MIDTERM EXAM

ANSWERS

EXPERIMENTAL BIOPHYSICS

FFFN20 & FYST23

SATURDAY, MARCH 16, 2013

0800-1200

VICTORIASTADION ROOM 3D

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THE QUESTIONS ARE ORGANIZED ALONG THE CONTENTS OF THE LECTURES. IF YOU CANNOT ANSWER ONE QUESTION, DO NOT DWELL TOO LONG. TRY THE NEXT QUESTION INSTEAD!

FEEL FREE TO DRAW FIGURES IN YOUR ANSWERS. OFTEN IT IS EASIER AND MORE EFFICIENT TO CONVEY A MESSAGE USING A FIGURE THAN IN TEXT.

TYPICALLY, FOR A PASSING GRADE [G], YOU SHOULD HAVE AT LEAST 50% CORRECT, AND A PASSING WITH DISTINCTION GRADE [VG] REQUIRES AT LEAST 75%. THE FINAL GRADING SYSTEM USED WILL DEPEND ON IN WHAT CONTEXT YOU FOLLOW THE COURSE.

MAKE SURE TO WRITE YOUR NAME ON EACH SHEET OF PAPER. USE A NEW SHEET OF PAPER FOR EACH NEW FIELD, *I.E.* ONE NEW SHEET OF PAPER FOR *BIOLOGY AND SCALES*, ONE NEW SHEET FOR *OPTICS ON THE SMALL SCALE*, ETC ETC.

NOTE THAT THE NUMBER OF POINTS ASSIGNED TO EACH QUESTION GIVES YOU INFORMATION ON HOW MUCH TO WRITE.

HAND IN YOUR ANSWERS TOGETHER WITH THE QUESTIONS.

A FEW USEFUL NUMBERS:

$1\ K_BT \approx 4$ PN NM $= 4\ 10^{-21}$ J AT ROOM TEMPERATURE

$N_A = 6\ 10^{23}$ MOLES^{-1}

$\eta_{WATER} = 1\ 10^{-3}$ KGM^{-1}S^{-1} AT ROOM TEMPERATURE

GOOD LUCK!

JONAS T

1. Biology and Scales [13p]

Use a new sheet of paper for these questions.

[a] The genetic information flows through three important groups of biomolecules in a living organism. What are they and how are they related [hint: The central dogma of molecular biology]? What is the relative stability of each one of the molecules? Why? [6p]

DNA, RNA and proteins [3p]. DNA stores the information. When a protein is needed, a gene is activated and a copy of the DNA is transcribed into an RNA. The RNA is then used as a template to assemble peptides into a polypeptide chain that is then folded into a protein [translation]. [2p]

DNA is most stable typically followed by proteins and finally RNA. DNA stores the blueprint and must be stable over the life span of the organism at least. Proteins are used in various parts of the body with a wide range of time scale. Their life time vary widely therefore. RNA is primarily an intermediate messanger from DNA where the information is stored to the protein. To be able to vary protein production, the messanger (RNA) must have a lifetime less than the timescale at which the regulation needs to take place. [1p]

The question can also be interpreted as referring to the underlying mechanism making the RNA less stable. Ribose instead of deoxyribose makes it more hydrolyzable. [1p]

[b] Diffusion is an important means of transport on the small scale. A typical human cell has a size of roughly 30μm, but some cells are up to 1 m in length. What would the diffusion time for a typical protein, virus and bacteria be across these two length scales? [5p]

$$\left\langle r^2 \right\rangle = \left\langle x^2 \right\rangle + \left\langle y^2 \right\rangle + \left\langle z^2 \right\rangle = 3 \cdot 2Dt = 6Dt$$

Stokes-Einstein relation gives (with f = the viscous drag; F_{drag}=f v)

$$D = \frac{k_B T}{f} = \frac{k_B T}{6\pi\eta a}$$

$$\Rightarrow t = \frac{\left\langle r^2 \right\rangle}{6D} = \frac{\left\langle r^2 \right\rangle 6\pi\eta a}{6k_B T} = \pi \frac{\left\langle r^2 \right\rangle \eta a}{k_B T}$$

With typical sizes: protein - 10nm; virus 100nm; bacterium 1µm (radii)

With typical numbers for water at 25°C we have

$$k_B T = 4 \cdot 10^{-21} J [= kgm^2 s^{-2}]$$

$$\eta = 1 \cdot 10^{-3} kgm^{-1} s^{-1}$$

$$\Rightarrow t = \pi \frac{\langle r^2 \rangle \eta a}{k_B T} = \pi \frac{1 \cdot 10^{-3} kgm^{-1} s^{-1} 10^{-6} m \, 1m^2}{4 \cdot 10^{-21} kgm^2 s^{-2}} \left(\frac{\langle r^2 \rangle}{1m^2} \right) \left(\frac{a}{1\mu m} \right)$$

$$\Rightarrow t = 785 \cdot 10^9 s \left(\frac{\langle r^2 \rangle}{1m^2} \right) \left(\frac{a}{1\mu m} \right) = 25000 years \left(\frac{\langle r^2 \rangle}{1m^2} \right) \left(\frac{a}{1\mu m} \right) = 785 ms \left(\frac{\langle r^2 \rangle}{1\mu m^2} \right) \left(\frac{a}{1\mu m} \right)$$

	size	*diff time (r=30μm)*	*diff time (r=1m)*
protein	*10nm*	*7s*	*250 yrs*
virus	*100nm*	*71s*	*2 500 yrs*
bacterium	*1000nm*	*12min*	*25 000 yrs*

This is a rough estimate. Values may differ if numerical prefactors are ignored.

[c] What alternative to diffusion as a means of transport do we see inside cells? Why? [2p]

Motor proteins. Diffusion is really slow compared to relevant time scales of human life! [2p]

OR

Ion pumps. Diffusion only takes place against the concentration gradient. [2p]

2. Optics on the small scale [20p]

Use a new sheet of paper for these questions.

[a] In a typical single-molecule imaging experiment you would see several bright spots using your camera. How do you tell whether these spots indeed represent the emission from single individual molecules? [3p]

- *Blinking, ON/OFF behavior – the intensity is either on or off, never in between*
- *Reasonable photon count*
- *Antibunching – the photons come one by one, not two at the same time.*

[b] There are several sources of noise that need to be considered when designing a single-molecule experiment. Assuming that the signal is given by the number of detected photons, *N*, explain the sources of noise and give an expression for the signal to noise ratio, *S/N*. [3p]

- *Shot noise - inherent noise due to the quantized nature of light. Light comes in packages that are Poisson distributed in time.*
- *Readout noise - each time the signal is read out from a pixel in the CCD, a finite amount of noise is added.*

- *Background noise - light in the room and fluorescence from contaminants and other sources.*
- *Dark-signal noise - thermally activated electrons give rise to noise.*

Note that the noise here refers to the fluctuations in the number of electrons measured, not the absolute level. The absolute level is also important, but only if the total charge in each pixel exceeds the dynamic range of the CCD.

$$\frac{Signal}{Noise} = \frac{N}{\sqrt{\sigma_{shot}^2 + \sigma_{readout}^2 + \sigma_{background}^2 + \sigma_{dark}^2}}$$

$$\frac{Signal}{Noise} = \frac{N}{\sqrt{N + n_{readout} + (C_0 + C_b P_0)T + c_d T}}$$

[c] What strategies can be employed to reduce the amount of noise and thereby enable single-molecule detection? What other actions must be taken to make it possible to see individual molecules? [6p]

- *Shot noise - just detect more photons through more efficient detection system, less losses in filters and optics, better dyes etc. Note that the excitation intensity should not exceed what would saturate the dye, as given by the fluorescence lifetime. Shot noise is a fundamental physical characteristic of light. It cannot be avoided. [1p]*
- *Readout noise - use an EM CCD that amplifies the signal in each pixel. This essentially removes the contribution of this noise source. Use one long exposure rather many exposures that are subsequently averaged. [1p]*
- *Background noise - work in a dark room; use clean buffers; use low fluorescence substrates such as quartz or fused silica; low fluorescence optics and immersion oil; minimize excitation volume through the use of TIRF, confocal, SNOM etc. Choose a more red shifted dye. Match the filter set to the dye: Select filters such that only the wavelengths to excite the dye are used and such that only the wavelengths of the emitted light from the dye is detected. This may remove most of the contribution of this noise source. [2p]*
- *Dark-signal noise - cool the CCD. This may essentially remove the contribution of this noise source. [1p]*

Low concentration of sample so that only one molecule is excited at once

[d] STORM is a type of microscopy that allows you to exceed the diffraction limit. What is the basic idea? How can high-resolution 3D imaging capabilities be implemented? [4p]

STORM (Stochastic optical reconstruction microscopy) is based on the idea that a random subset of fluorophores is activated. The density of the fluorophores is such that the fluorophores can be resolved optically. In this way, using FIONA[4], the position of each fluorophore can be determined to a high accuracy. By repeating the activation several images is the result each with its fluorophores accurately determined. By combining the images a high-resolution image is the result.

In practice for the dye system used in the early paper[5]:

-strong RED light to deactivate all fluorophores

subsequently

-weak GREEN light to activate a small subset

-weak RED to image the activated fluorophores until switched off

- go back to weak GREEN and repeat the process until most of the fluorophores have been imaged at least once and/or most of the fluorophores are bleached so that no further measurements are possible.

Obviously the dye system must be switchable. In the first paper a system of Cy5 and Cy3 in close proximity is used. The Cy3 must be as close as possible to the Cy5 to ensure high recovery rate.

Note, that this is not a question of resolving two spots. Instead the position of a point-spread function is determined to arbitrarily high precision. By measuring the position at two different times or using two different wavelengths, the diffraction limit can be superseded.

3D imaging is realized using a cylindrical lens in the emission light path. This makes the image astigmatic. From the orientation and size of the resulting point spread function the position in z can be calculated.

[e] STED is a powerful optical technique. Describe its major features. What resolution can it achieve? [4p]

Principle: Excitation in a standard Gaussian mode. Stimulated emission in a ring-like mode. The stimulated emission is at a wavelength that is filtered out and does not reach the photodetector. In effect this means that the central spot is decreased in size. To go beyond the diffractin limit it is important that the stimulated emission is depleted, i.e. that the STED beam has an intensity greater than saturation.

Performance: High-resolution, sub-diffraction limit imaging in the far-field.

Basicly the standard diffraction limit is improved by the STED factor.

In practice ~30nm resolution, but requires skill and expensive equipment.

3. Basic Fluidics [18p]

Use a new sheet of paper for these questions.

[a] In the world as we humans see it, most fluid transport is realized by pressure driven flow. Why is that not suitable for micro- and nanofluidics, the world as bacteria see it? Describe in a few words each three alternative techniques to transport of sample and/or fluids! [6p]

Surface to volume ratio is high leading to a high fluidic resistance such that for a given pressure difference we have $v \sim a^2$ and flow rate $\sim a^4$. In other words, the rate of fluid transport decreases rapidly with decreasing channel size.

Another type of problem associated with pressure driven flow is that a sample plug is spread out because of the parabolic flow profile.

Alternative techniques to avoid the strong size dependence, $v \sim a^2$:

- *Electrophoresis – charged particles will move in an applied electric field.*

- *Electroendosmosis – due to surface charge of the channel, the loosely bound ions in the diffuse double layer will move, thereby dragging the fluidi with them.*
- *Capillary force – surface energy drives the water into a hydrophilic channel.*
- *Dielectrophoresis – polarizable particles (charged microbeads, viruses, DNA etc) can be moved in an electric field gradient.*

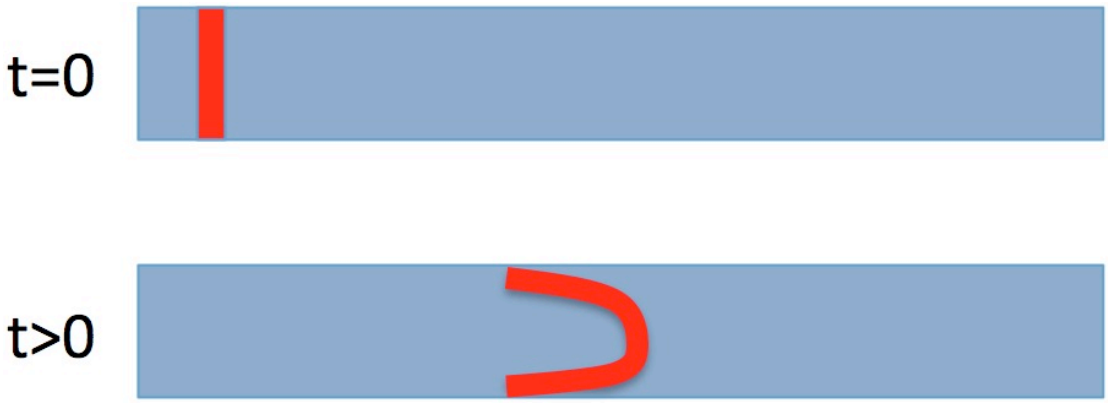
To avoid the spreading of a sample plug:

- *Taylor diffusion (see question 3c).*
- *Confine the sample in a droplet (see question 4d)*

[b] Although pressure-driven flow is not ideal for small channels it is nevertheless used. Assuming a simple channel with one end at atmospheric pressure it can be implemented in two ways: either vacuum or over pressure can be connected to the other end. Discuss any disadvantages and advantages of the two approaches. [4p]

- *Overpressure: device can break apart. Mtrl leak out of device. Risk that the particles cannot stand the pressure. Any magnitude of pressure can be obtained. Less bubbles.*
- *Vacuum: bubbles can form in the device; maximum pressure difference is 1 atm. Air can be sucked into device. The device can collapse or the device is held together by the vacuum.*

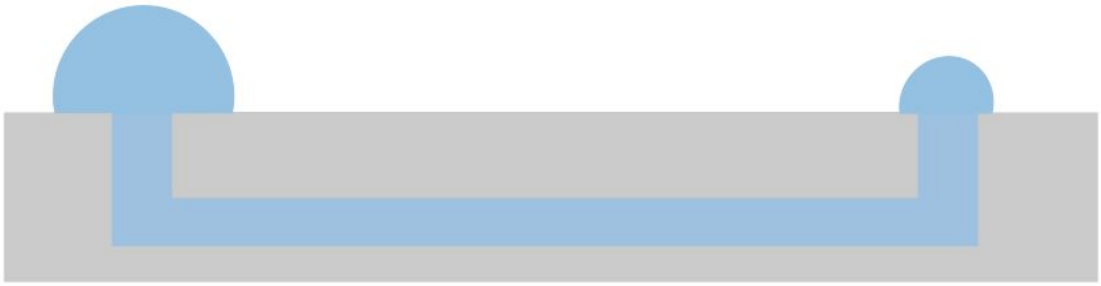
[c] A plug of sample in a fluidics channel is normally deformed due to the parabolic flow profile of the liquid. How can diffusion help to minimize the distortion of a sample plug in a pressure-driven flow? [4p]



Parabolic flow profile spreads out the plug ~time.

The solution is Taylor diffusion: Combined with lateral diffusion the spread of the plug is instead ~sqrt(time).

[d] The capillary effect will result in the filling of water in a small channel (made in silicon oxide). Explain why! What is the fundamental reason? In the case shown in the figure, what will the end result be (at time-> inf; assuming no evaporation)? Why? [4p]



Surface energy will drive the liquid to the end of the tube. The interfacial energy of the air-solid interface exceeds that of the liquid-solid interface. To minimize its total energy the system therefore rearranges such that the air-solid interfacial area is minimized.

To minimize the total interfacial energy, the area of the water-air interface must be minimized. For a given volume of water, a single semisphere has the smallest area. Therefore in the end there will be one drop of water on the left side side.

This can be shown by calculating the total energy of the system using the surface energies for the three different interfaces (solid liquid, solid air, liquid air). Then considering the total volume of the liquid outside the channel, the relationship between the sizes of the two semidroplets can be established. Finally, using Yong's equation together with the known contact angle, we can eliminate two of the surface energies.

It can now be shown that the derivative with respect to the radius of the larger droplet is always negative, i.e. the energy decreases with increasing size of the droplet.

If the droplets start off as strictly equal, the derivative with respect to the radius of the droplet is zero, but this is not a realistic physical situation. There are always small fluctuations, and as soon there is an inequality, transport of liquid will start to amplify this inequality.

The phenomenon is known as Ostwald ripening.

4. Fluidics Applications [20p]

Use a new sheet of papers for these questions.

[a] The H-filter, pinched flow device and the bumper array (deterministic lateral displacement) are all three particle-sorting devices. Very briefly, what are the basic ideas of each one of them? How are their functions connected? What are their limitations? [6p]

- *H-filter – see fig 10 in ref [4]. Sorting based on diffusion and can therefore not be run arbitrarily fast. If Pe>1 for all of the particles, no separation occurs. A fluidic arrangement shaped like an H. A sample solution is run from one side to the other in one pair of reservoirs. In the other reservoirs plain buffer is run. The plain buffer will pick up particles in the sample stream if the particles diffuse sufficiently. Large particles will not diffuse and so exit in only one reservoir. Small particles will diffuse and exit in both reservoirs. Low precision.*
- *The bumper array is deterministic and does not rely on diffusion. It can therefore be run at high speeds without any loss in performance (as long as laminar flow conditions are maintained). High precision. Risk for clogging.*

- *Pinched flow – see ref [5]. Two flow streams (sample and buffer) are squeezed through a narrow constriction and then expanded. Any particles in the sample stream with radii larger than the minimum width of the sample flow stream will migrate over to the buffer stream. It corresponds essentially to the bumper array, except that in this case there is only one "bump".*

The bumper arrays is based on the exclusion of beads with radii larger than a critical size from one flow stream, just like in pinched flow. On the other hand, in the bumper array the size selection happens multiple times as opposed to in the pinched-flow device where it happens just once.

[b] The mechanical properties of cells is relevant as a gauge to measure for example their metastatic potential. Give an example of a microfluidic device that can be used to fractionate cells based on their softness. [4p]

The bumper array can be used to sort cells in space based on their softness. The shear flow in the bumper array causes the particles to deform, thereby obtaining a smaller effective size. Equally sized particles with different deformability will therefore have different trajectories.

[c] Normally microfluidics implies dominance of viscous effects. However, running at higher speeds may result in useful focusing of microparticles. Under what conditions does this happen? Explain qualitatively the basic mechanisms involved and what overall design is required. [6p]

Focusing of the streams is the result of three interacting components: inertial lift due to the "wall effect" from the wall; inertial lift due to the shear gradient from the center towards the wall; Dean vortices. An asymmetricly curved channel will result in the focusing of one single stream. The focusing is strongly dependent on the size of the particles so that by careful extraction of the focused stream size-based sorting can be implemented.

A Dean vortex is formed in a curved channel (radius of curvature R) where the inertial forces act on the fluid. Since we have a parabolic flow profile, the centrifugal force will not be equal for each part of the fluid. The fluid at the center will move at the highest speed and so will experience the greatest centrifugal force. See fig 4 in Squires' and Quakes' review paper[9].

Dean vortices are formed because of the centrifugal force on the fluid. The inertial effects refer to effects of the fluid, not on the particles. This was shown by running particles in buffer solutions of different densities where the result was the same independent on whether the particles were denser or less dense than the surrounding fluid.

[d] "Digital fluidics" is another name for microfluidics based on small droplets. This can be useful for genomics or single cell biology. Explain how these droplets are formed. [4p]

Viscous stress [unit force per unit area], $\sim \frac{\eta U_0}{h}$*, tends to tear apart droplets, making them smaller, whereas the capillary stress,* $\sim \frac{\gamma}{R}$*, (essentially the energetically unfavorable formation of additional oil-water interface) drives the size of the droplets*

upwards. The equilibrium radius is thus given by: $R \sim \frac{\gamma}{\eta U_0} h = \frac{h}{Ca}, \quad Ca = \frac{\eta U_0}{\gamma}$, *where Ca is the capillary number.*

5. NanoSafety [13p]

Use a new sheet of paper for these questions. Draw figures!

[a] Which are the principal two cell types involved in a foreign-body reaction in the brain? [4p]

- The microglial cells (ED1-positive cells), which are the resident macrophages of the brain and phagocytize any infectious agents and intruders. They are part of the immune response of the brain. Upon brain injury, there are also recruited macrophages (from outside the brain). [2p]

- The reactive astrocytes (GFAP-positive cells): these cells are involved in tissue repair. [2p]

[b] Many studies of nanoparticle toxicity give conflicting results. What could the reason for such conflicting results be? [4p]

The detailed structure and arrangement of the nanoparticles may differ in different studies without being mentioned in the text.

- *Degree of aggregation.*
- *Size distribution.*
- *Shape.*
- *Surface charge.*
- *Penetration in tissue*
- *Different tissues used*

[c] With what mechanisms may nanoparticles cause cell damage? [2p] Under what circumstances may this be a good thing? [1p]

In total [3p].

- *Upon photo-oxidation of the NP, Cd^{2+} ions are produced, which bind to mitochondrial proteins, diminish cellular respiration and cause cell death.*
- *In general, Cd is a carcinogen (interference with DNA repair processes) and a neurotoxin (influencing lipid peroxidation).*
- *It has persistent effects in biological systems.*
- *Cd binds to metallothionein protein, disrupting zinc metabolic pathways in the liver and kidney.*
- *Formation of reactive oxygen species*
- *Nanoparticles could be used as anticancer drugs killing nearby cells. Also in combination with photoactivation (photodynamic therapy, PDT)*

[d] An important regulatory problem is how to define safety thresholds over which a user needs to take specific precautions. Typically for standard chemicals the threshold is defined by a certain amount of material. For example, in Europe if more than 10kg

of a new chemical is handled per year, detailed testing needs to be done of the toxicity of the compound.
However for nanoparticles this may not be appropriate. Explain why.
[2p]
Nanoparticles have much higher surface area per unit mass. Combined with the fact that the greater surface area to volume ratio is an important factor contributing to the nanotoxicity, this makes the mass criterion irrelevant. Instead a surface area criterion should be used.

6. Molecular Motors [16p]

Use a new sheet of paper for these questions.

[a] Define "processivity" and "duty ratio". What tasks in the cell require processive and non-processive motors? [4p]

Answer: processivity: the degree by which a motor takes several steps along the track (note that this number is a stochastic quantity). Several steps on average – processive

non-proc: force and speed; proc: cargo transportation

Duty ratio: fraction of time that a motor spends attached to the filament

[b] For myosin moving along actin, one may consider two hypothetical ways that the myosin takes the steps. What are these two? Explain one experiment how these two hypotheses can be tested? [4p]

Two ways of interpreting this question: could be (A) myosin V (a bipedal motor) or (B) myosin II (as in skeletal muscle, with only one head)

Interpretation A:

Possible answer 1: Two hypotheses: hand over hand and inchworm.

Attach a dye on one of the legs of the myosin molecule. Determine the location of the dye with high accuracy using FIONA.

Hand over hand: two step sizes are seen: short - long - short - long etc…

Inchworm: one step size is seen

Possible answer 2: One can discuss the relative importance of a "power stroke" (directed motion) and of diffusion in stepping. To help resolve this, one can try to resolve the pattern of motion during the stepping itself (looking for substeps) with FIONA or similar, and determine the characteristic time constants. For myosin V one finds a fast motion (consistent with a power stroke) followed by a slower phase (consistent with diffusion).

One can also use modeling to test various models against real data (where again substep information is highly useful)

Interpretation B:

Because myosin II has only one head, the inchworm vs hand-over-hand question doesn't come up. However, the relative role of diffusive and "power-stroke" like motion has been discussed (and to some, is unresolved).

[c] Assume that the mean square fluctuation $\langle x^2 \rangle$ of a bead in a laser trap is 4 nm^2. When an actin filament is attached to this bead and a myosin motor exerts force on the filament the bead is displaced 4 nm away from the centre of the trap. Which is the force exerted by the motor? (Hint: 1 $k_BT \approx 4$ pN nm) [4p]

Answer: Equipartition - (1/2) k_BT = (1/2) $k\langle x^2 \rangle$ where k is trap-stiffness. Now it is clear that k = 1 pN/nm. Thus, a displacement of 4 nm corresponds to a force of F = kx= 4 pN

[d] Applying sufficient force to stop the motion of a motor is known as the *stall force*. How does the motion of a molecular motor and an everyday macroscopic electric motor or combustion engine differ? [2p]

- *molecular motor: microscopically equal motion in two directions*
- *combustion engine: complete stall even at microscopic scale*

[e] The velocity of one single actin-myosin pair is on the order of 10μm per second. How come we can move our muscles much faster than that? [2p]

Many contractile subunits (each engaging many motors) coupled in series. The individual displacements of all subunits add up and give a much larger total displacement in the same time, thus faster.