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## Notes &amp; Tips

## Miniaturized device for agitating a high-density yeast suspension that is suitable for in vivo nuclear magnetic resonance applications

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## ABSTRACT

In vivo nuclear magnetic resonance (NMR) monitoring requires a high-density cell suspension, where cell precipitation should be avoided. We have designed a miniaturized cell agitator that fits entirely into an 8-mm NMR probe but that, being mounted into the instrument, is situated outside of the sensitive area. The device consists of two glass tubes connected in a way that, when gas flow is blown through them, creates influx of cell suspension into the device that returns through apertures. This flow creates continuous circular vortex of the cell suspension in the whole sample volume, whereas there are no moving mechanical parts or gas bubbles crossing the instrument's sensitive area. The gas flow controls conditions of the cell suspension and removes volatile waste metabolites.

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Some methods allow on-line in vivo time-series measurements (i.e., monitoring) of cellular metabolism. For example, nuclear magnetic resonance (NMR)<sup>1</sup> spectroscopy can monitor some important intracellular metabolites, such as phosphorus-containing compounds (e.g., phosphonucleotides, phosphosugars, polyphosphates), in vivo by <sup>31</sup>P NMR. With NMR, it is possible to determine in vivo intracellular metabolite concentrations, pH levels, and kinetics of enzyme reactions as well as to identify metabolic pathways (see, e.g., Refs. [1–4]). However, NMR spectroscopy has relatively low sensitivity to physiological concentrations of cellular metabolites. Therefore, limitations of in vivo metabolite concentrations can be overcome only by a high concentration of cells.

Campbell-Burk and Shulman [3] stated that to distinguish molecular species during the course of in vivo NMR measurements, the experimental setup should employ high-resolution spectroscopy, which is possible only if the following criteria are satisfied: (i) high cell density (10–50% wet weight/volume), (ii) a wide-bore NMR instrument (e.g., 20 mm), and (iii) a stirring setup that maintains the constant physiological state of cells within the NMR instrument during the whole measurement period. Narrow-bore <sup>31</sup>P NMR spectroscopy (8 mm) also brings the additional advantage of measurements of in vivo kinetics for some reactions using magnetization transfer (see, e.g., Refs. [1,2,4]). However, this method requires long-term accumulation of the signal; therefore, cell pre-

cipitation during the course of the measurement must be avoided. Cell precipitation results in heterogeneity of nutrient supply, such as gases and carbon, nitrogen, and phosphorus sources, resulting in variation of the cellular physiological state across the population. Consequently, the cellular precipitation of the suspension must be prevented and, at the same time, all of the required nutrients must be distributed homogeneously to avoid excessive variation of metabolic changes during the NMR monitoring. In addition, the stirring device should not disturb the NMR magnetic field.

A number of approaches have been developed to prevent cell settling during the course of in vivo NMR measurements to achieve a high resolution [3], including a double-bubbler apparatus [5,6], a perfused system by immobilization of cells in an agarose gel matrix [4,7] or other porous materials, a hollow-fiber dialysis system [8], and an NMR bioreactor (e.g., from Bioengineering AG, Switzerland). Of course, an NMR bioreactor directly integrated into the NMR instrument is the best solution for the on-line, in situ, and in vivo measurement of fermentation systems with NMR monitoring of cell cultures growing in a liquid phase (e.g., bacteria, yeast), but this solution is quite expensive.

Yeast *Saccharomyces cerevisiae* strain CEN.PK 122 (from the EUROSCARF yeast collection, <http://web.uni-frankfurt.de/fb15/mikro/euroscarf>) were grown aerobically in CBS medium [9] in a glucose-limited chemostat at a dilution rate of  $D = 0.05 \text{ h}^{-1}$  with  $18 \text{ g L}^{-1}$  glucose in the feeding medium,  $30 \text{ }^\circ\text{C}$ , and  $250 \text{ rpm}$ . Under these conditions, the biomass density reached  $5.36 \pm 0.05 \text{ gDW}$  (grams dry weight)  $\text{L}^{-1}$  (or  $60.92 \pm 2.22 \text{ gWW}$  [grams wet weight]  $\text{L}^{-1}$ ). The yeast culture from the chemostat was condensed 40-fold, for that 90 ml of the culture were pelleted by filtering through a Sartorius cellulose acetate filter ( $\varnothing = 0.2 \text{ }\mu\text{m}$ ) under vacuum and washed three times with working buffer (25 mM Mops [pH 7.0],

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<sup>1</sup> Abbreviations used: NMR, nuclear magnetic resonance; gDW, grams dry weight; gWW, grams wet weight.

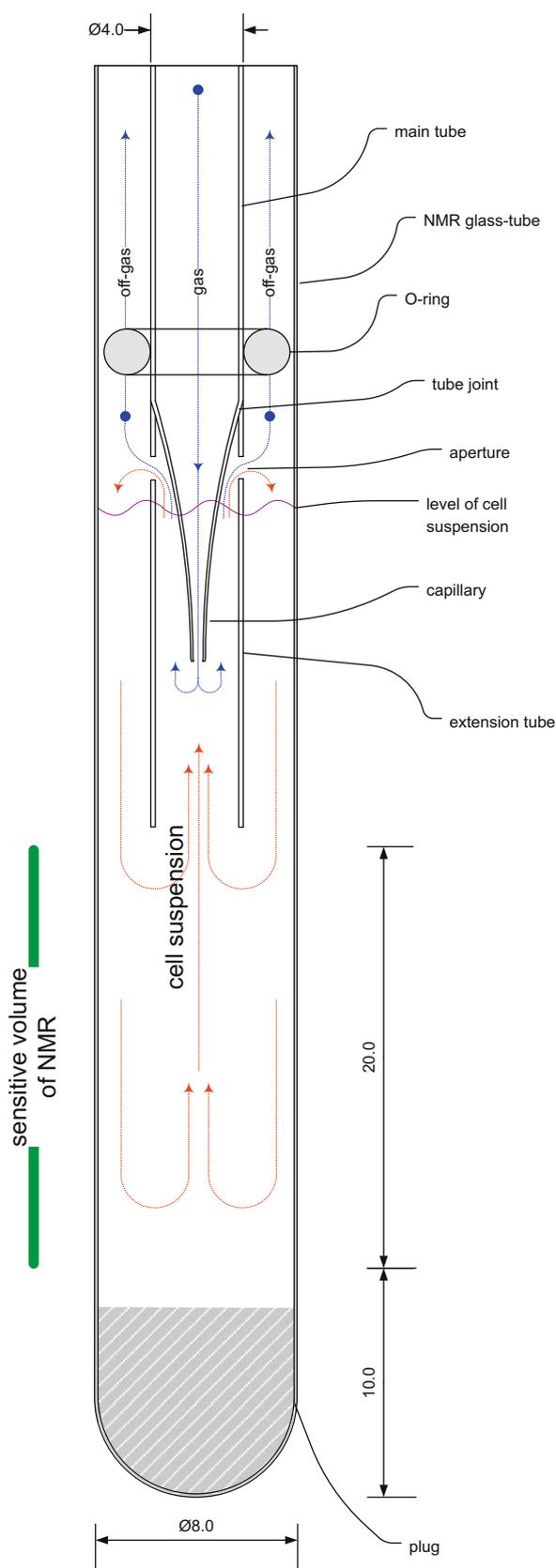
2 mM MgSO<sub>4</sub>, 1.7 mM NaCl, 2 mM KCl, and 100 mM glucose). Then filtered cell pellet was resuspended in 2 ml of the working buffer and 250  $\mu$ l of D<sub>2</sub>O was added (final D<sub>2</sub>O content of 11.1%). This was a very high-density cell suspension that would have precipitated within a minute without agitation. After that, 750  $\mu$ l of this suspension was transferred to an 8-mm NMR probe equipped with an agitating device (Fig. 1).

The cell agitating device was assembled completely from dielectric materials such as glass and silicone tubes, rubber O-rings, and plastic fasteners. The device includes a 200-mm-length glass tube with an external diameter of 4 mm (called the main tube). One of the ends of this tube is stretched out to the capillary with a diameter of 0.1–0.05 mm at the tip. The capillary end of the main tube is inserted into another glass tube with the same external diameter of 4 mm (called the extension tube), and the joint is firmly glued in place (Fig. 1). The length of the extension tube is 60 mm, and it has several 1-mm-diameter apertures close to the glued joint. All apertures must be above the capillary tip (Fig. 1). The main tube is then connected to the gas flow, and the device can be inserted into an 8-mm-diameter NMR tube (8  $\times$  230 mm, Wilmad Labglass, USA). The device is vertically centered within the NMR tube using three 6-mm-diameter rubber O-rings located above the joint between the main and extension tubes (Fig. 1). The device must be immersed in the cell suspension such that the tip of the capillary is under the surface of the cell suspension and the apertures are above the surface of the suspension. When immersed, the main tube of the device sticks out of the NMR tube, and a plastic fastener can be used to secure the depth of the device immersion in the NMR tube. It is important to note that the lower end of the extension tube must be above the sensitive volume of the particular NMR instrument (Fig. 1).

In vivo <sup>31</sup>P NMR spectra were acquired at 161.97 MHz on a vertical 9.4T wide-bore NMR spectrometer (Bruker Avance 400 Ultra-shield) using an 8-mm 1H/BBI probe with the following parameters: bp pulse, 14 ms (pl 4.6); relaxation delay, 1 s; spectral width, 8090 Hz (corresponding to 50 ppm); time domain, 4K; number of acquisitions, 512 or 1024, with resulting scan time of 11 or 22 min, respectively. Spectra were processed automatically by applying a user's program with size of 16K, line broadening of 5 Hz, and an automatic baseline correction.

Gas (in this case air) was blown through the main tube toward the capillary. The gas passes through the capillary tip and forms a bubble that immediately escapes upward through apertures in the extension tube. The 1-mm gap between the internal wall of the NMR tube and the O-rings of the device is sufficient for the unrestricted gas flow out of the NMR tube. Consequently, it does not cause a buildup of pressure. The movement of gas bubbles pulls the cell suspension into the apertures. After passing the apertures, the gas escapes from the NMR tube, whereas the cell suspension drops back into the tube. The suction force of the agitating device is dependent on the rate of gas flow and is sufficiently strong to result in stirring of the cell suspension across the whole volume of the NMR tube. As a result, there is no cell sedimentation over the measurement period. It is important to note that there are also no gas bubbles crossing the sensitive volume of the NMR instrument (Fig. 1); this is extremely important for the homogeneity of the magnetic field within the sensitive volume of the NMR instrument. In addition, the bottom of the NMR tube was filled with plastic filler (Fig. 1) to reduce tube's internal volume and consequently increase the homogeneity of the suspension.

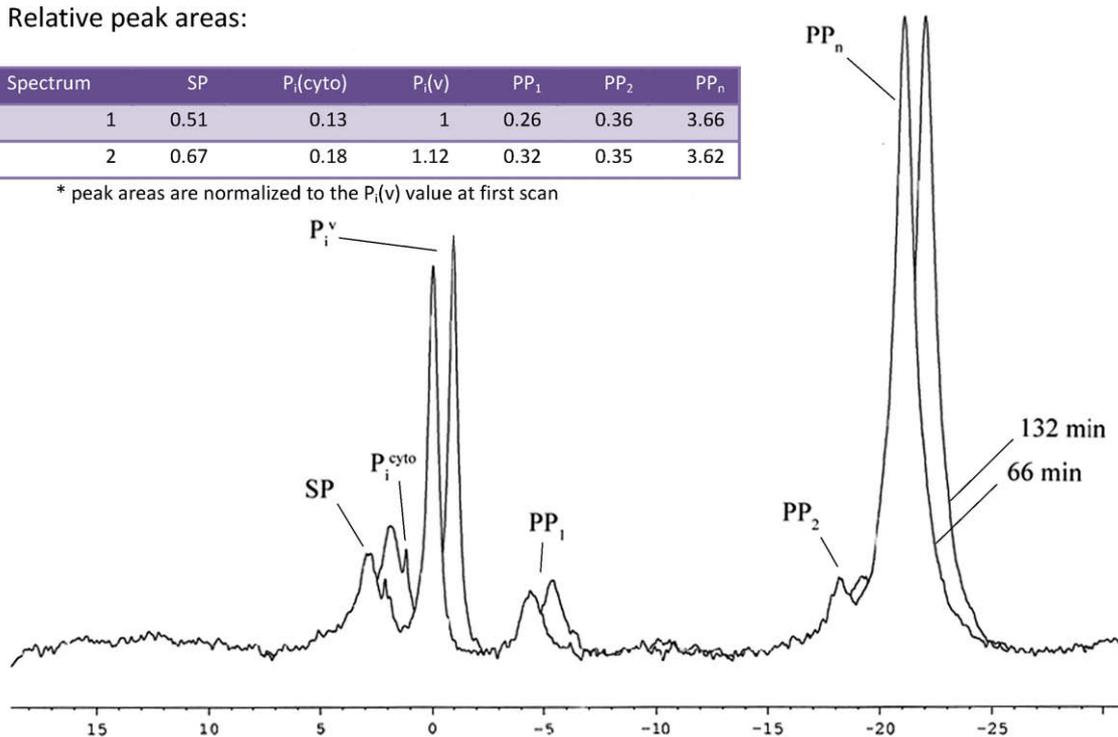
Furthermore, the gas flow controls experimental conditions (e.g., degree of oxygenation) and also removes volatile metabolites (e.g., CO<sub>2</sub>, ethanol) from the cell suspension, whereas nonvolatile metabolic waste products (e.g., glycerol) are either innocuous or nontoxic until they reach high concentrations. In addition, the cell suspension can be fed with concentrated solutions of nutrients through another supply line. Under such conditions, high-density yeast suspensions remain viable and physiologically intact for



**Fig. 1.** Sketch of the cell agitating device inserted into a conventional 8-mm NMR glass tube. Blue arrows represent gas flow, and red arrows represent movement of the cell suspension. See the text for further explanations and notation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Relative peak areas:

Spectrum	SP	P <sub>i</sub> (cyto)	P <sub>i</sub> (v)	PP <sub>1</sub>	PP <sub>2</sub>	PP <sub>n</sub>
1	0.51	0.13	1	0.26	0.36	3.66
2	0.67	0.18	1.12	0.32	0.35	3.62

\* peak areas are normalized to the P<sub>i</sub>(v) value at first scan

**Fig. 2.** In vivo  $^{31}\text{P}$  NMR monitoring of high-density yeast *S. cerevisiae* CEN.PK 122 suspension agitated using the described device. Shown are two consecutive spectra, each integrated from 3072 scans (66 min). SP, phosphosugars; P<sub>i</sub>(cyto), cytoplasmatic inorganic phosphate; P<sub>i</sub>(v), vacuolar inorganic phosphate; PP<sub>1</sub> and PP<sub>2</sub>, oligophosphates; PP<sub>n</sub>, polyphosphates.

2 h, which is sufficient time to run high-resolution in vivo NMR measurements in narrow-bore instruments (Fig. 2).

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