GUIDE - CARS microscope

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1 CARS setup

The video-rate CARS microscope setup is placed in the F-5428 room. The scheme of the setup is shown in Fig. 1.

1.1 Illumination

Pump and Stokes beams are generated with the OPO and the picoTRAIN lasers respectively. The APE Levante OPO is adjusted to generate a 816.8 nm beam (6 ps pulses) with a power of approximately 30-40 mW at the sample plane in the CARS microscope [1].

The 1064 nm at the output of the picoTRAIN Nd :Vanadate (6 ps pulses) is adjusted at a power of approximately 60 mW under the objective. Note that the optimal power ratio for pump ($\approx$ 816 nm) and Stokes (1064 nm) lasers should be around 1 :2.

The combination of a half-WP and a polarizing beamsplitter at the output of both lasers allows the control of the beam power (Fig. 1).

1.2 Recombination of the Stokes and pump beams

The two beams recombine at the dichroic D1. To generate CARS signal at the sample, they must recombine at the same *time* and at the same *place*. Given these two requirements, two things in the setup need to be adjusted (explained in the next subsections).

To confirm that the recombination of the two lasers is good and assess its efficency, one can use a BBO cristal for second-harmonic generation (SHG) and sum-frequencies generation (SFG). In order to do that, a new lens (with a focal length of 75 mm, as an example) and the BBO cristal are placed after dichroid D1. This new lens is used to focus both lasers on the BBO cristal. With proper adjustment of the angle of the cristal, one will see that the 1064 beam generates a 532 nm beam (green), and that the 816 beam generates a 408 nm beam (purple), both via SHG. When the two beams recombine correctly in space and in time, with proper adjustment of the cristal's angle, there will be a third beam generated by sum frequencies with a wavelength of approximately 466 nm (blue).

For more information about the manipulations in the lab for SHG and SFG, watch this video : `https://www.youtube.com/watch?v=-xbaCfr3kO8&list=PLUxTghemi4FsBotHd4CRZLjeoNeLokKpA`.

1.2.1 Delay adjustment

The delay can be adjusted in the picoTRAIN line with the translation of the mirror M1. Since the pulses of the picoTRAIN are not very short (6 ps), finding the right spot in the delay line is not too difficult : there is a large range (a few μm) for which the time adjustment will be good. In the case of lasers generating fast pulses (femtosecond pulses, as an example), the adjustement of the delay line must be more precise.

1.2.2 Wavefront curvature matching

The wavefront curvature of the two beams must also match. Otherwise, if one beam has a divergence that is a little bit different from the second beam, they won't focus exactly at the same place after passing through a lens. Focalization at different spots reduces the efficency of the generation of the coherent signal at the sample. The wavefront curvature of one beam can be adjusted by changing the space between the lenses of its 4f system (composed of lenses L1 and L2 for the OPO or L3 and L4 for the picoTRAIN).

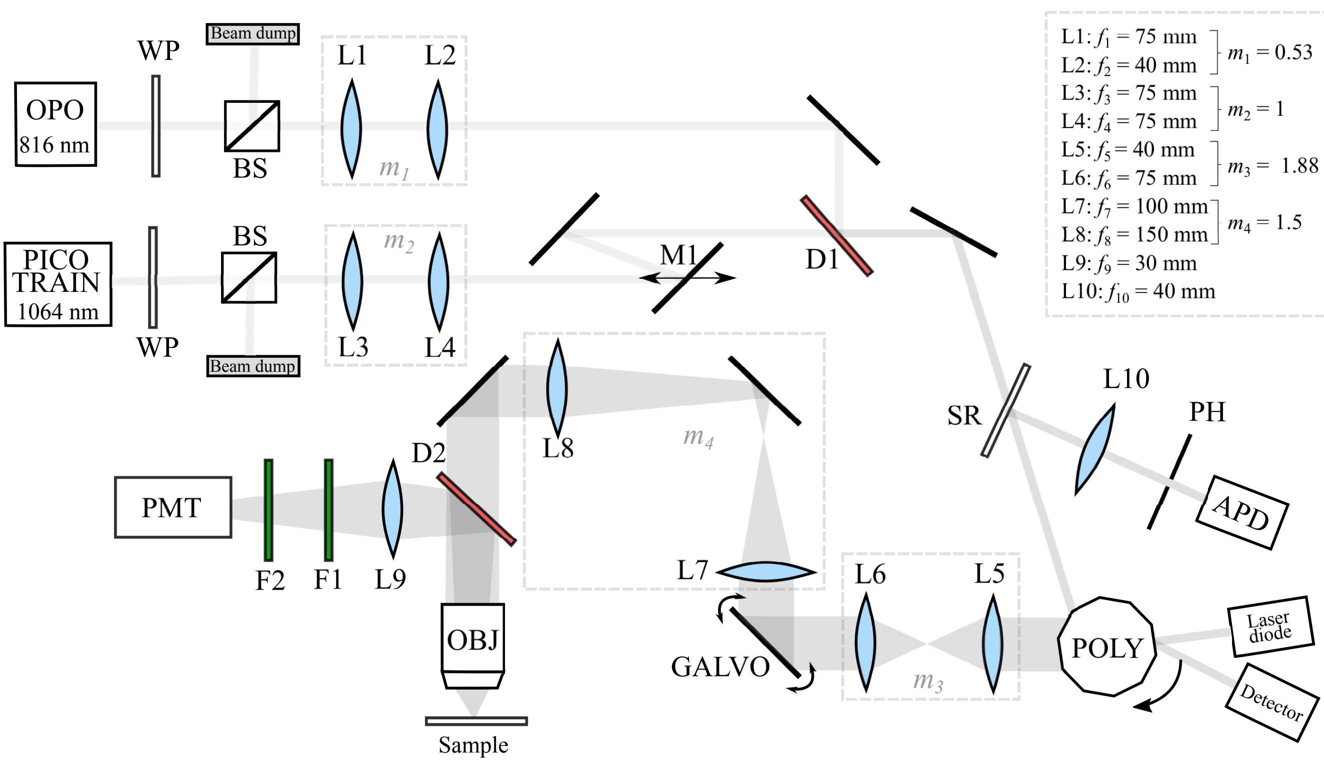

FIGURE 1 – CARS setup scheme. L : lens, D : dichroic mirror, WP : waveplate, BS : beam splitter, M : plane mirror, POLY : polygon, GALVO : galvo mirror, SR : semi-reflective plate, OBJ : mircoscope objective. Lenses that are included in the same dashed box are components of a 4f system, with a specific magnification m.

1.3 Scanning system

The CARS microscope works at video-rate, thanks to the scanning system, composed of a 36 facets polygon (Lincoln Laser, DT-36-290-025) and a galvo mirror (Cambridge Technology, model 6240H) [2].
The system acquires images of 1024×512 pixels at a rate of 30 frames per second.

1.4 Backreflections detection

On the laser path before the polygon, a semi-reflective plate is added to allow the backreflections of the sample to be detected. The backreflections are detected with a Thorlabs APD410C [3].

Note that the multiple reflections of the laser on the lenses can also be detected by the APD, resulting in bright spots on the backreflection image. To avoid this, the 4f systems can be adjusted to be vonlontary a little bit off-axis (the lenses must be on x-y translation mounts). If the first lens is moved a little bit on the left, then the second lens has to be moved the same amount on the right to compensate. The reflection of the laser will therefore be deviated from the incident path and won't reach the APD.

There is also out-of-focus light that can deteriorate the obtained image of backreflections. To avoid out-of-focus light to be detected, a pinhole is added right before the APD. The pinhole size can be calculated using both the following equation and the Python raytracing module [**?**] :

$$r_{Airy} = \frac{1.22\lambda}{2NA_{objective}} \tag{1}$$

The latter equation determines the spot size radius at the focal spot. This equation is valid for a 1-photon absorption, which is not the case in the CARS microscope, which is a non-linear process. In this case, the rule of thumb for a good approximation is to use the same equation, but with the emission wavelength instead of the illumination wavelength. Using $\lambda = 661$ nm and $NA = 1.2$ leads to a spot size radius r_{Airy} of 0.33 um.

The code used in the present case is shown in Appendix 1. One can easily find that the required pinhole diameter for a spot size radius of 0.33 μm is $\approx$ 50 μm.

1.5 Synchronization with HSYNC

The synchronization of the polygon with the acquisition system requires the presence of a small laser diode that reflects on the polygon and that is detected by a small photodetector. This system, that we name HSYNC, needs to be aligned properly : otherwise, the obtained image can be shaky or even reversed (the HSYNC determines "where" the image begin on the software Nirvana).

1.6 Focussing on the sample

The beam is focussed on the sample with a water-immersion microscope objective Olympus UPlanSApo 60x/1.20 [4]. A sample holder controlled with a Sutter Intrument 3-axis stage allows the positionning of the sample with respect to the objective [5]. Note that the working distance is 0.28 mm.

1.7 Detection

The CARS signal is emitted at a wavelength given by the following equation :

$$\lambda_{CARS} = \frac{1}{\frac{2}{\lambda_{\text{pump}}} - \frac{1}{\lambda_{\text{Stokes}}}} \tag{2}$$

With a pump signal at 816 nm and a Stokes signal at 1064 nm, one can easily find the CARS signal to be emitted at 661 nm. The CARS signal coming from the sample is detected with a PMT. Two filters are added in front of the PMT. The filter F1 is a bandpasse filter at 655 nm with a bandwidth of 40 nm. It is used to block everything except the CARS signal. The second filter F2 is a shortpass filter at 750 nm, which is perfect for blocking the OPO and picoTRAIN laser lines.

Be careful, the PMT must not be used under bright ambiant light. When the PMT is plugged in and have a non-null gain, the lab lights should be closed and the setup should be covered with a black drape. The setup should be light-tight, otherwise the ambiant photons will produce noise in the image.

1. Turning on the setup
   - (a) Turn on the acquisition box on the upper shelf.
   - (b) Turn on the 3D translation stage on the upper shelf (Sutter Instruments, MPC-200).
   - (c) Turn on Nirvana 2.x on the computer.
     - i. Connect BLIQ VSM (top right of screen). The polygon should start rotating.
   - (d) PUT ON OPTICAL GLASSES (Thorlabs LG9, at the entrance of the lab).
   - (e) Open the two shutters at the back of the picoTRAIN laser. The green laser beam that pumps the OPO will appear and the second beam is the 1064 nm output (only visible with a detection card).
   - (f) With a detection card, make sure that you can see both beams (1064 nm and 816.8 nm) at the entrance of the polygon by opening the shutters one at a time.
   - (g) Verify with the detection card that there is a rectangle beam shape after the top right mirror on the vertical part of the optical setup (after both the polygon and the galvo).
   - (h) With the Thorlabs power meter, verify that the power at the sample is 25 mW for the 1064 nm (picoTRAIN laser) and 40 mW for 816.8 nm (OPO laser). For both lasers, if needed, adjust the power at the sample with the polarisers which are right after the beam splitter (at the output of both lasers).
2. Imaging
   - (a) Place the sample under the microscope (do not scratch the tip of the objective with the slide or the coverslip).
   - (b) Put a drop of water on top of the coverslip.
   - (c) Lower the objective as low as possible near the sample (without touching the coverslip).
   - (d) Turn off the lights in the lab.

(e) In Nirvana...
   i. Open the PMT with the slider on the right panel.
   ii. Click on the "Live on" button. A uniform black image will be displayed.
   iii. Rise the gain of the PMT to approx. 90-95%. A little bit of noise should appear in the image.

(f) Move the objectif up until the signal of the sample appears in Nirvana.

IMPORTANT : Never open the lights in the lab while the PMT is ON. Also, DO NOT use any light source near the PMT/sample while the PMT is ON.

EMERGENCY : Turn off both lasers by shutting both shutters on the picoTRAIN laser.

2 Pro tips

Here is a bunch of tips and facts about the CARS microscope setup and acquisition :

1. Verify that the galvo mirror is placed in the right side. The mirror typically has only one side that is coated properly. If it is on the wrong side, big losses of power may happen.
2. Verify the orientation of the PMT inside the housing. It may be wrong, or the thread might by loussy. If it's not in the right direction, the detected signal will be weak or inexistant.
3. Here is a trick to remove the noise of the PMT. The PMT housing is not perfectly grounded, which causes the noise. To solve this problem, one can buff the housing to remove the anodizing coating, and place aluminium tape to electrically join all the parts of the case together. In that way, all the housing is at the same ground.
4. The wavefront curvature matching with the two laser's 4f system is critical to get a good signal. Many people don't do it, but they should.
5. The aligment of the HSYNC is a little tricky. One must try many configuration to get the most stable image. It is also important to get the "beginning" of the image at the right spot in Nirvana. This can be adjusted using a sheet of paper with things written on it placed on the lens L7. The image of the sheet will appear in the backreflection window in Nirvana. The sheet should be fully imaged, without any interruption. Otherwise, if a dark vertical spot appears in the image, the position of the beginning of the image has to be adjusted with the HSYNC.
6. Why do we use long (picosecond) pulses and not short (femtosecond) pulses ? Spectra of femtosecondpulses (about hundreds of cm^{-1}) are much wider than the Raman bands (approx. 10 cm^{-1}) [6]. As a result, many spectral components of the sample will be excited at the same time, thus creating unwanted background signal. Also, as mentioned above, the delay adjustment would be more difficult with fs pulses than ps pulses.
7. Turn on the box before opening Nirvana on the computer.
8. If horizontal black lines (1 pixel large) appear in the PMT image in a periodical way, this is probably caused by defects on a face of the polygon. These black lines can be removed on computer with post-processing of the image.

9. To confirm that the setup is properly adjusted and produces a good CARS signal, one can test a butter sample or Polystyrene beads (sandwiched between two microscope slides). They should generate plenty of CARS signal. Other well-known samples like a mouse spinal cord can also be tested.
10. Always use a BBO crystal to assess the efficinecy of the two beam combination for second-harmonic generation (SHG) and sum-frequencies generation (SFG) as explained in the section 1.2.
11. Always check the OPO display to see if there is a signal with an appropriate power. If there is no signal, the small button in the front side of the OPO should be turned slightly (to the both sides) until a signal is obtained.

3 Lab problems and ways to solve them

This section regroups the problems encountered in the lab and explains their meaning and how to solve them. Each subsection is a problem of its own

3.1 Monitors are turned off, P_{OPO} does not go up

This can happen after a power cut or outage.

Characteristics of the problem :

— The OPO monitor is turned off.

— The LD1 parameter is at 0 on the AMP monitor of the picoTRAIN laser (Fig. 2).

— The P_{OPO} parameter on the OPO monitor is extremely low and doesn't go up even by changing the delay line in the OPO laser.

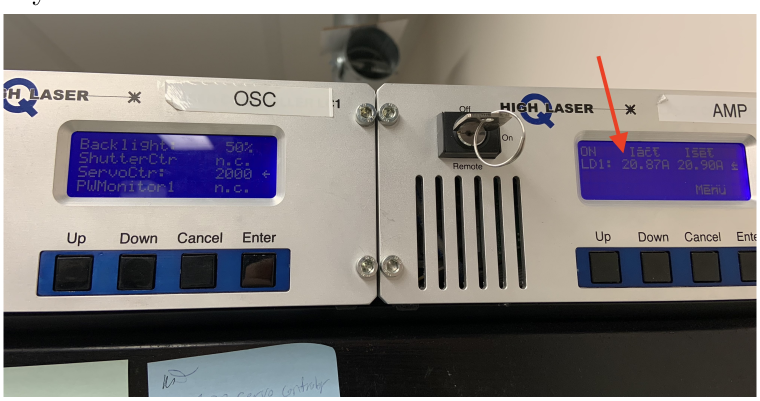

FIGURE 2 – PicoTRAIN laser monitors (OSC and AMP). LD1 should not be at 0 (red arrow).

How to solve the problem :

1. Go check if the AMP and OCS monitors of the picoTRAIN laser. They must both be turned on with the keys when the lasers are used. If there is a value in the AMP that is at 0 (Fig. 2), then the AMP monitor will not work even if it is turned on. With the *DOWN* and *UP* buttons, get to this 0 value and blast the *enter* button. It should work at some point.

2. Make sure that the cooling system is up and running. If it is beeping, add distilled water (go to this link to know how to do that `https://www.youtube.com/watch?v=IkXRXbdo0EQ`).
3. At the back of the OPO monitor, disconnect the cable *Thermo* (Fig. 3).

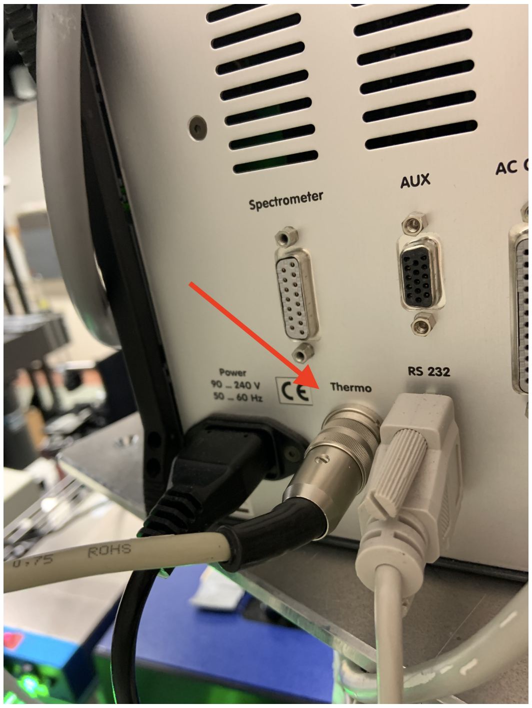

FIGURE 3 – Back of the OPO monitor. The *Thermo* cable should be unplugged and plugged before turning on the monitor.

4. Reconnect the cable *Thermo* at the same place.
5. At the front of the OPO monitor, keep your finger on the *START* button until the screen turns on (Fig. 4)

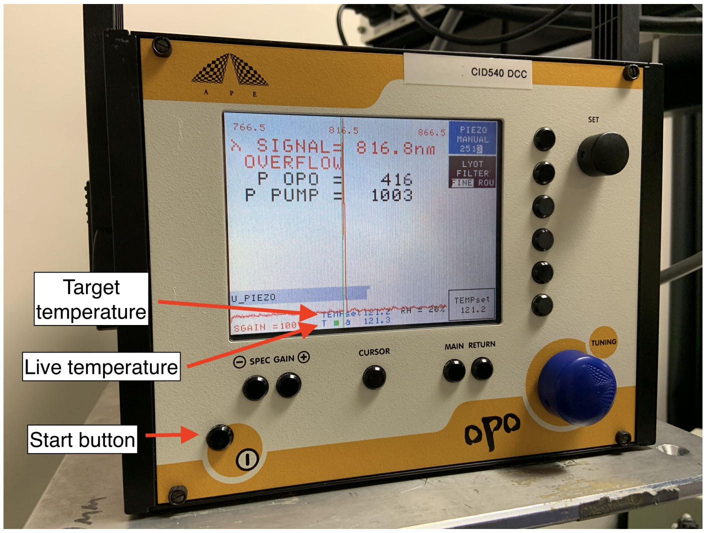

FIGURE 4 – Front of the OPO monitor.

6. The live temperature should then start to go up until it reaches the value of the target temperature (Fig. 4). This can take up to 30 minutes to be stable.
7. By adjusting the delay line of the OPO laser, you should get back your signal (P_{OPO} should go up).

3.2 P_{OPO} does not go up

Characteristics of the problem :

— P_{OPO} on the OPO monitor does not go up when changing the delay line of the OPO laser, even after turning the AMP and OSC monitors on and stabilizing the temperature of the OPO laser on the OPO monitor.

How to solve the problem :

1. Verify that *ServoCtrl* on the OSC monitor is at the value that you want. It might be possible that the OPO laser is not pumped enough to produce an intense signal at the output. Follow figure 5 to select the right *ServoCtrl* value for your application.

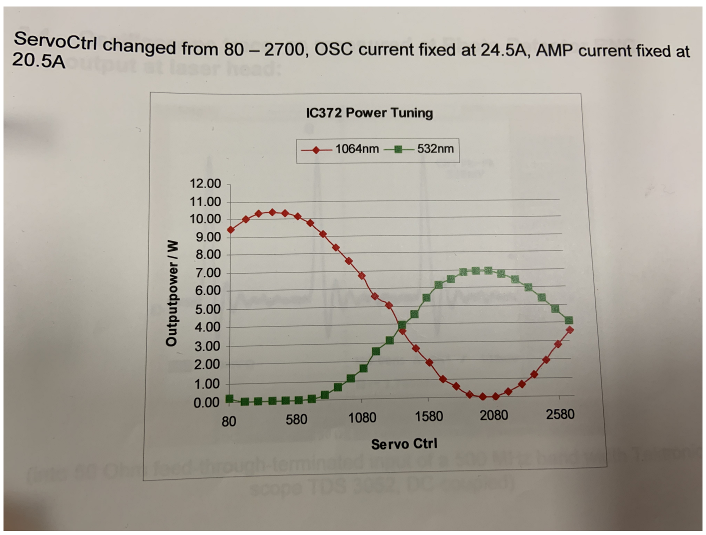

FIGURE 5 – *ServoCtrl* adjustments.

2. By changing the delay line in the OPO laser, you should be able to recover the signal at the ouput (increase P_{OPO}).
3. If this still does not work, the black screws on top of the OPO laser (2 on the right and 2 on the left) can be tuned, but **be careful**. Those screws adjust mirrors for the amplification inside of the laser. Turning them slightly on one side or the other by a couple of degrees should allow you to recover the output signal of the OPO laser. Adjust the delay line of the OPO laser at the same time.
4. If this still does not work, then the OPO laser should be opened and realigned. Follow steps in the OPO guide. Be **patient** and **meticulous**.

Références

[1] APE. *Levante Emerald ps.* https://www.ape-berlin.de/en/opo-optical-parametric-oscillator/levante-emerald/.

[2] Cambridge Technology. *62xxH Series Galvanometer Scanners.* https://www.cambridgetechnology.com/sites/default/files/Datasheet%20-%20Galvos-62xxH%20Series-DS00003_R1_v4.pdf.

[3] Thorlabs. *APD410C - InGaAs Variable-Gain Avalanche Photodetector.* https://www.thorlabs.com/thorproduct.cfm?partnumber=APD410C.

[4] Olympus. *UPLSAPO-S/UPLSAPO-W Super Apochromat Objectives.* https://www.olympus-lifescience.com/en/objectives/uplsapo/.

[5] Sutter Intruments. *MP-285 micromanipulator system.* https://www.sutter.com/manuals/MP-285_OpMan_ROE_BasicOp.pdf.

[6] Potma, Eric O., and X. Sunney Xie. 2004. "CARS Microscopy for Biology and Medicine." Optics & Photonics News 15 (11) : 40–45.

[7] title=Designing a large field-of-view two-photon microscope using optical invariant analysis, author=Bumstead, Jonathan R and Park, Jasmine J and Rosen, Isaac A and Kraft, Andrew W and Wright, Patrick W and Reisman, Matthew D and Côté, Daniel C and Culver, Joseph P, journal=Neurophotonics, volume=5, number=2, pages=025001, year=2018, publisher=International Society for Optics and Photonics

Appendix 1

```python
import envexamples
from raytracing import *
import matplotlib.pyplot as plt
"""
To obtain and plot the intensity of a point source at the pinhole of a confocal
    microscope (with variable pinhole size)
 as a function of position of focal spot by sending a large number of rays in
    the system (changing the position of the
 focal spot provides an optical sectioning process).
"""
# Focal spot radius (Airy disk radius)
focalRadius = 0.00054
# Dictionary of pinhole factors with an empty list which will subsequently
    contain the transmission efficiency
# for each focal spot position
pinholeModifier = {1 / 3: [], 1: [], 3: []}
# list of all relative positions from the ideal focal spot position in nm
positions = [1000, 800, 500, 300, 150, 100, 50, 25, 0, -25, -50, -100, -150,
    -300, -500, -800, -1000]
# Number of total rays produced by the focal spot
nRays = 1000
# Production of rays from a focal spot with a radius determined by focalRadius
inputRays = RandomUniformRays(yMax=focalRadius, yMin=-focalRadius, maxCount=
    nRays)
# Focal length of the objective
objFocalLength = 0.57
def path(delta=0):
```

```python
    illumination = ImagingPath()
    illumination.append(Space(d=delta))
    illumination.append(System2f(f=objFocalLength))
    illumination.append(System4f(f1=150, f2=100))
    illumination.append(System4f(f1=75, f2=40))
    illumination.append(Space(d=40))
    illumination.append(Lens(f=40))
    illumination.append(Space(d=40))   # Path finishes at the pinhole position
    return illumination
def optimalPinholeSize():
    """
    Finds the magnification of the optical path and use it to find the optimal pinhole size when the focal spot is at one
    focal length distance of the objective.
    Return
    -------
        pinholeIdeal : Float
            Returns the optimal pinhole size
    """
    # Dictionnary of the position and magnification of all conjugate planes of the focal spot.
    planes = path().intermediateConjugates()
    # The last conjugate plane is the pinhole. The magnification of this position is saved in mag.
    mag = planes[-1][1]
    # Calculates the pinhole size that fits perfectly the focal spot diameter.
    pinholeIdeal = abs(mag * (focalRadius * 2))
    return pinholeIdeal
def illuminationPath(pinholeFactor=None, delta=None):
    """
    Determines the amount of rays emitted from the object that are detected at the pinhole plane.
    Parameter
    ---------
        pinholeFactor : Float
            Factor changing the pinhole size according to the ideal pinhole size.
        focalSpotPosition : float
            Position of the focal spot according to the objective (first lens)
    Returns
    -------
        illumination : object of ImagingPath class.
            Returns the illumination path
    """
    illumination = path(delta)
    pinholeSize = optimalPinholeSize() * pinholeFactor
    illumination.append(Aperture(diameter=pinholeSize))
    # Counts how many rays make it through the pinhole
    outputRays = illumination.traceManyThrough(inputRays, progress=False)
    return outputRays.count / inputRays.count
for pinhole in pinholeModifier:
    print("\nComputing transmission for pinhole size {0:0.1f}".format(pinhole))
    efficiencyValues = []
    for z in positions:
        print(".",end='')
        deltaPosition = (z * 0.000001)
```

```
        efficiency = illuminationPath(pinholeFactor=pinhole, delta=deltaPosition)
        efficiencyValues.append(efficiency)
    pinholeModifier[pinhole] = efficiencyValues
plt.plot(positions, pinholeModifier[1 / 3], 'k:', label='Small pinhole', linestyle='dashed')
plt.plot(positions, pinholeModifier[1], 'k-', label='Ideal pinhole')
plt.plot(positions, pinholeModifier[3], 'k--', label='Large pinhole', linestyle='dotted')
plt.ylabel('Transmission efficiency')
plt.xlabel('Position of the focal spot (nm)')
plt.legend()
plt.show()
print(optimalPinholeSize())
path.display()
```