that is present in each organism, will offer the possibility to first-hand evaluate *similarities* and *differences* between bacterial and eukaryotic photosynthetic components. **The interpretations of such patterns will lead students to draw evolutionary (endosymbiosis theory) and ecological (photosynthesis) inferences while appreciating the colorful panel of biological pigments.**

MATERIAL

- Dried Spirulina powder
- Dried Chlorella powder
- Dried Persil (food seasoning is OK)
- Ethanol 100%
- distilled H₂O
- Petroleum Ether
- Acetone
- Heating block/Bunsen
- Precision balance
- 15 mL tubes (3x)
- 50 mL tubes (3x)
- 500 mL becker + cover lid
- Silica plates on aluminium support
- P20
- Mortar and pestle
- Ruler
- Pencil
- Tweezers
- 15 mL tube holder
- 50 mL tube holder

PIGMENT'S EXTRACTION

This part of the experience aims to extract the pigments from the dried cellular material of the different organisms. To do this, pestling and a gentle treatment of ethanol will be enough to solubilize the biological pigments in the liquid solvent (EtOH).

- POWDER PREPARATION

- Weight 10g of dry material (Spirulina, Chlorella and Persil) and put it in the mortar
- With a pestle, reduce the dry material in a very fine powder (3'-4' of energetic pestling should be enough)

- PIGMENTS SOLUBILIZATION (1)

- Pour 200 mL of tap water in the 500 mL becker and place it on the heating block. Set the heat block intensity at mid high power (the water should be on the point of starting boiling, but not yet boiling *i.e.* around 75-80 °C).
- Add 2 mL of EtOH to a 15 mL each tube
- Weight 500 mg of the fine powder and transfer it to the 15 mL tube (save the remaining powder for the next steps, see below). Repeat the step for each organism.
- Close the tubes with the lid and mix thoroughly the content by inversion. Be sure that all the powder is dissolved in the EtOH. At the end, "arm-spin" the tubes so that only few material remains on the tube walls.

- Once the water will be at the right temperature, remove the lids from the 15 mL tubes and place them into the becker. Close the becker with its glass lid and leave the tube into it for 3'-4'
- Remove the tubes from the water and firmly close the lid. Leave rest the tubes for 10', if possible in the dark.

- PIGMENTS SOLUBILIZATION (2)

- Fill three 50 mL tube with roughly 30 ml of distilled water
- Pour the remaining powder, and mix energetically.
- Repeat the steps for each organism.
- Leave the 50 mL tubes on the rack until the end of the experience without moving it, so that the fine powder may deposit on the bottom of the tube

THIN LAYER CHROMATOGRAPHY (TLC) MIGRATION

Thin Layer Chromatography (TLC) is an analytical technique mainly used to separate coloured substances as, for example, biological pigments. Starting from a mixture of different pigments, they can be separated on the basis of their differential adsorption (*i.e.* ability to adhere to a certain compound without forming chemical bounds) for the substrate on which they are placed (*e.g.* TLC silica plates). This separation is possible thanks to the migration via capillarity of a solvent over the surface of the chromatographic plate. During its "run" the solvent will solubilize the pigments mixtures that have been previously spotted on the surface. The solubilisation of the different pigments will take place according to their degree of solubility in the specific solvent, which, in turn, is defined by their polarity (*i.e.* partial charge due to electrons' dipole of a chemical structure). In this context, pigments which will be more soluble in the solvent will loosely adsorb to the substrate, whereas those which will not be soluble in the solvent will adsorb more tightly to the silica plate.

In general terms, pigments with a high polarity (*e.g.* phycoerythrin) will adsorb strongly to the silica plate and will not migrate with the solvent. On the contrary, pigments with a low polarity (*e.g.* β -carotene) will be solubilized by the solvent and will migrate at the top of the silica plate. Eventually, the chromatographic run will concentrate pigments with the same polarity in crispy bands that will migrate at different speeds on the silica plate as a result of their solubility/adsorption rate. The final result will be a pattern of bands, characteristics for each pigment's mixture.

TLC PLATE PREPARATION

While waiting for the 10' dark extraction of the pigments, you can already start the preparation of the TLC silica plate. NOTE: hold the silica plates with tweezers and handle it always using gloves, never with bare hands.

- Take a 4 x 7 cm silica plate and measure 1,5 cm from the bottom (*i.e.* the 4 cm border) on each side of it. With a pencil, delicately draw a line without scratching the silica surface.
- On the pencil-drawn line, draw three equidistant dots.

TLC BOX PREPARATION

Since the solvents used (Petroleum Ether and Acetone) are very volatile, inflammable and potentially harmful, **these steps should ONLY be handled by the teacher**. When possible, solvents shall be handled under a chemical hood to prevent volatile evaporation to spread through the classroom. If this is not possible, make sure that the classroom is well ventilated.

- Pour 10 mL of Petroleum Ether in the TLC box
- Pour 2 mL of Acetone in the TLC box
- Close the box with the lid and delicately mix the two solvents.

TLC PIGMENTS MIGRATION

Once you will remove the tubes from the dark, you will be clearly able to observe for each extraction two different phases: a lower one made of cellular debris and an upper one of a dark green colour where the pigments are dissolved in ethanol.

- Using the P20, take 3 uL from the upper phase (pay attention not to dip the tip within the cellular debris, if so, the tip will be obstructed by the debris) of *Spirulina* extracts and delicately spot it on the first of the three equidistant signs without touching the silica plate with the tip.
- Wait for 1' for the spot to dry and take again 3 uL of extract and spot it on the same spot previously done.
- Repeat the first two steps for the second (*Chlorella*) and third (*Persil*) extracts obtaining three equidistant spots. Change the tip every time that you pass to the next extract.
- Wait for the last drops to dry for 1'

NOTE: from the next step onwards, due to the presence of slightly hazardous solvents, the protocol should be performed by the teacher only.

- Pick the TLC plate with tweezers on the top of it
- Gently put the TLC plate in the TLC box maintaining the bottom horizontal
- Close the TLC box
- Leave it run until the solvent front reaches 1 cm from the top of the TLC plate (8-10')
- Remove the TLC from the box and let it dry for 3'-4'.
- Observe the results and take pictures (pigments will oxidate and will not be visible anymore).

RESULTS AND DISCUSSION

• Technical considerations

The main factor that defines pigments' pattern of migration is the degree of their solubility within the solvent. This is characterized by the chemical properties of pigments, but it is influenced as well by the type of solvents that are used. One of the most commonly used solvent mix in TLC experiments is a mixture of petroleum ether, acetone and chloroform (3:1:1). This mixture allows a neat migration and separation of the different pigments (Fig. 3A). However, chloroform is an extremely toxic solvent that cannot be used for our purposes *i.e.* within a classroom experience. On the other hand a solvent mix made up of only petroleum ether and acetone (5:1) can similarly separate the pigments with comparable patterns (Fig 3B).

• Migration patterns

Once the migration will be completed, the different patterns will show some features that are shared among the different organisms, whereas others are characteristic of a specific organism (Fig.3). In all the patterns, the fastest band reaching the top of the TLC plate is the carotenoids' band. Furthermore, all the organisms show an intense green/blue band. This band represents the green pigment chlorophyll a, **a pigment that, as been said in the introduction, is crucial for the photosynthetic process.** Provided that cyanobacteria evolved much earlier than green algae and higher plants (according to fossil evidences), the presence of chlorophyll a in spirulina and in the chloroplasts of chlorella and persil is a good **piece of evidence for the proposed endosymbiosis theory.** Evidently it was not spirulina itself that was engulfed by the ancient eukaryotic cell, but a further primitive cyanobacterium.

The results of the TLC migration also evidence a very similar pattern between chlorella and persil, with a strikingly different pattern for spirulina. As all other cyanobacteria, spirulina, doesn't have chlorophyll b and, moreover, compared with persil and chlorella it shows the presence of phycobilins (*e.g.* phycoerythrin). **Phycobilins are universally diffused through primitive species as rhodophytes (*i.e.* red algae) and cyanobacteria. Their absence in green algae and higher plants, suggests that phycobilins may have been ancestor pigments eventually lost through**

chloroplast's evolution. In this TLC experiments, phycoerythrin, due to its high polarity, doesn't solubilize in the solvents mixture and it remains tightly adsorbed to the silica surface. It remains however clearly visible in the place where the extraction solution has been spotted. On the other hand, phycobilins cannot be efficiently extracted by ethanol and therefore are not detectable on the TLC plate. For the purposes of our experiments, phycobilins can be easily extracted in water (see *Phycocyanin fluorescence* chapter).

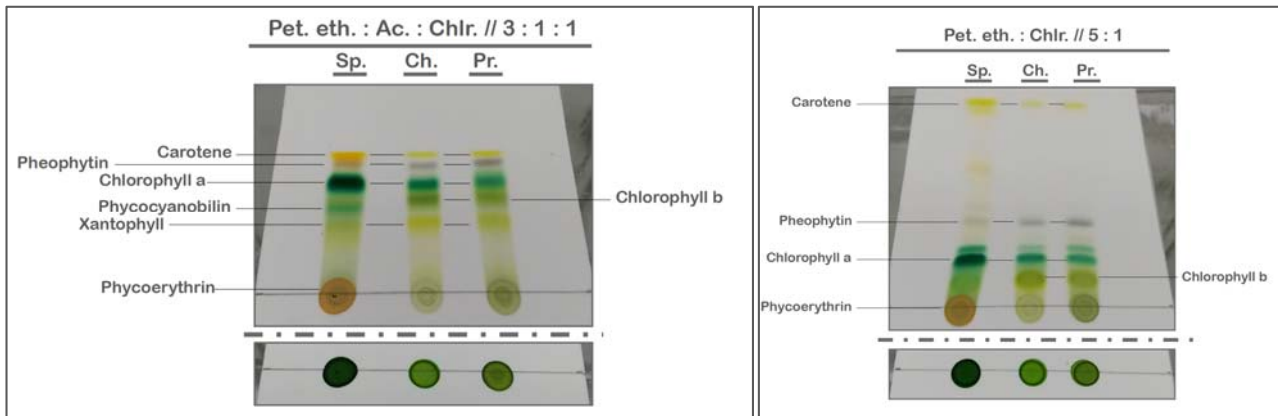


Fig. 3: TLC migration of pigments extractions from spirulina (Sp.), chlorella (Ch.) and persil (Pr.). The lower box represents the spotting of the different extraction on the silica plate, before the solvent migration. The upper boxes show the final result of the pigments separation, using different solvent mixtures. In the left box, a mixture of Petroleum ether (Pet. eth), Acetone (Ac.) and Chloroform (Chlr.) has been used with the 3:1:1 ratio. In the right box, a mixture of Pet. eth and Ac. with a ratio 5:1 has been used.

• Phycocyanin fluorescence

In order to proceed with the discussion, at this stage it should follow the observation of the extractions that have been made using only distilled water (see PIGMENTS SOLUBILIZATION (2)):

- WATER DISSOLVED PIGMENTS OBSERVATION

- Gently pick up the 50 mL tubes without shaking them.
- Observe the tubes in the light. Take pictures (without using the flash!)
- With an intense source of light (the LED light of any smartphone will work) illuminate the solution within the tubes. Take pictures (using the flash!).

What it can be observed in solution is the blue coloured pigment phycocyanin. When illuminated with a strong emitting source of light *e.g.* LED light, this will excite its electronic structure that, in order to return to its ground state, will emit a red wavelength, characteristic of its chemical structure (Fig. 6). In physiological conditions where the intracellular lamellae are intact, chlorophylls would collect the energy quanta emitted by phycocyanin (*i.e.* fluorescence) channelling it further to the photosystems. As a matter of fact, this is the role of several accessory pigments as it is the case of phycobilins.

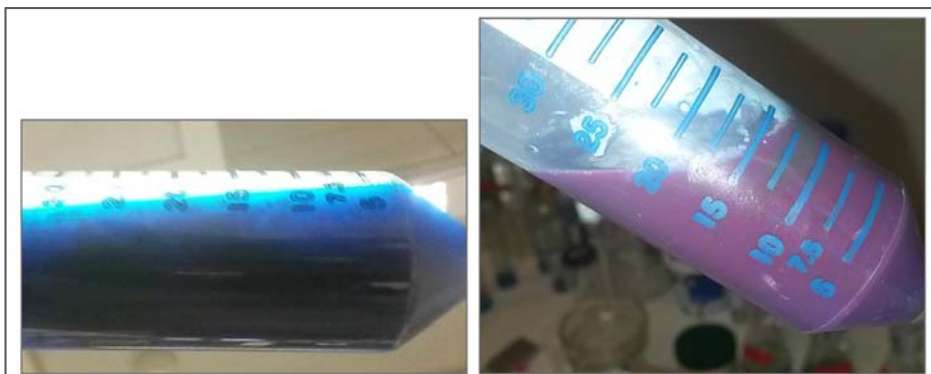


Fig. 4: the phycobilin pigment, phycocyanin in aqueous solution. When light is observed through the solution, this it looks bright blue, due to the absorption spectra of phycocyanin (see fig. 2). When a strong light is flashed on the solution, the emitted fluorescence will turn the solution red and it will not be translucent anymore.

EXTENDED ACTIVITY

In order to complement the exercise with a further engaging activity, live spirulina and live chlorella can both be observed under the microscope, they both look awesome!