Technological challenges for the advanced study of deep subseafloor life.

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Introduction: During the past decades, scientific ocean drilling has explored the subseafloor biosphere at some representative drilling sites: on the ocean margins, organic-rich anaerobic sedimentary habitats (e.g., Shimokita coalbeds) harbor sizable numbers of microbial cells at least over 1,000 meters below the seafloor whereas microbial populations in ultra-oligotrophic aerobic sedimentary habitats of the oceanic gyre (e.g., South Pacific Gyre) are several orders of magnitude lower. Previous molecular ecological studies have demonstrated that microbial communities in deep sedimenary habitats consist largely of phylogenetically diverse microbes, which are distinct from previously known isolates. Hence the metabolic functions of individual microbial components, as well as the strategy for long-term survival under the energetically and geophysically severe condition, have remained largely unknown. To tackle these significant questions, technological development for the advanced microbiological and biogeochemical analyses is of our essential challenges.

Discriminative detection of microbial cells: One of the most fundamentally significant techniques is the precise detection and enumeration of indigenous subseafloor life. We established a highly efficient and discriminative detection and enumeration technique for microbial life in sediments using automated image analysis after staining microbial cells with DNA specific dye SYBR Green I (SYBR-I)[1]. Acid wash treatment of sediment slurry with hydrofluoric acid significantly reduced non-biological fluorescent signals and enhanced the efficiency of cell detachment from the sediment particles. We found that cell-derived SYBR-I signals can be distinguished from non-biological backgrounds by dividing green fluorescence (band-pass filter: 528/38 nm [center-wavelength/bandwidth]) by red (617/73 nm) on images. A newly developed automated microscope system could take a wide range of high-resolution image in a short time, and subsequently enumerate the absolute number of cell-derived signals by the image calculation [2].

Quantitative cell separation and combination with flow cytometry: The methodological constrain on cell detection and enumeration is the limitation of sediment amount that can be placed on observation membrane for microscope. One of the clues to solve this problem is to separate microbial cells from geological matrix. We have standardized an improved cell separation method, which effectively detached the cells from mineral grains of the sedimentary habitat, by applying multiple density gradient layers [3]. Similar to the microscopic detection, we could discriminatively recognize cell-derived fluorescence, and the separated cells can be enumerated without a significant deviation between automated fluorescent microscopic system and flow cytometry.

The combined use of these new techniques allows us to separate the cells for single cell genomics and secondary ion mass spectrometry (e.g., an ion imaging by NanoSIMS ion microprobe). We also established clean sample prepration procedures, which are capable of very low biomass sample down to 10^{1}~10^{2} cells cm^{-3}. The systematic analytical scheme currently applies to some representative deep-biosphere samples such as the South Pacific Gyre and Shimokita coalbeds (i.e., IODP Expeditions 329 and 337, respectively).

References: [1] Morono Y et al. (2009) *ISME J* 3:503-511. [2] Morono Y and Inagaki F. (2010) *Sci Drilling* 9:32-36. [3] Morono Y et al. (2013) *Environ Microbiol* 15:2841-2849.