# DATA S1: SUPPLEMENTAL MATERIALS

## Simultaneous Raman microspectroscopy and fluorescence imaging of bone mineralization in living zebrafish larvae

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## A. SUPPLEMENTAL MATERIALS AND METHODS

**Fish husbandry:** Zebrafish were raised according to standard methods(1, 2), and handled according to the guidelines of the Weizmann Institute Animal Care and Use Committee. Handling of zebrafish was also done in compliance with German and Berlin state law, carefully monitored by the local authority for animal protection (Lageso, Berlin-Brandenburg, Germany). The following fish strains were used: *Tg(fli:EGFP)*$^{y1}$(3), *nac* mutants(4).

**Sample preparation:** In order to monitor zebrafish bone formation *in vivo*, a calcein green fluorescent marker [CAS number 1461-15-0 Sigma-Aldrich] and calcein blue [CAS number 54375-47-2 Sigma-Aldrich] were used. Calcein binds to free calcium ion and stain newly formed bones. To visualize and analyze the mineral content of newly formed bones, we used 20-27 days post fertilization (dpf) zebrafish. The fish were immersed 30 minutes in calcein solution (0.2% wt)(5) with calcein green, or overnight in calcein blue, and then rinsed in fresh water three times in order to allow diffusion of the free calcein. After rinsing in water, the fish were anesthetized in 0.16% tricaine-methanesulfonate (MS222) solution, and mounted on depression slides using methyl-cellulose [CAS M0387 Sigma-Aldrich (6%)] or low-melt agarose (9012-36-6) before non-invasive imaging. This protocol allows for visualization of bones and endothelial cells with two different green fluorescence markers or green and blue fluorescence markers within the same animal, enabling identification of the region of interest, prior to the Raman analysis.

**Correlative fluorescence-Raman setup:** The setup is built on the commercially available confocal Raman microscope (alpha300, WITec, Ulm, Germany) equipped with a Helium Neon (HeNe) (633 nm) laser excitation and piezoscanner (P-500, Physik Instrumente, Karlsruhe, Germany). The spectra were acquired with a thermoelectrically cooled CCD detector (DU401A-

BV, Andor, UK) placed behind a grating (600 g $mm^{-1}$) spectrograph (UHTS 300; WITec, Ulm, Germany) with a spectral resolution of 3 $cm^{-1}$. The laser beam was focused through a 10× (Nikon, NA = 0.2) and 60× water immersion (Nikon, NA=1.0) microscope objectives. The ScanCtrlSpectroscopyPlus software (version 1.38, Witec) was used for measurement and WITec Project Plus (version 2.10, Witec) for spectra processing. The Raman signal is spectrally filtered by a longpass filter (filter 3).

The implementation of the fluorescence modality relies on the substitution of some of the original optics, integration of excitation light in addition to the white illumination light, and the addition of a module in the imaging arm. Details of the changes made are shown in Fig.1 and summarized in Table 1. The excitation for the fluorescence is held in the illumination port at the back of the microscope and spectrally selected by the filter positioned in the filter holder (filter 1). The excitation light is reflected by the dichroic mirror (DM1) and the sample illuminated by the objective in place. The signal from the sample is collected in the epifluorescence mode, transmitted through the dichroic mirror (DM1) and reflected by the second dichroic mirror (DM2) placed in the first module of the microscope imaging arm. The signal is spectrally filtered at the desired wavelength (filter 2) and imaged onto a fluorescence camera by an achromatic doublet lens. Because DM1 and DM2 transmit the Raman laser (red to deep-infrared) and the scattered light in the Stoke region of the spectra, the setup allows simultaneous live visualization in the fluorescence mode and live collection of the Raman spectra. The dichroic mirror (DM1) and excitation filter (filter 1) allow a choice for the excitation wavelength for the fluorescence. This can be made at 400 or 490 nm, without modifying the current optical setting. These two wavelengths can excite a major part of the fluorescent markers and proteins used in traditional fluorescence imaging. Furthermore, the setup can be easily tailored to other wavelengths.

Table 1: Details of the components added to the microscope.

| **Added Components** | **Details** |
|---|---|
| LED | pE-100, 400 or 490 nm,CoolLED |
| Filter 1 | F69-390, HC Tripleband Exciter 482/587, AHF |
| Dichroic Mirror 1 (DM1) | F38-495, Strahlenteiler HC BS 495 AHF |
| Dichroic Mirror 2 (DM2) | F48-567, Strahlenteiler T 565 LPXR AHF |
| Filter 2 | F67-446, HC Tripleband Sperrfilter 446/532/646, AHF |
| Lens | Achromatic Doublet, Imaging Lens, Thorlabs |
| Fluorescence Camera | NeoSCMOS, Andor |

## B. SUPPLEMENTAL FIGURE

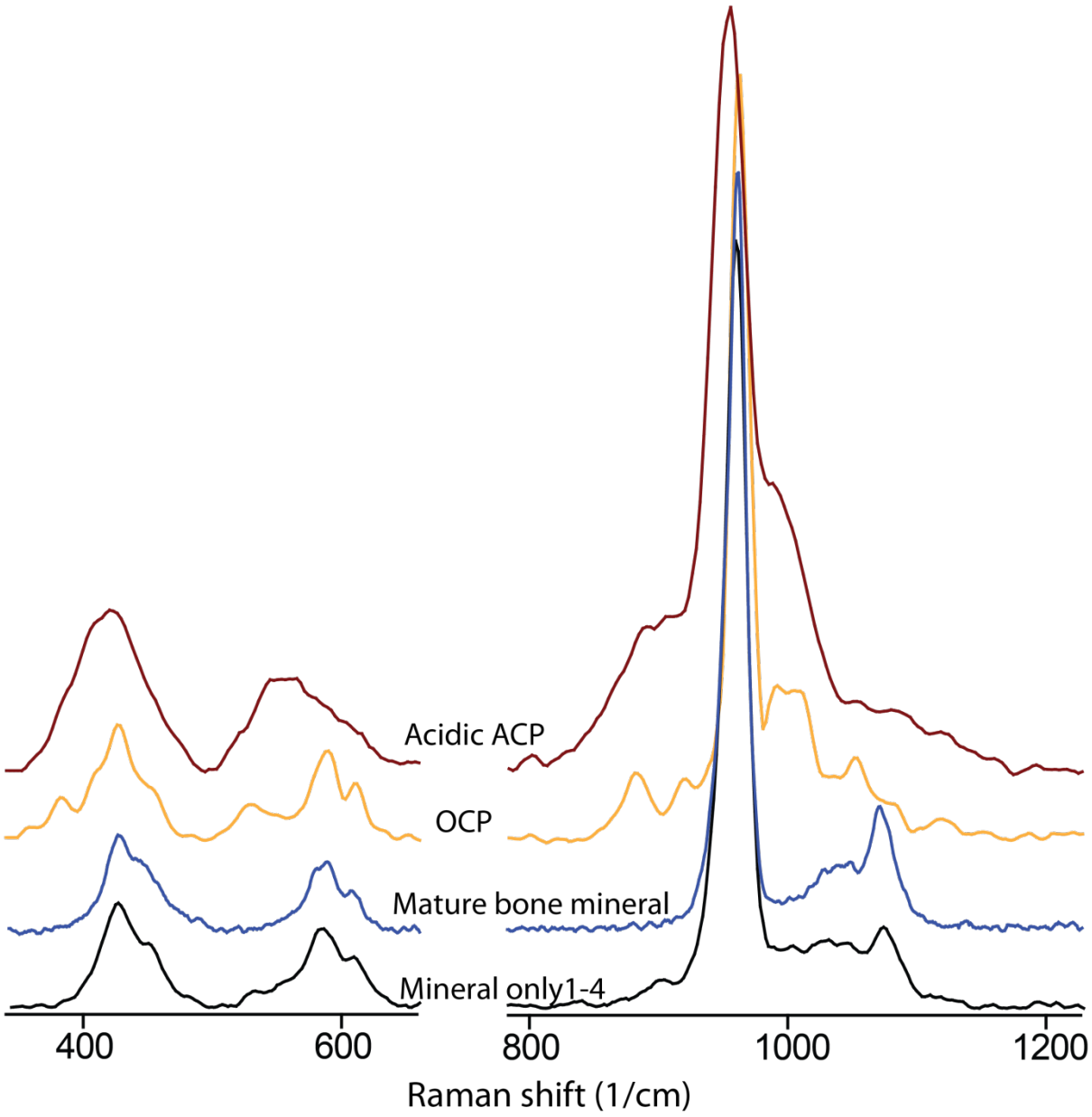


Figure S1: Reference spectra of acidic amorphous calcium phosphate (acidic ACP), octacalcium phosphate (OCP), mature zebrafish mineral, and zebrafish bone mineral phase shown in Fig. 3b.

**References**

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