| ESRF | **Experiment title:** X-ray scattering study of excitable membrane : correlation between membrane structure and nerve physiological states | **Experiment number**: SC 1681 |
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| **Beamline**: ID02 | **Date of experiment**: from: February 4, 2005 to February 6, 2005 | **Date of report**: August, 23, 2005 |
| **Shifts:** 9 | **Local contact(s)**: S. Finet & . Narayanan | Received at ESRF: |


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## Report:

The X-ray scattering experiments carried out at LURE from 1998 to 2004 on pike olfactory nerves - the first X-ray scattering study ever performed on excitable membranes - yielded highly valuable information on the structure and the thermodynamic properties of the axonal membrane (Luzzati et al, J.Mol.Biol.2004**, 343**, 187-197 and 199-212). The nature and the quality of the results emboldened us to address to the experimental facilities available at the ESRF to tackle the putative structural events associated with the propagation of action potential.

After a few test experiments performed in November 2004 we were allocated 9 shifts from February 4 to 7, 2005. We report here on the results of these experiments and on the present state of the analysis (that is still underway).

**Technical aspects and experimental set-up**

i) - *Animals handling and nerve dissection.* The problems of purchasing, transporting and keeping the fishes alive and of dissecting the nerves at the pace of the X-ray scattering experiments were all solved with the skilled and friendly collaboration of the staff of the ESRF animal house.

ii) - *Sample holder.* We designed a humidity- and temperature-controlled sample holder allowing us to perform electrophysiological and X-ray scattering experiments - namely to expose the nerve to the X-ray beam and to record the scattering spectrum over time windows of variable width set at different times after the onset of the electrical stimulation (see Fig. 1).

iii) - *Electrophysiological experiments.* The experimental set-up is represented in Figure 1. The nerve is supported by a comb of platinum electrodes: G is a grounding electrode, a 15 V stimulation of short duration (2 ms) is applied *via* the electrode S, action potential is recorded between the electrodes $R_1$ and $R_2$. The X-ray beam is centred midway between $R_1$ and $R_2$. The signal recorded displays a maximum (phase 1) and a minimum (phase 2). Phase 1 involves a portion of the nerve that is not exposed to the X-ray beam; its amplitude was thus used to estimate the physiological state of the nerve during the overall time of experiments. Phase 2, in contrast, is recorded beyond the irradiated region of the nerve and was thus used to assess radiation damage.

iv) - *X-ray scattering experiments.* The spectra were recorded from s=0.0055 to 0.085 $Å^{-1}$ ($s=2\sin\theta/\lambda$, $2\theta$ is the scattering angle and $\lambda$ the wave-length), a wider range than in the previous LURE experiments ($0.0045<s<0.04$ $Å^{-1}$). The spectrum of the axonal membrane (the signal) was separated from the noise issued

from the cytoskeleton via an algorithm based upon the notion that the noise is nerve-dependent whereas the signal is the same in all the nerves. When dealing with one and the same nerve it is thus possible to explore the time-course of the structure of the axonal membrane and to assess the relative amount of the cytoskeletal elements.

v) - *Error analysis.* The X-ray scattering spectra undergo mathematical treatments whose issue hinges upon a reliable assessment of the statistical errors that affect the data. We approached the problem empirically, by comparing different spectra recorded in the same conditions.

vi) - *Time resolved experiments.* We greatly benefited from the sub-millisecond beam shutter recently developed for muscle diffraction experiments. Synchronization of nerve excitation, shutter opening, X-ray and electrophysiological data recording were easy and effective.

vii) - *Beam collimation and air scattering.* Two drawbacks had unfortunate consequences on the experimental results. One was the radiation scattered by the air column ahead of the nerve, whose effects could have been reduced by a proper design of the sample holder and of the collimator. The other was the narrowness of the entrance window on the detector side.

**Results**

i) - *Radiation damage.* The brilliance of the beam –beam exposures at ESRF were more than $10^4$ times shorter than at LURE - allowed us to record reasonably good spectra in a few milliseconds and excellent ones in 100 ms, but raised the problem of radiation damage. In order to keep damage under control we resorted to electrophysiological and X-ray scattering criteria. For this purpose we performed series of 100 ms X-ray scattering experiments, keeping the electrophysiological activity of the nerves under control throughout the entire cycle.

From the electrophysiological viewpoint the amplitude of phase 1 of action potential remained unchanged during the first 45 minutes and then decreased progressively to approximately 50% in approximately 2 hours. In contrast the amplitude of phase 2 - sensitive to radiation damage - remained constant for only 500ms and decreased to approximately 50% of its initial value in the following 2s (Fig. 1).

The scattering spectra remained unaltered for X-ray exposures not exceeding approximately 1s and then underwent subtle alterations: a) - it took a few seconds before the axonal membrane structure became perceptibly altered; b) - the variance of the scattering data increased with exposure time, suggesting that the structural effects of X-ray exposure are heterogeneous; c) - the cytoskeleton contribution to the spectra seemed to be more sensitive to radiation than that of the axonal membrane.

The main conclusion was that X-ray exposure must be short, and that the effects of radiation damage must be taken into account when exposure times exceed 500 ms.

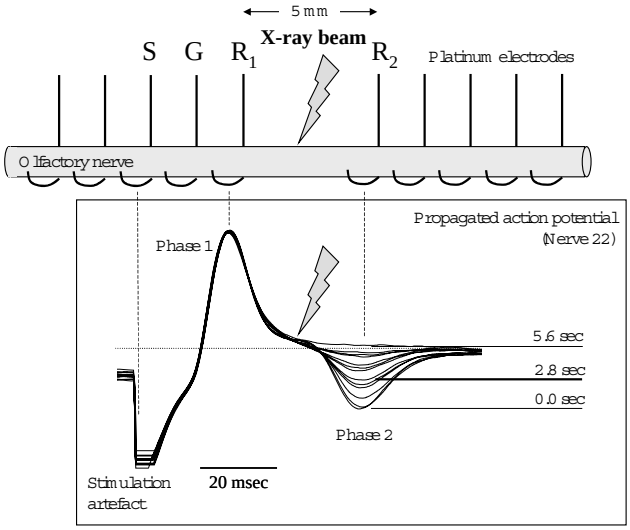


**Figure 1.** Radiation damage from an electrophysiological viewpoint.

ii) - *Membrane pairing.* An unexpected and puzzling finding of our previous work was that upon isotonic-KCl-induced depolarization a large fraction of the axonal membranes associates in pairs by apposition of their extra-axonal faces. This phenomenon was observed in 6 out of 10 experiments performed at LURE. The factors ruling the phenomenon and its possible biological significance were not clearly understood. We have now collected 17 spectra of paired membranes that are all almost identical to each other. Pairing thus seems to correspond to a stable and reproducible state of the axonal membranes and the structure of the pairs deserves a more careful analysis.

We also tested the effects of a variety of physiologically relevant agents. The membrane-pairing effect of isotonic KCl does not seem to be sensitive to external $Ca^{++}$ concentration (or to that of other divalent cations), nor to the presence of tetrodotoxin, a drug known to block the opening of $Na^+$ channels and thus the transmembrane $Na^+$ flux. In contrast, no pairing was observed in the presence of. tetraethylammonium chloride, a drug known to block the opening of $K^+$ channels and thus the transmembrane $K^+$ flux. It thus

appears that isotonic KCl-induced depolarization leads to membrane pairing both in the presence and in the absence of the transmembrane flux of $Na^+$ ions, whereas the flux of $K^+$ ions is required for the phenomenon to occur. This observation may have promising physiological implications.

iii) - *Time-dependent X-ray scattering experiments* (Figs 2 and 3). We performed different types of time-dependent experiments. Disentangling the effects of action potential from those of radiation damage and of electrical stimulation in 900-odd noisy spectra turned out to be a tricky endeavour still underway. At the present state of the analysis two results are firmly established: *a*) - the space correlation of the membranes in the axon bundles - as revealed by the very-small angle part of the spectra - is time-dependent, with a maximum centred at approximately 55 ms from the onset of action potential (apparently in pace with the closing of $Na^+$ channels and the opening of $K^+$ channels since this is the time required for the spike of action potential to reach the irradiated area of nerve). *b*) - The internal structure of the axonal membrane - as revealed by the outer part of the spectra ($0.04<s< 0.08$ $Å^{-1}$) - is also time-dependent. The process seems to be bimodal, with two events centred at approximately 20 and 80 ms from the onset of action potential (apparently in pace with the opening of respectively the $Na^+$ and the $K^+$ channels).

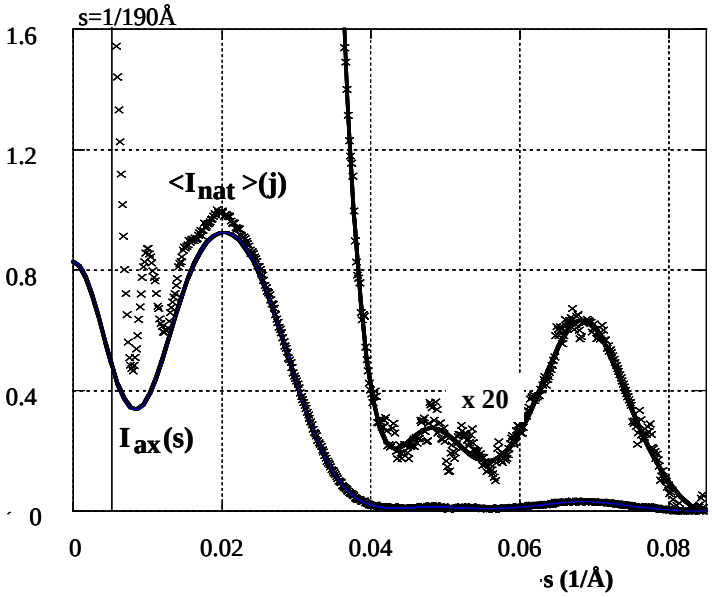


**Figure 2.** $<I_{nat}>(j)$ is the average spectrum of 21 native nerves, recorded at the channel j. $I_{ax}(s)$ is the continuous intensity curve of the axonal membrane.

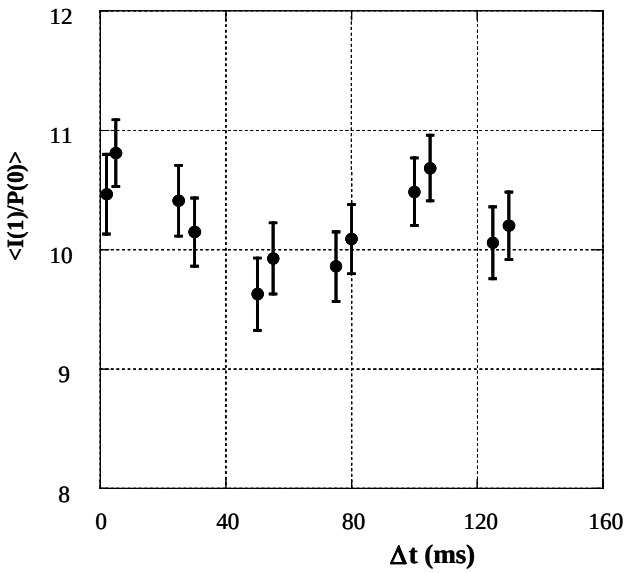


**Figure 3.** $<I(1)/P(0)>$ is the normalized intensity at the point s=1/(190 Å) (see Fig. 2) averaged over 6 nerves. $\Delta t$ is the time elapsed since the onset of the action potential. Note the presence of a minimum in the vicinity of $\Delta t$=55 ms.