MSc in Photonics

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MASTER THESIS WORK

Use of single-walled carbon nanotubes for near-infrared fluorescence within inner tissue in C.elegans

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Presented on date 21st December 2011

Registered at

Escola Tècnica Superior d'Enginyeria de Telecomunicació de Barcelona

Use of single-walled carbon nanotubes for near-infrared fluorescence within inner tissues in *C.elegans*

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Abstract. Imaging and tracking long distance transport of proteins such as molecular motors with conventional fluorescence methodologies is nearly impossible mainly due to photobleaching. In this work we demonstrate that DNA-SWNTs are very convenient tagging particles for *in vivo* tracking of proteins due to their low toxicity and bright and stable fluorescence. Also, soft lithography is used to build a microfluidic trap for *in vivo* fluorescence imaging in *C.elegans*.

Keywords: SWNT, fluorescence, elegans, microfluidics, nanotube

1. Introduction

1.1. Aim

Unlike most cells, neurons are highly polarized with asymmetric axonal and dendritic processes. An average eukaryotic cell is approximately spherical with a diameter around 20-50 µm. Therefore a protein in the cell centre only has a distance up to 25 µm to any other part of the cell. Within these distances of few µm, diffusion of proteins is a fast process. But neurons' axons usually are much longer (they can be longer than one meter) and its synapses need a continuous supply of nutrients, membrane lipids and proteins as well as retrograde transport of signalling molecules to the soma.
A well-described way to transport axonal cargo from the soma to the synapses is by means of motor molecules travelling along microtubules[1]. This transport is mainly facilitated by kinesin and dynein motor proteins (anterograde and retrograde direction respectively). A convenient and elegant way to study this transport is by attaching a fluorescent protein to the transporting molecules, exciting the fluorescence of the protein and observing it with a microscope[2]. One drawback of this method is the fluorescent proteins. These proteins can blink and bleach too fast on the order of the observed measurement time (begin fading within a few minutes, sometimes seconds)[2] preventing the study of long distance transport. Besides, *in vivo* studies need the excitation and emission light to penetrate the tissue well; thus, the light should be in the infrared range (optical window range of biological tissues), but infrared fluorescent proteins are still not well established[3]. All those challenges make it interesting to search for an improved labelling method for molecular motors.

1.2. The nematode C.elegans

Caenorhabditis elegans (*C.elegans*) is a nematode (round worm), which lives in temperate soil environments and is about 1 mm long. It is widely used as an experimental model organism, especially in genetics and developmental biology. *C.elegans* was chosen for this study because of its transparency, its fast growth rate, its easy handling, maintenance and storage, its short life cycle as well as because it is one of the simplest organisms with a nervous system[4].

The short life cycle of *C.elegans* (~2 days from egg to adult and ~17 days lifespan) permits the possibility of growing the worms with a mixture of nutrients and fluorescent particles achieving a fast absorption of the latter within the worm's tissue[5]. *C.elegans* feeds by an automatic pumping and isthmus peristalsis (swallowing) of the bacteria in liquid suspension of its surroundings. Its transparency facilitates the excitation of the fluorescent particles within the worm's body. And, in a continuation of this study, *C.elegans*' nervous system is a good candidate for *in vivo* experiments imaging motor proteins labelled with fluorescent particles.

1.3. Single-walled carbon nanotubes

1.3.1. What are SWNTs?

Single-Walled Carbon NanoTubes (SWCNTs, usually SWNTs) have novel and unique mechanical, electronic and optical properties that make them potentially useful in a wide range of fields[6]. They can be conceptualized as a graphene sheet (monoatomic layer of carbon with honeycomb crystal lattice) rolled with a certain chiral angle to form a seamless hollow cylinder[6]. SWNTs usually have a diameter of about 1 nm and lengths on the order of microns. That makes them materials with extremely high aspect ratio and it is possible to consider SWNTs as one-dimensional materials[6].
This chiral angle previously mentioned can be represented by a vector described by a pair of indices (n,m). These indices denote the number of unit vectors along two main directions in the 2D graphene crystal lattice. Depending on its value one can roughly say that the SWNTs are metallic when ($2n+m$) = $3k$ (where k is integer) and the rest semiconductors with different band gaps[6].

1.3.2. Fluorescence

Individual and chemically intact semiconducting nanotubes fluoresce in the near-infrared (NIR) region (reminder: optical window range of biological tissues) between 900 to 1600 nm. This fluorescence is intense enough to be detected from within small organisms and biological tissues[5]. It also does not blink or bleach, allowing long and continuous measurements of it[7]. The difference between the excitation and emission wavelengths is larger than that of organic fluorophores allowing a proper optical filtering for an efficient detection[7]. Nanotubes with different chiral angles emit in different wavelengths permitting their differentiation[7]. Due to their high aspect ratio, SWNTs' fluorescence is excited just if light's polarization has a component parallel to the nanotube. These properties make SWNTs a very promising tool in the field of proteins or molecules labelling, particularly for long imaging.

1.3.3. Preparation

Nanotubes, because of their high surface area combined with van der Waals attraction, tend to clump forming bundles and ropes. That is a problem since interactions within such aggregations quench fluorescence; for obtaining fluorescence they have to be disaggregated. This problem can be solved by surface functionalization and physical separation of the nanotubes[8].
Surface functionalization process consists of dilute nanotube product in an aqueous solution of a surfactant. That lowers the interfacial surface tension between the solution and the nanotubes, counteracting the tendency of nanotubes to aggregate. Then, physical separation of the SWNTs is obtained by tip sonication. Once the nanotubes are separated, the presence of surfactants prevents further aggregation. Right after the sonication the solution is centrifuged to separate SWNTs from bundles. Finally, it is possible to decant the supernatant to extract the nanotube solution[8].
There are many different surfactants –there are also substances which are not surfactants but can be used to functionalize SWNTs– and some of them can be poisonous for living organisms. Although we mainly used DNA to functionalize SWNTs used with worms, which is not poisonous, we tested how poisonous for *C.elegans* are the following commonly used surfactants: Pluronics F108 (known to be "biocompatible"[9]), SDBS and NaDOC (both known for obtaining good fluorescence with SWNTs functionalized with them[10]). The results appear in **Figure A1**.
In this thesis, SWNTs functionalized with surfactant X are referred as X-NTs and when the surfactant is not specified DNA-NTs are implied. Unfortunately, nanotubes functionalized with DNA have to be ultrasonicated for about 90 minutes in order to obtain enough individual nanotubes and not waste too much material. The previous mentioned surfactants are very cheap thus it is not a problem to

ultrasonicate shorter and lose some material. During the ultrasonication process nanotubes get broken. For this reason, DNA-NTs are usually shorter than the other ones.

1.4. Soft lithography microfluidics
Microfluidics refers to the process or manipulation of small amounts (10^{-9} to 10^{-18} litres) of fluids constrained in small (generally sub-millimetre) spaces. Its small size makes the behaviour of the fluid different from "macro-scale" fluids, making it even counterintuitive. Probably the most notable difference is that the flow through its microchannels is laminar, meaning that the fluid flows in parallel layers without lateral mixing. These novel characteristics offer the possibility to control concentrations and molecules in space and time[11].
Soft lithography refers to a series of lithography techniques which use elastomeric materials (soft and deformable materials at ambient temperatures), normally PDMS. These techniques have some advantages with respect to other kinds of lithography such as its low cost, the possibility to obtain a resolution up to several nanometres and the possibility to use them for biology applications[12]. Also, PDMS is transparent. Soft lithography microfluidics has been proven useful for a wide range of applications from channel fabrication to pattern generation[13].
In vivo C.elegans imaging typically involves the immobilization of the worm using glue or a microfluidic devise. The use of a microfluidic biochip is advantageous because it reduces the manipulation effort and offers a controllable, chemical-free microenvironment to image the worm. It also gives the possibility to deliver food or chemicals to the worm while imaging it[14]. Furthermore, it permits to control which part(s) of the worm to immobilize, the width of the movement of the rest of the body and its position. All that can be done keeping the worm alive for further experiments.

2. Methods

2.1. Worm handling
C.elegans culturing is described in detail in Brenner's 1974 paper[15]. For all the experiments we used the N2 strain of *C.elegans*, which corresponds to the wild type of this nematode. Worms are kept at a constant temperature of 15 or 20ºC (depending on the growth speed needed) in Petri dishes with NG Agar (Agar plates) seeded with OP50 *E.coli* bacteria which the worms feed on. Due to its small size, *C.elegans* handling has to be done with the help of binoculars; in our case we used binoculars MZ6 and S6E from Leica. For some experiments it was necessary to keep the worms in an aqueous solution but *C.elegans* thrive only in a relatively small pH range. In these cases an M9 buffer solution, unlike other more saline buffer solutions, is optimal for *C.elegans*' physiology. A buffer solution is an aqueous solution with the property that the pH of the solution changes very little when a small amount of strong acid or base is added to it. M9 buffer could not be used mixed with NaDOC because the mixture gelificated, in this case we used DI-water instead.
The most common way to obtain worms for an experiment is by taking them from the plate with a platinum stick (Pick) one by one. A very convenient way for unloading the worms from the Pick is by putting a drop of M9 buffer over the destination surface and introducing the Pick in the drop. Then, the worms begin to swim away leaving the Pick free. It is convenient to sterilize the Pick by introducing it into a little flame before loading and after unloading the worms. This method for moving the worms is precise but laborious and it is potentially harmful for them. As *C.elegans* have a fixed number of cells and cannot heal, it is important to assure the health of the worms. For this reason we used fluorescein dye for proving the health of the worms when moved with the Pick by me. When not ingested, fluorescein dyes are known to enter inside healthy worms just through the chemosensory organ (nose) showing fluorescence basically in a small area around this entrance[16]. In injured worms fluorescein enters also through the wounds showing fluorescence in other parts of the worm's body. The test showed no evidence of injures.
An easier way to obtain a large quantity of worms is by washing away the worms from a plate with M9 buffer (or DI-water if it is necessary). The procedure for washing a plate away consists in sprinkling the entire surface of it with the liquid using a pipette while holding the plate in an inclined position. The plate should be re-sprinkled using the liquid from inside of it as many times as needed to drag all the worms. Then, use the pipette to transfer all the liquid with the worms (from now on called worm solution) to an Eppendorf tube (Eppi). The Eppi with the worm solution has to be centrifuged

and the supernatant taken out with a pipette until getting the desired amount/concentration of worm solution. Finally it has to be mixed to have a homogeneous distribution of worms in the solution. Depending on the needs, the amount of liquid and the centrifuge speed may vary, but for a standard 6 cm Petri dish we used a 100-1000 µL pipette (blue tip), 2000 µL of buffer, a 2000 µL Eppi and we centrifuged it at 18ºC, 1800 rpm during one minute in an Eppendorf Centrifuge 5415R. This procedure is much faster than Pick taking and almost all the worms from a plate are taken. Besides, most of the bacteria and some eggs are also taken. Taking the bacteria can be an advantage in some experiments and a drawback in others. In the latter case it is possible to get rid of most of it by washing the bacteria from the solution out. This is done by adding new liquid after discarding the supernatant and further centrifuging. As bacteria are almost weightless, the centrifugation almost does not affect them thus a large fraction of them is taken out with the supernatant every time this is discarded. This process should be repeated at least twice in order of having a very low concentration of bacteria.

2.2. Feeding SWNTs to the worms

The preparation of the nanotube solutions used to feed the worms consisted on diluting normal stock nanotube solution with M9 buffer. The concentration we normally used was 1:100, although we also sometimes used 1:10, 1:1000 or other concentrations. For some experiments we used stock nanotube solution which was not as concentrated, in this case we used just 1:10 and 1:100 concentrations.

2.2.1. At the Petri dish

This is probably the most "natural" way to let the worms ingest nanotubes because they ingest the nanotubes while eating bacteria from the Agar plates in normal laboratory conditions. For this kind of feeding 3.5 cm Petri dishes are seeded with 70 µL of OP50 and let rest overnight. Next day 70 µL of the desired concentration of nanotubes solution is added carefully over the seeded bacteria. It is important to drop all the bacteria at the same spot to be able to cover all of it with nanotube solution. When the nanotube solution is soaked in the Agar (~1h), worms are transferred to the plate with a Pick. After a short time the worms go to the region seeded with OP50 to feed, ingesting nanotubes together with the bacteria. After the desired time (normally 1h, 4h or overnight) the worms are transferred to a new seeded Agar plate during ~0.5h in order to get rid of the faeces and superficial nanotubes before preparing the sample.

2.2.2. In an Eppi

Another way to make the worms ingest nanotubes is by filling an Eppi with M9 buffer and introducing worms in it with the Pick. Then, adding nanotube solution until obtaining the desired concentration and letting it rest in a shaker (we used an Eppendorf Thermomixer Comfort at 350 rpm, normally at 18ºC) for the time needed. After that, the solution has to be washed out (as described at the end of the section *2.1.*) one time, discard the supernatant and transfer the worm sediment to a new seeded Agar plate. When manipulating worms with a pipette, a 100-1000 µL pipette (blue tip) should be used in order to not harm them (the aperture of smaller pipettes' tip is too small). The worms should be deposited separated from the bacteria and wait for ~1h. During this time the remaining nanotube solution soaks and the worms get rid of the faeces and superficial nanotubes, being ready to prepare the sample. With this method it is very easy to lose some worms while pipetting solution in and out the Eppi, thus it is recommended to introduce an excess of ~15-20 worms.

We used this method because it exposes the chemosensory organ (which gives access to neurons) directly to the nanotube solution. We expected the nanotubes to enter through this organ and stain neurons. In order to facilitate the visualization of neurons' fluorescence we reduced the interference of intestinal fluorescence by keeping the worms at 4ºC while being in contact with nanotube solution because chilled worms do not feed[16]. Although intestinal fluorescence was dramatically decreased we still could not observer neurons' fluorescence. For that reason we repeated the same experiment using fluorescein (0.04, 0.2 and 0.4 mg/mL) instead of nanotube solution to check the possibility to stain neurons by this method.

2.3. Experimental setup description

The basic setup that was used for imaging SWNTs inside the worms consisted of a Coherent's Compass 561-40 laser (diode-pumped and continuous-wave yellow light (λ = 561 nm) with

P = 40 mW) as light source followed by a beam expander which was made with Thorlabs' doublets with f_1 = 40.0 mm and f_2 = 300.0 mm. Then, a third lens (Thorlabs doublet f_3 = 75.0 mm) preceded an AHF beamsplitter 630 DCXR. The beamsplitter directed the light to the objective (Zeiss Plan – APOCHROMAT 100x/1,46 Oil) and let the fluorescence coming back from the objective pass to the camera. A 900 nm-longpass filter (AHF ET 900LP) let only pass the infrared fluorescence light. The images were taken with an Andor's iXon^{EM} + DU-888 back illuminated EMCCD camera. The objective we utilized has to be used with a Zeiss specific tube lens (f_A = 164.5 mm) in order to correct all aberrations. This path was used to obtain high magnification (~100x) images. In order to have a larger field of view for the experiments with the trap we added a Photometrics camera (QuantEM:512SC) to the setup. The lens used to focus the beam to this camera was a Thorlabs doublet with f_P = 60.0 mm. The oil we used for the objective was Zeiss Immersol^{TM} 518F. The stage for the slide holder had movement in x, y and z directions with a high-precision positioning for the z direction. As the polarization of the laser light was not aligned with the beamsplitter, the sample was illuminated with elliptically polarized light. This way all the semiconducting SWNTs illuminated fluoresced regardless of their direction at the sample. The software used was Andor SOLIS for Imaging for Andor's camera and RS Image for Photometrics' camera.
Although we marked the position of the worms with a marker it was very useful to use a table lamp illuminating the sample indirectly to find them. With this method it was relatively easy to find the worm's x-y position. For the fluorescence imaging it was very important to keep the room dark in order to have a better signal-to-noise ratio from the fluorescence emitted by the SWNTs.

2.3.1. Sample preparation and imaging
The usual way to prepare a sample was to put a drop of ~5 µL of M9 buffer on a glass-slide prepared with a thin layer of agarose at the centre (agarose pad). Then, transfer between 5 and 15 worms to the drop with the Pick and add another ~5 µL of 1% sodium azide in order to kill the worms (it prevents the production of ATP inside the cells). Finally, cover the drop with worms with a coverslip and seal it with nail polish. It was convenient to mark the position of the worms with a marker in order to facilitate to find them using the setup. The thin layer of agarose avoids any movement of the worms due to gravity or transportation. It was important to wait until the nail polish was completely dry before using the sample in the setup, in order not to smash the worms while searching for the focal plane.
The inexistence of NI autofluorescence in *C.elegans* was tested by finding the x-y position of worms which were never in contact with SWNTs with a table lamp and subsequently shining them with the excitation laser. It was impossible to detect any fluorescence. That demonstrates that the fluorescence obtained in the experiments came only from SWNTs.

2.3.2. Trap experiment add-ons
Besides Photometrics' camera, to be able to proceed with the Trap experiment we had a Leica S6E binocular and a syringe pump by Harvard Apparatus (model: 55-2226). We also used two 2 mL plastic syringes (BD Discardit II) with a 21G needle (Terumo) and Polyethylene tubing (0.023" ID x 0.038" OD from VWR). The tubing was introduced in the in/outlets from the trap without the need of any kind of connecting pin and was connected to the syringes through the needles. One syringe was used by means of the syringe pump and the other one was used by hand. We also had a 2 mL Eppi filled with nanotube solution. The Eppi was sealed with parafilm. One needle was used to make a hole to the parafilm and one end of a piece of tubing was introduced right to the bottom of the Eppi and fixed with tape.
The binocular was used to be able to see the position of the worm when introducing it into the trap. It was introduced through the *worm inlet* with the syringe used by hand. When the worm was at the correct position, the pressure applied with the syringe was released. Then, the syringe pump, which was connected to the *outlet* of the trap, was turned on at 35 µL/min. Finally, the tubing from the Eppi with the nanotube solution was connected to the *food inlet* of the trap. After ensuring that the worm was still at the correct position, the trap was transferred to the slide holder in order to begin to take images. This was a crucial step and easily the worm could move. Sometimes this movement was small enough and it was possible to use the syringe to bring back the worm to its position. Otherwise the procedure had to be started again. When everything was in order and it was possible to begin to

image, the Eppi with the nanotube solution had to be kept ~20-30 cm higher than the trap to facilitate the flow. As the head of the worm was exposed to this flow, it was automatically ingested staining the intestines of the worm. **Figure A1** in appendix shows the path of a worm during the trapping process.

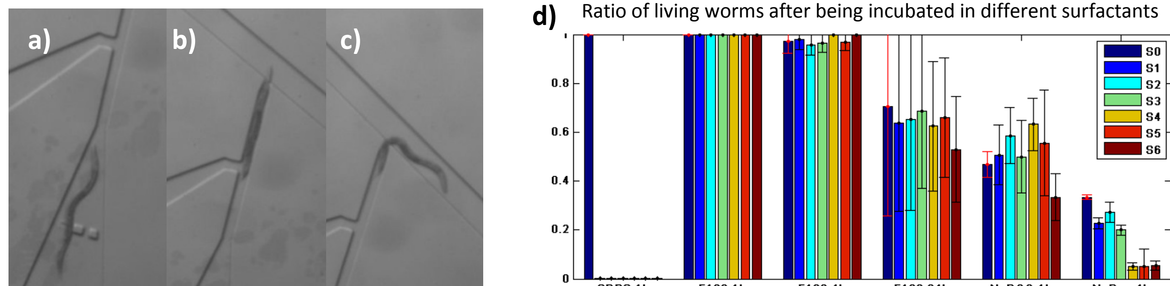

Figure A1: Worm's path during trapping: a) introduction, b) worm in position and c) extraction. d) Ratio of living worms after being incubated in different surfactants. Error bars correspond to standard deviation.

2.4. Microfluidic chamber

The route to build the microfluidic chamber with soft lithography consists of making the design, printing the mask from it, then making a mold and finally use this mold to build the chamber with PDMS.

2.4.1. Design & Mask

To design the microfluidic chamber AutoCAD Mechanical 2011 was used. The design is based on the microfluidic biochip from references[14, 17] but with some simplifications, see **Figure 1**. It has one *food inlet* (1) connected to the *food channel* (2), which ends at the *outlet* (3). The *worm inlet* (4) leads to two arrays of *micropillars* (5). These *micropillars* facilitate the proper head-to-tail position of the worm when heading to the *worm trap* (6). The end part of the *worm trap*, the *head aperture* (7), is designed to match the shape and size of the worm's head. The *microcolumns* (circular dots) were implemented to ensure the thickness of the larger parts of the chamber. One must take into account that PDMS is a deformable material thus it could bend at the big cavities. The design also has an *expel inlet* (8) which leads approximately to the middle of the *worm trap*. This part of the design was thought for the possibility to use a worm solution as a source of worms instead of introducing them one by one. In this case, pressure would be applied from the *expel inlet* when a worm had to be discarded, avoiding the introduction of a new worm during this process. At the end, this part of the design was not used, but it was not a disadvantage for the use of the microfluidic device at all.
A base design was prepared and from this design, a number of other designs with minor changes in the sizes of the different important parts (*head aperture*, *worm trap*'s width and distance between *micropillars*) were made. This way, we ensured to obtain a suitable trap for the experiment. To facilitate the introduction and the extraction of the worm, a step-architecture was implemented. The thickness of the immediate neighbourhood of the *worm trap* had to be smaller than that of the worm in order to trap the worm correctly. The rest of the chamber could be wider and this facilitated the movement of the worm in these areas. For this reason, two mask designs were needed for the construction of each chamber. One corresponds to the wide parts (dark blue in **Figure 1**) and the other embraces the entire chamber (dark blue + bright blue in **Figure 1**). The molds were fabricated on a 3" silicon wafer, thus each trap was small enough to prepare four traps on each wafer (**Figure 1**).
The design was processed and the mask printed in emulsion film with super high resolution by JD Photo (JD Photo-Tools, Oldham, UK).

2.4.2. Mold

The process of building the trap's mold had to be done at the cleanroom to avoid dust contamination. The mold is prepared on a silicon wafer with SU-8 photoresist. Photoresists are light-sensitive materials which crosslink (negative resists) or depolymerize (positive resist) upon light exposure. As the trap had a step-architecture, the mold had to be prepared with two layers. The bottom layer's thickness corresponded to that of the *worm trap* part. This part had to be ~28 µm thick to squeeze a bit the worm. To obtain this thickness SU-8 2025 (68.55% of solid content) is optimal[18]. The second layer had to be ~15-20 µm thick to let the worm move easily through the channels of the biochip (the layers superimpose). To obtain this thickness SU-8 2010 (58% of solid content) is optimal[19]. SU-8

2150 (77% of solid content) was used to prepare solutions matching the solid content these photoresists. That was done by thinning it with SU-8 2000 Thinner (MicroChem Corp); 12.33g of SU-8 thinner every 100g of SU-8 2150 and 32.76g of SU-8 thinner every 100g of SU-8 2150 respectively. The mixture had to be prepared in the cleanroom to avoid dust contamination of the photoresists.

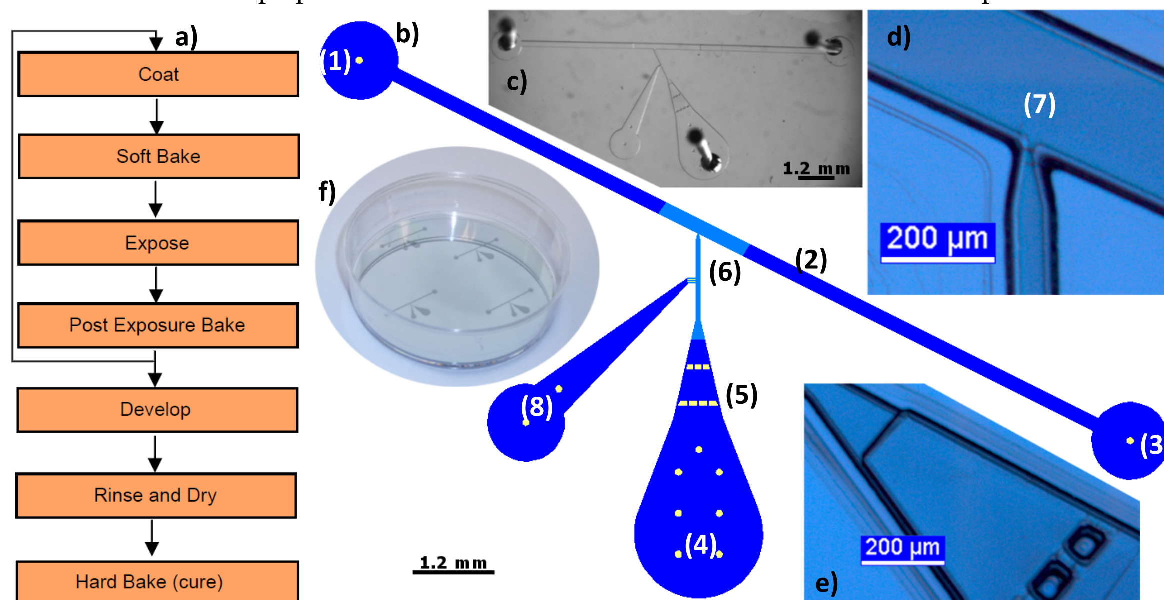

Figure 1: a) Flowchart for the production of the molds. b) Sketch of mold's design. Different tones of blue illustrate different layers of step-architecture: dark blue + bright blue base layer, dark blue extra layer. c) Image of a finished trap with its in/outlets. d) Detail of the *head aperture* of a mold. e) Detail of slight misalignment between layers of a mold. f) Image of a wafer with the 4 molds.

The process to build the mold is shown in the flowchart in **Figure 1**.
The settings for the 28 µm layer were (prior pouring of ~2 mL SU-8 2025 over the wafer):

Spin-coater: spin at 500 rpm for 15 s with acceleration of 100 rpm/s followed by 45 s at 2500 rpm with acceleration of 300 rpm/s.
Soft bake: 1 min at 65ºC followed by 5 min at 95ºC.
Expose: 7 s exposure of 365 nm light at constant intensity with low vacuum contact.
Post exposure bake: 1 min at 65ºC followed by 1 min at 95ºC.

The settings for the thin layer were (prior pouring of ~2 mL SU-8 2025 over the wafer):

Spin-coater: spin at 500 rpm for 15 s with acceleration of 100 rpm/s followed by 45 s at 4200 rpm with acceleration of 300 rpm/s.
Soft bake: 2.5 min at 95ºC.
Expose: align previous layer with the mask and 5.5 s exposure of 365 nm light at constant intensity with low vacuum contact.
Post exposure bake: 4 min at 95ºC.

Exposure was done with a Karl SUSS (MJB4) mask aligner. To extract the excess of material, the mold had to be developed (~7 min) with mr-Dev 600 (Microresist Technology). Then, washed by rinsing isopropanol and dried with nitrogen. The traps were examined with a Leica DM 4000M microscope to verify proper alignment and quality. Due to small differences in the amount of photoresist poured over the wafer and laboratory conditions, the thickness of the photoresist layers might vary from those expected. Thus it was needed to measure the thickness of both layers at different parts of each trap from the wafer with a Veeco Dektak 6M profiler. The hard baking process consisted in heating the wafer slowly up to 180ºC and decreasing the temperature also slowly to room temperature. If this process is done too fast, instead of sealing existing cracks it produces additional ones. Finally, the mold was put inside of a Petri dish to preserve it from dust.

2.4.3. PDMS chamber

To prepare the PDMS we used Sylgard 184 Silicone Elastomer (Dow Corning) which is supplied as two-part liquid component, base and curing agent. It was mixed 10 parts base to one part curing agent,

by weight[20]. Then, it was placed in a desiccator ("vacuum pot") until the vast majority of bubbles were gone. After that, the PDMS mixture was poured into the Petri dish with the mold inside and placed in a desiccator until there was no bubble left. The PDMS was cured overnight at ~50ºC.
When the PDMS was cured it was taken from the Petri dish and the holes for the in/outlets made with a cutting tip (Harris' Uni-Core) with a diameter of 0.75 mm. The four traps were cut off. Then, one by one, each trap was rinsed with isopropanol to ensure its cleanness, dried with Techspray ultra pure duster and by letting it rest for some seconds at 95ºC. Afterwards the trap and a 21x26 mm coverslip were put in a plasma oven (Harrick scientific corporation, model: PDC-002) at 200W for ~40-50 s and right after that joint together permanently. See image in **Figure 1**. This procedure was not done in the clean room but taking care not to contaminate the samples with any particle.

2.5. Surfactants' survival rate experiment
Solutions of the different surfactants were made to have a concentration of 2wt% of surfactant in M9 buffer (DI-water in the case of NaDoc) solution. This was made by putting 0.2 g of the surfactant in a little laboratory bottle and filling it in with the liquid until 10 g were reached (or using multiples of these values). For each surfactant experiment 7 samples were prepared with 200 µL of worm-solution, 2X µL of surfactant-NT solution and 2·(100-X) µL of M9 buffer (DI-water):

S0	X = 0	Blank sample	S4	X = 60	0.6% surfactant
S1	X = 10	0.1% surfactant	S5	X = 80	0.8% surfactant
S2	X = 20	0.2% surfactant	S6	X = 100	1.0% surfactant
S3	X = 40	0.4% surfactant			

The best way to prepare the samples was to first fill the Eppis in with the M9 (DI-water), then put the surfactant and finally prepare the worm solution and fill it in. When the samples were made, they were put in a shaker (350 rpm) at 18ºC during one hour, four hours or over night (~18h). After this time, the samples were mixt in order to have a homogenious distribution of worms and 100 µL of the solution was taken out and put in a fresh agar plate to determine the percentage of dead worms. This percentage was found by counting the total number of worms and of dead worms under the vision area of the MZ6 binocular at maximum magnification (4x). This was done various times per sample.
Due to the high mortality in the SDBS experiment, two more samples with 0.05 and 0.025% of surfactant were tried afterwards with no living worm found. See **A1** graph.

2.6. Progeny experiment and fluorescence in time
In order to know if there is transmission of SWNTs from the worms to their progeny worms were fed in a plate for 1h with 1:100 nanotubes. After this hour the worms were transferred to new plates two times (1h and overnight incubation respectively). Then, they were transferred to a new plate during 1h. After that, they were left to lay eggs in another plate for 1h and put back to a new plate. During the following days the worms were changed once a day. The progeny was imaged during adulthood.

3. Results

3.1. General
The fluorescence of SWNTs in *C.elegans* is present basically at the digestive system of the nematode regardless the kind of SWNTs, its concentration and the feeding way used. The larger accumulation appears to be in the pharynx, fluorescence is a bit less intense along the intestine and normally increases again in the rectum. See **Figure 2**. The intensity of this fluorescence makes it possible to see other parts of the worm such as *in uterus* eggs, the mouth cavity or even the tail. We were normally able to differentiate the different growth stages of the not laid eggs of imaged worms. As expected, F108-NTs emit less fluorescence inside the worm than SWNTs functionalized with the other surfactants. Fluorescence of DNA-NTs inside a worm is not dimmer than that of SDBS, and NaDOC's fluorescence intensity is a bit lower. Also, fluorescence in worms fed with SWNTs at 4ºC was significantly reduced. In several occasions individual SWNTs were seen inside *C.elegans*; **Figure 3**.

3.2. Microfluidic trap
It was possible to image the fluorescence of SWNTs inside living *C.elegans*. The flow of SWNTs at the top part of the *head aperture* was sometimes visible. It was also possible to see the movement of

the worm through the fluorescence of the SWNTs inside it. The distribution of SWNTs was similar to that of the other experiments. There was a leakage of nanotube solution inside the *worm trap* cavity, producing an accumulation of SWNTs around the trapped worm. Images are shown in **Figure 4**.

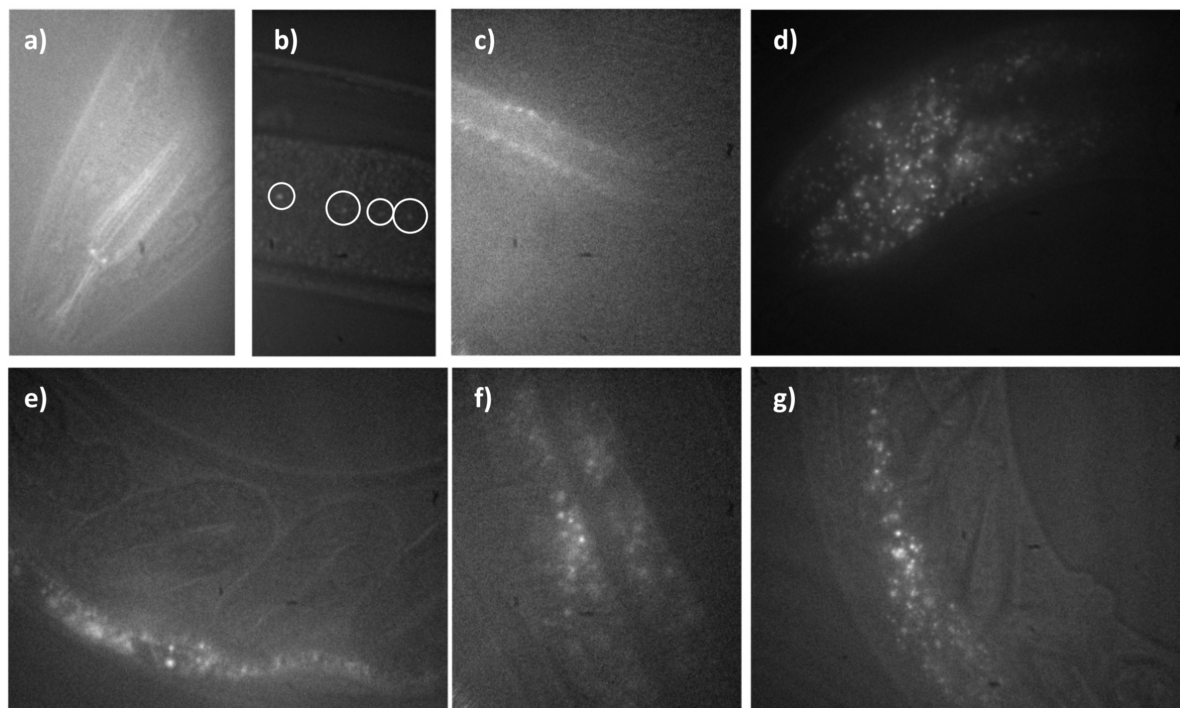

Figure 3: a) Mouth cavity; 1:100 during 5h. b) Four individual SWNTs; 1:100 during 1h at 4ºC. c) Fluorescence of 1:100 NaDOC-NTs during 4h. d) Fluorescence of 1:1000 DNA-NTs during 23h. e) Intestine fluorescence illuminating eggs; 1:100 SDBS-NTs during 3h. f) Dim fluorescence of 1:100 F108-NTs during 25h. g) Fluorescence of 1:100 SDBS-NTs during 4h.

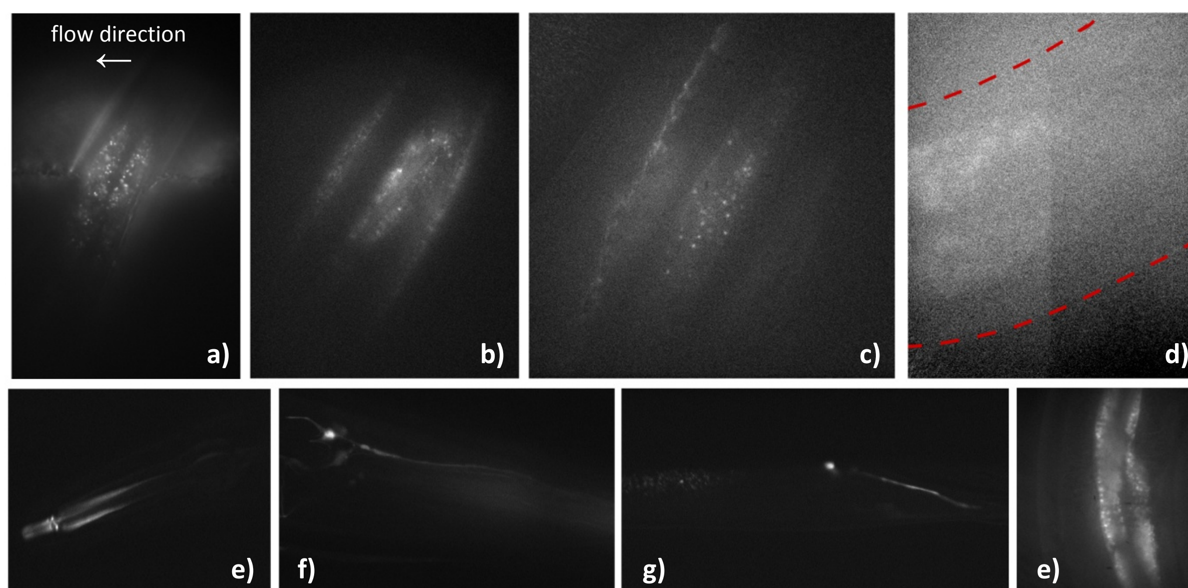

Figure 4: First three images are of an alive worm fed with 1:100 solution of SWNTs inside the microfluidic trap. a) Pharynx part. Connection between *food channel* and *head aperture* is visible due to SWNTs accumulation. b) Top part of the intestine. Accumulation of SWNTs between worm and chamber's wall is visible. c) Detail of the middle part of the intestine with the Andor camera. d) Pharynx image of a worm fed with 1:100 DNA-NTs during 1h 5 days before. Next three images are of fluorescein neuron's experiment. e) Nose entrance. f) Neuron coming from the chemosensory organ. g) Further part of the neuron. e) Fluorescence in the intestine showed by a progeny experiment worm with not enough plate changes.

3.3. Fluorescence in time and progeny

C.elegans expel progressively, although not completely, the nanotubes from within their entrails. Some days after the ingestion of the nanotubes, the remaining fluorescence is clearly decreased; see **Figure 4**. Progeny from worms fed with nanotubes do not show fluorescence, demonstrating that there is no *in utero* transference of nanotube to the eggs.

The nanotube elimination process is slow. This is, in part, because the worms reingest excreted nanotubes. This reingestion process is very effective. In previous progeny experiments, the worms were less often changed from plates and both, progeny and worms fed with SWNTs previously showed intense fluorescence. The fluorescence showed by the progeny came from the digestive system, suggesting that those nanotubes were ingested rather than transferred *in utero*. See **Figure 4**.

3.4. C.elegans' neurons experiments

Images obtained with fluorescein suggest the possibility to stain *C.elegans*' neurons with SWNTs. See **Figure 4**. Various attempts to visualize this fluorescence were done by using different concentrations of nanotubes, and exposure times. Also, nanotubes functionalized with different surfactants were used. The results with the Eppi method of feeding worms were not substantially different from those fed in a plate. The most remarkable result was that we were able to confirm that ice-cold (4ºC) worms do not feed, or at least feed very little. The fluorescence showed by worms fed in 4ºC nanotube solution was very dim, even in worms fed overnight.

4. Conclusions

It is demonstrated that DNA-NTs are very suitable for biologic fluorescence applications due to their bright fluorescence inside biologic tissue and low toxicity. That makes SWNTs very good candidates for motor labelling experiments. SWNTs are very persistent inside *C.elegans*; worms get rid of them slowly but when eaten again SWNTs still fluoresce with the same intensity. Due to their good fluorescence, with low concentrations of nanotubes good images can be taken.

It was also demonstrated the convenience of using microfluidic traps for *in vivo* fluorescence imaging of *C.elegans*. It is possible to stain *C.elegans*' neurons through its chemosensory organ, but in order to be able to do it with SWNTs in a reproducible way, more research has to be done.

Acknowledgements

I would like to thank Christian Doppler Laboratory for Cavitation and Micro Erosion and especially Robert Mettin for giving me a job and flexibility to do it. I would also like to thank the help of my advisors Nikta Fakhri and Dieter Klopfenstein and of the entire working group.

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