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X-ray fluorescence microscopy of olfactory receptor neurons

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Abstract. We report a x-ray fluorescence microscopy study of cells and tissues from the olfactory system of *Xenopus laevis*. In this experiment we focus on sample preparation and experimental issues, and present first results of fluorescence maps of the elemental distribution of Cl, K, Ca, P, S and Na both in individual isolated neural cells and in cross-sections of the same tissue.

1. Introduction
The sense of smell is concerned with the parallel and simultaneous detection of a multitude of molecular structures. Olfactory signal processing includes transport of odorants through the mucus to the transduction compartments, either stereocilia or microvilli, issuing from the dendrite of an olfactory sensory cell into the mucus [1]. As both stereocilia and microvilli have diameters between 100 and 200 nm, conventional optical microscopy is not sufficient to elucidate their fine structure. The olfactorial signal pathways presents a tremendous analytical challenge, regarding chemical and spatial resolution. The aim of the present investigations was to see to which extent x-ray fluorescence microscopy and x-ray spectromicroscopy is suitable at the presently achievable resolution and sensitivity to address this problem. In order to understand complex biomedical issues involving inorganic elements, the chemical species (chemical specification) of the elements must be considered: oxidation state, coordination, and/or complex or molecular structure [2, 3].

2. Sample preparation x-ray microscopy experiments
X-ray fluorescence and x-ray spectromicroscopy are in principle well suited methods to achieve these high requirements to study the biological structures of interest. However, a major challenge, is given by the need to prevent highly mobile molecular species such as ions, from rearrangement during sample fixation and preparations. Preservation of biological samples from X-ray damage during the measurements is a second important issue, excluding high resolution experiments in aqueous solution without fixation. While cryo microscopy is the best solution towards the goals of structural isomorphism and reduced radiation damage, it is also technically the most challenging one. Here we used freeze drying method after rapid cryogenic plunging as well as embedding samples in styrene-methacrylate mixture and cutting 1 µm thin slices for analysis, see Fig 1. As experimental model we used cells and tissues from the olfactory system of *Xenopus laevis*, in which both kinds of transduction compartments are co-exist.
Figure 1: Two analytical procedures for cell preparation and X-ray fluorescence measurement at room temperature. After freeze-drying protocol, cell and tissue samples were prepared in two different ways: direct measurement without any fixation or with the classical electron microscopy embedding preparation using a styrene-methacrylate mixture.

Cryo-preserving and freeze drying method: The sample of the olfactory receptor neurons (ORNs) were freshly isolated from the nasal epithelium of larvae *Xenopus laevis* (stage 54-56) using mechanical trituration in divalent free buffer solution and short incubation in papain [4, 5]. Isolated cells were carefully transferred into a Ringer solution containing 0.05 M ammonium acetate buffer, pH 7.8 (1:1 w:w). The cells were suspended in 5 µl solution were placed on a 200 nm thick Si₃N₄ membrane, which had been coated previously with 0.1 % laminin and 0.01 % poly-L-lysine. All cells used in this study were clearly identified, prior to the measurements as ORNs on the basis of their characteristic morphology, having a single thick dendrite with a knob-like swelling from which emanated 3-10 cilia. Cells naturally attached to the substrate after approximately 3 min. The samples were rapidly frozen in liquid ethane cooled in liquid nitrogen, and subsequently quickly transferred in liquid nitrogen until freeze-drying at -70°C for two days. Samples are then very slowly warmed up to room temperature over 24 h and stored over silica gel in a desiccator.

Embedding the neural tissue for X-ray microscopy: Samples isolated from nasal epithelium of larvae *Xenopus laevis* were rapidly frozen in a mixture of propane: isopentane (2:1) cooled with liquid nitrogen to -196°C in an aluminum mesh and freeze-dried at -70°C for three days and stored at room temperature over silica gel in a desiccator. Freeze-dried samples were infiltrated with ether in a vacuum-pressure chamber and embedded in styrene-methacrylate using a technique specifically developed for analysis of diffusible elements. 1 µm thick sections were cut dry by glass knives, mounted on adhesive 100-mesh hexagonal Cu grids (G-100 hex, Canemco and Marivac Inc.), coated with carbon, and stored over silica gel.

X-ray fluorescence: The elemental distribution and elemental speciation of isolated neural receptor cells with intact ciliae was carried out by scanning x-ray microscopy, using the combined capabilities of the scanning transmission microscope (STXM) at ESRF ID21 beamline, as well as the nanoprobe imaging system at ID22NI beamline, in two separate beamtimes. The specification of these beamlines are given elsewhere [6, 7]. The following ions are of special importance in the neural receptor cells: Cl, K, Ca, P, S, Na as well as the trace elements Zn, Fe and Cu. The best fluorescence results, without x-ray damage, have been obtained at a photon energy of 4.1keV energy at ID21. Typical fluorescence maps of individual cells (analyzed with PyMca software) and cross section of the embedded olfactory epithelium are shown in
Figure 2: Na, P, S, Cl, K and Ca distribution and overlapped map of K (red) and Cl (blue) of the embedded tissue of neural receptor cells on the left part (marked with black letters); the zoom in the small part (26 x 19 µm), image 130 x 96 pixels and a dwell time 400 ms per pixel at E=4.1 keV. On the right side (marked with white letters) is shown the element distribution a individual isolated neural receptor cell; image 25 x 15 µm 100 x 70 pixels with a dwell time 900 ms per pixel at E=4.1 keV. Typical sum spectra (a), the distribution and overlapped map of K (red) and Cl (blue) in the embedded tissue (b) and individual isolated neural receptor cell(c).

Fig. 2. The distribution and concentration of chloride in this system is relevant in view of signal transduction [4]. The main result of this study was the localization of Cl, mainly in the soma of the receptor cell, but also in patches along the dendrite. We hypothesise that these patches of high chloride concentration may correspond to acidic intracellular compartments. This can be deduced in particular from the single cell maps (Fig. 2). While Cl was expected to be located primarily to the cilia, it appears an intracellular gradient and possibly a transport mechanism exist in this kind of receptor cell. This is consistent with recent immunocytochemical results indicating $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ co-transporters to be located to the somata and dendrites [8]. Chloride was also observed to accumulate in globular structures close to the plasma membrane. The formation of spurious NaCl salt as an artefact of the preparation can be excluded from the simultaneously measured Na map. In our analysis Na, K and Ca were distributed to variable extents in the different parts of the cell, their distributions showing some overlap (Fig. 2). These differences show that the distribution is not dominated by a single functional architecture such as homogeneously expressed and membrane associated transporters or co-transporters. The comparison of the two presented methods of sample preparation shows that individual ORNs are better suited for elemental mappings than tissue slices. On the other hand, thin tissue slices can potentially help to address questions such as the odorant transport within the mucus. Even if the preparation of dissociated ORN is more invasive, it certainly gives reliable results as to the element distributions.

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References