



# Nanobacteria: Are They or Aren't They Alive? A Case Study on What It Means to Be a Biological Organism

by

**Merri Lynn Casem**

Department of Biological Science  
California State University, Fullerton

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## Part I—What Does It Mean To Be Alive?

Biology is the study of living things. Whether a single cell or a Sequoia tree, a humpback whale or a human being, you have an intuitive sense of what it means to be a biological organism. Sometimes, however, the designation of something as a living thing is not so obvious. A recent example of this is the discovery of *nanobacteria*.

Bacteria are *prokaryotic* cells. Prokaryotes lack the internal, membrane-bound structures associated with *eukaryotic* cells (your body is made up of eukaryotic cells). Bacteria are extremely abundant and versatile, occurring in every environment on Earth (including inside and outside your body). Many bacteria can cause diseases. The name, *nanobacteria*, refers to the very small size of these organisms (on the order of 0.2 $\mu$ m to 0.5 $\mu$ m). This class of bacteria was originally isolated from human and cow blood. It has been proposed that these bacteria function to stimulate a process called *biomineralization*.

### *Biomineralization:*

The formation of inorganic crystalline structures in association with biological macromolecules. This process is responsible for the production of bone and dental enamel. This process is also referred to as *calcification*.

Biomineralization is a good thing when it occurs in the correct location, but often this process occurs in the wrong place at the wrong time. The formation of kidney stones is a good example of this kind of pathological (disease-related) form of biomineralization. Nanobacteria have been isolated from within human kidney stones, leading to the suggestion that these bacteria may be the cause of this disease.

**Assignment for Part I:**

The fundamental issue under consideration is whether nanobacteria are alive. How would you decide this question? To answer this you need to think about the properties common to all living things and how you would test whether the nanobacteria possessed these properties.

What are the properties of a biological organism?

Think of at least THREE properties of life.

Fill out the table on the [Work Sheet for Part I](#).

Choose ONE property of life and propose a way you could test for that property.

## Work Sheet for Part I

**QUESTION: What are the properties of a biological organism?**

Property of Life	Is this property common to ALL living things?	How would you test for this property?

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### Part II—What Is the Evidence that Nanobacteria Are Alive?

Nanobacteria were originally discovered by two researchers from Finland, Drs. E. Olavi Kajander and Neva Ciftcioglu. They isolated very small (0.2 to 0.5 $\mu$ m) coccoid (round) particles from human and cow blood. They found that they were very difficult to work with and did not behave like typical bacteria. They reported: "Nanobacteria are poorly disruptable, stainable, fixable and exceptionally resistant to heat" (i.e. none of these standard techniques worked on the nanobacteria).

The researchers determined that a culture of nanobacteria will double in size in three days and high doses of gamma radiation or antibiotics will prevent this multiplication. They claim to have isolated a "16S rRNA gene sequence that falls within the  $\alpha$ -2 subgroup of Proteobacteria," a class of bacteria that includes several human pathogens.

In a research report published in the *Proceedings of the National Academy of Sciences, USA (PNAS 95: 8274-8279, 1998)*, Kajander and Ciftcioglu present additional information about nanobacteria. The data presented on [Data Sheet 1](#) and [Data Sheet 2](#) are excerpted from this article.

#### **Assignment for Part II:**

Scientists conduct experiments in an attempt to answer specific questions. Once they have analyzed their results, they write up their findings for publication. Scientific information is shared through publication in scientific journals. Other scientists can then read and evaluate the research. The scientific process can be complicated by the use of specialized language.

Read over the summary information presented above and examine the data.

What terms or concepts are new or unclear to you?

What questions do you have?

List these on the [Work Sheet for Part II](#).

## Data Sheet 1 for Part II

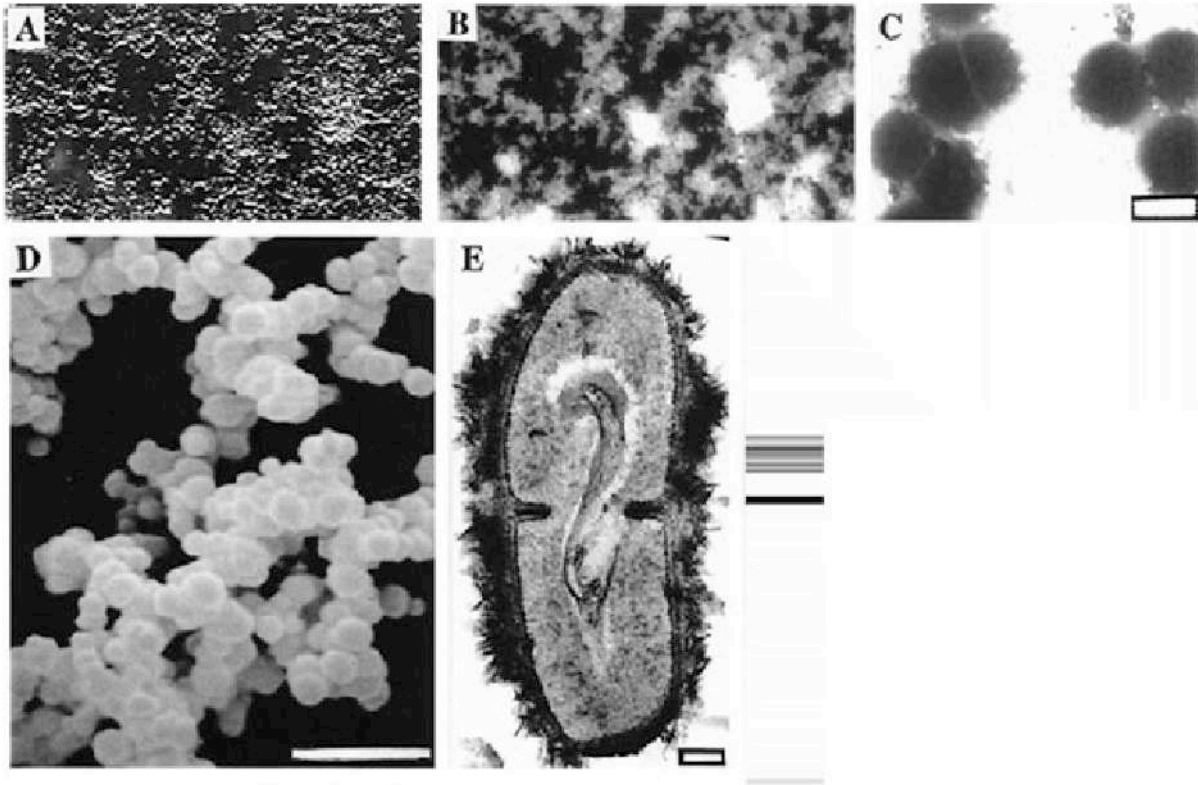


Fig. 1. Light and electron microscopic images of nanobacteria.

(A) DIC image of bottom-attached nanobacteria after a 2-month culture period.

(B) DNA staining of the same area (X1600) with the modified Hoechst method.

(C) Negative staining of nanobacteria isolated directly from FBS. (Bar = 200 nm.)

(D) SEM micrograph showing their variable size. (Bar = 1  $\mu\text{m}$ .)

(E) A dividing nanobacterium covered with a "hairy" apatite layer. (Bar = 100 nm.)

*Source: Kajander and Ciftcioglu 1998 (PNAS 95: 8274-8279).*

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## Data Sheet 2 for Part II

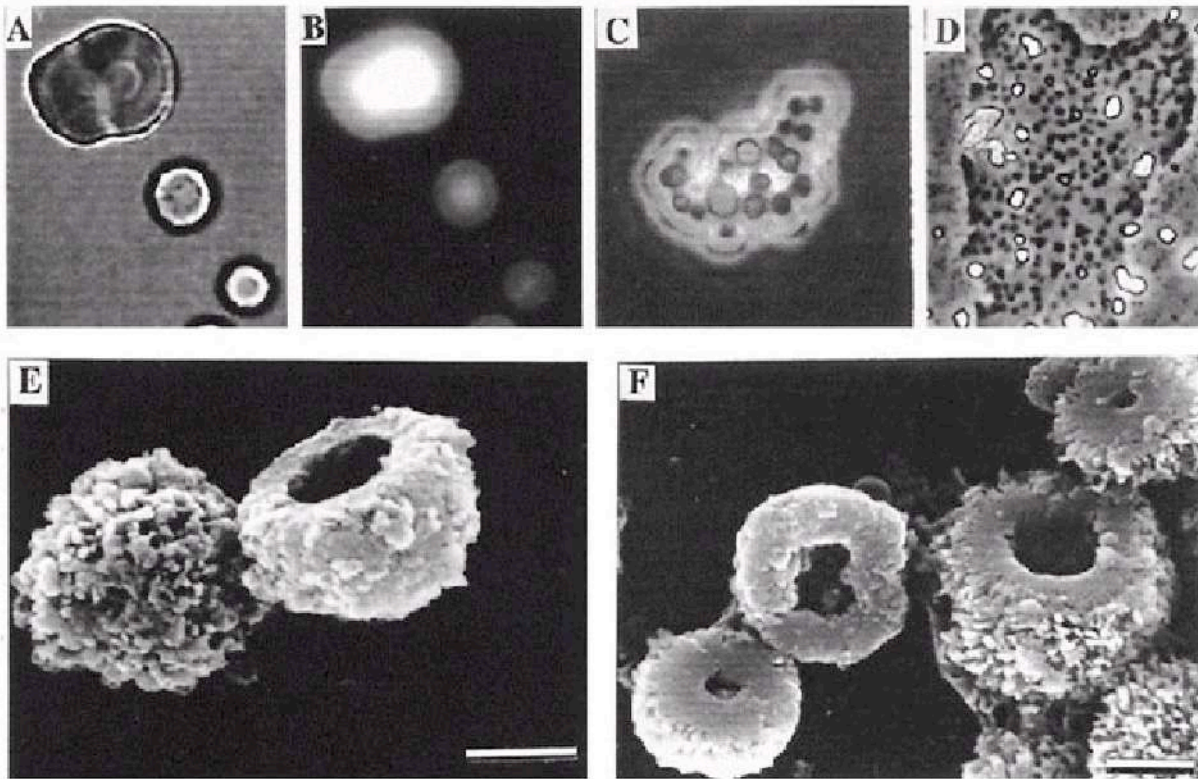


Fig. 3. Nanobacteria cultured under SF conditions and their interaction with cells.

(A) Light microscopic micrograph.

(B) DNA staining of the same area with the modified Hoechst staining method.

(C) DIC images of nanobacteria inside a common apatite shelter.

(D) A partly demineralized nanobacterial group (A-D, X860).

(E and F) SEM micrographs of nanobacterial dwellings detached from the culture vessel.  
(Bars = 1  $\mu\text{m}$ .)

*Source: Kajander and Ciftcioglu 1998 (PNAS 95: 8274-8279).*

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## **Work Sheet for Part II**

**TERMS AND CONCEPTS I DON'T KNOW:**

**QUESTIONS I HAVE:**

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### Part III—More Evidence of Life

In their 1998 paper, Kajander and Ciftcioglu describe various experimental results to support their hypothesis that nanobacteria are living organisms. In addition to the evidence you have already considered, these authors describe three key experiments that they feel greatly strengthen their hypothesis.

#### Experiment 1—Transferability

When nanobacteria are cultured for a period of time (1 month), the process of biomineralization that they trigger results in the formation of a "biofilm" on the surface of the culture container - much like a hardwater deposit around a faucet. It is possible to scrape up this biofilm, dilute the components (1:10), and *transfer* the nanobacteria into a new culture container. After another month, the culture container is once again coated with a biofilm.

The authors report that they were able to repeat this 1:10 dilution and transfer protocol on a monthly basis for five years. They describe this property as "transferability."

#### Experiment 2—Gamma Radiation

Nanobacteria could be isolated from culture as described above. When these isolated cells were exposed to high energy, gamma radiation and then added to a culture container, it was observed that *no* growth or formation of a biofilm was observed.

#### Experiment 3—Kidney Stones

Kidney stones were examined from 30 different human patients. When these stones were treated to slightly dissolve them, it was possible to isolate nanobacteria-like particles. When placed in culture, these particles behaved exactly like nanobacteria isolated from serum. That is, they formed a biofilm on the surface of the culture container.

#### Assignment for Part III:

A very important aspect of science is knowing how to interpret the results you get from an experiment. What does the data tell you? How much can you conclude from an experiment?

#### Activity:

Consider the results from each of the three experiments described above. What does each experiment tell you? How does the experiment support the hypothesis that nanobacteria are living? Use the table on the [Work Sheet for Part III](#) to record your thoughts.



## Work Sheet for Part III

	What can you conclude from this experiment?	Does this experiment support the hypothesis?
Experiment #1  Transferability		
Experiment #2  Gamma Radiation		
Experiment #3  Isolation from Kidney Stones		

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### Part IV—Corroborating Evidence (?)

A key requirement in the process of scientific investigation is the repetition of experimental results by other scientists. If others can repeat your work, then it is likely (although not guaranteed) that your conclusions and hypotheses are correct. In October of 2000, Cisar *et al.* (*et al.* means "and others") published a paper (*PNAS* 97:11511-11515; 2000) that examined the original work of Kajander and Ciftcioglu.

Cisar's team repeated the experiments described by Kajander. They isolated and cultured the nanobacteria in the same way and observed many of the same behaviors. Despite this, Cisar *et al.* believe that their evidence *does not* support the hypothesis that nanobacteria are living and play a role in the development of kidney stones in humans.

One difference between the papers focuses on the evidence for DNA. DNA can be identified by its staining properties (Hoechst or ethidium bromide) or by its ability to absorb light at a wavelength of 260nm (ultraviolet). Another method is to use the technique of *Polymerase Chain Reaction* (PCR). This technique uses short sequences of DNA called *primers* to trigger a chemical reaction that results in the amplification or increase in the concentration (number) of pieces of a specific region of DNA from a sample. In this example, the primers were specific for 16S rDNA and the sample was the isolated nanobacteria. Following the PCR reaction the authors could use other techniques to see the PCR product (agarose gels) and they could isolate and sequence the product to determine the exact genetic code or language associated with that PCR product.

The data from these and other experiments are presented on [Data Sheet 1](#), [Data Sheet 2](#), and [Data Sheet 3](#).

#### **Assignment for Part IV:**

The critical analysis of data becomes even more important when different groups reach conflicting conclusions. Scientific results are meaningless if they cannot be repeated and validated. The inability to repeat results could arise from unknown variables (quality of water, etc.), from minor changes in technique or procedure, from differences in interpretation (researcher bias), or from serious flaws with the original research.

#### **Activity:**

Consider the data from the work by Cisar *et al.*

Which terms or techniques are new or unclear to you?

How does this data compare to that of Kajander and Ciftcioglu? (To help you answer this question, refer back to Parts II and III of this case study).

Which of the new results are supportive of Kajander and Ciftcioglu?  
Which of the new results contradict Kajander and Ciftcioglu?  
Use the table on the [Work Sheet for Part IV](#) to critique the work of Cisar *et al.*  
Circle the result that you believe is most damaging to the hypothesis that nanobacteria are living organisms. Explain why you think this.

## Data Sheet 1 for Part IV

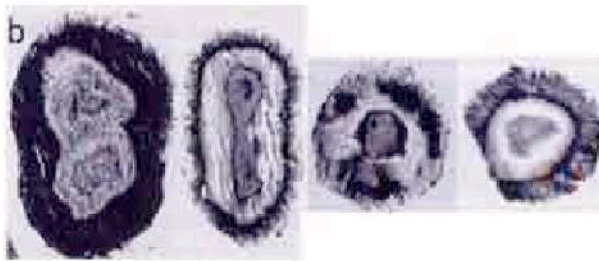
Experiment	Result
Culture of Nanobacteria	Nanobacteria maintained in culture generate a biofilm on the surface of the culture container within 3 weeks.
Gamma Radiation	Exposure to gamma radiation prevents the formation of a biofilm.
Transferability	When a biofilm (nanobacteria) isolated by scraping the surface of an established culture was diluted 1:10 and transferred into a new culture container, it grew - generating a new biofilm. This could be repeated for several months.
Cell-like appearance	The nanobacteria isolated from the biofilm has a coccoid (round) appearance. See Figures 2a and 2c on <a href="#">Data Sheet 2 for Part IV</a> .
DNA staining	Hoechst staining is diffuse (not focused) - does not appear to specifically localize to the cells. See Figure 2d on <a href="#">Data Sheet 2 for Part IV</a> . There was no ethidium bromide staining material following standard DNA isolation techniques (not shown).
DNA Isolation	There is no evidence of DNA based on absorption at a wavelength of 260nm. See Figure 3a on <a href="#">Data Sheet 3 for Part IV</a> .
Protein Isolation	Protein gel electrophoresis (a technique that allows you to see all the proteins in a sample) show only a few proteins. See Figure 3b on <a href="#">Data Sheet 3 for Part IV</a> .
P CR for 16S rDNA	<p>PCR reactions amplified a product of the expected size and with a sequence that was 85% identical to the published nanobacteria sequence.</p> <ul style="list-style-type: none"> <li>-The same PCR reaction product was found in samples that lacked the nanobacteria.</li> <li>- The sequence of the PCR product was 99% identical to that of <i>Pseudomonas</i>, a common bacterial contaminant.</li> <li>- The published sequences of 16S rDNA from nanobacteria are 99% identical to 16S rDNA from <i>Phyllobacterium</i>, another common contaminant.</li> </ul>

## Data Sheet 2 for Part IV

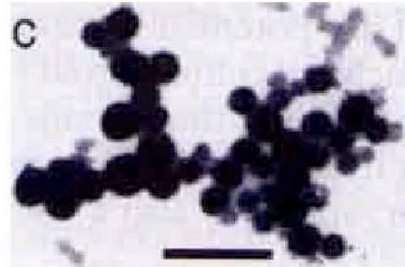
Fig. 2. Electron and light microscopic images of nanobacteria-like particles scraped from DMEM-containing subcultures of 0.45- $\mu\text{m}$  membrane-filtered saliva. Source: Cisar *et al.*, 2000 (*PNAS* 97: 11511-11515).



(a) SEM of biofilm material. (Bar = 5  $\mu\text{m}$ .)



(b) TEM of nanobacteria-like particles observed in thin sections of biofilm material. (Bar = 1  $\mu\text{m}$ .)



(c) TEM of negatively stained biofilm material showing small coccoid-shaped particles. (Bar = 0.2  $\mu\text{m}$ .)

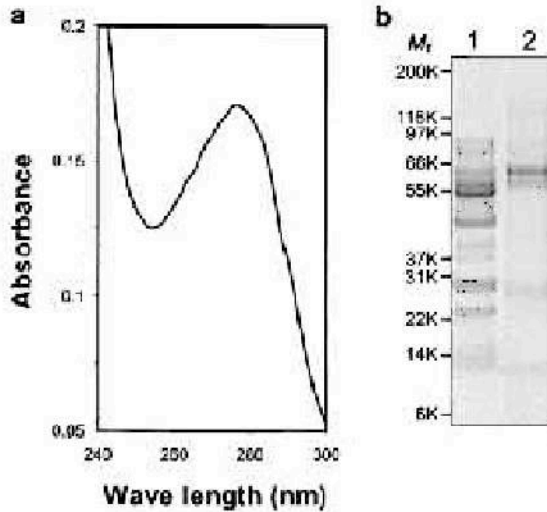


(d) Light micrograph of biofilm material stained with Hoechst 33258 showing nonspecific surface fluorescence of coccoid-shaped particles. (Bar = 1  $\mu\text{m}$ .)

### Data Sheet 3 for Part IV

Fig. 3. Biochemical examination of biofilm-associated macromolecules. Biofilm material from subcultures of 0.45- $\mu$ m membrane-filtered saliva was washed with PBS and solubilized by dialysis against excess EDTA followed by PBS.

Source: Cisar *et al.*, 2000 (*PNAS* 97: 11511-11515).



(a) UV absorbance spectrum of the dialyzed preparation.

(b) SDS/PAGE of the dialyzed preparation showing the Coomassie-stained profiles of membrane-filtered, whole human saliva (lane 1) and a comparable amount of biofilm-associated protein (lane 2). Mr of each molecular weight marker is indicated in thousands (K).

## Work Sheet for Part IV

**Check** the correct box to indicate whether you believe that the corresponding experiment by Cisar *et al.* supports or contradicts the hypothesis that nanobacteria are alive.

Experiment	Supports	Contradicts
Culture of Nanobacteria		
Gamma Radiation		
Transferability		
Cell-like Appearance		
DNA Staining		
DNA Isolation		
Protein Isolation		
PCR for 16S rDNA		

**Circle** the experiment that you think is most damaging to the hypothesis that nanobacteria are living. **Explain** your answer below.

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### Part V—Final Chapter (or is it?)

When Cisar *et al.* tried to repeat the experiments described by Kajander and Ciftcioglu, they did not feel that the results they obtained supported the hypothesis that nanobacteria were living. Cisar *et al.* claim to provide evidence that (1) there is no DNA associated with the nanobacteria based on DNA staining and lack of absorbance at 260nm, (2) that the number and type of proteins isolated from nanobacteria are insufficient for a living cell, and (3) that evidence of nanobacterial 16S rRNA is likely a result of contamination of the PCR results by other common bacteria.

While these results seemed to support the idea that nanobacteria are not biological organisms, there was a problem. Cisar *et al.* were able to repeat some of Kajander and Ciftcioglu's data. Specifically, Cisar *et al.* found that:

1. Nanobacteria maintained in culture would generate a biofilm
2. Exposure to gamma radiation prevented the formation of the biofilm
3. The ability to form a biofilm could be transferred (transferability)

What could account for these results if nanobacteria were not alive? Cisar *et al.* needed to explain these results if they wanted their conclusion to be accepted by the scientific community. They attempted to do this by designing an additional set of experiments.

#### **Assignment for Part V:**

It is not enough to simply suggest that someone else's research is wrong. The finding of "negative evidence" (not finding something) is usually not sufficient. You must provide compelling, positive evidence that offers an alternative explanation of the published observations.

#### **Activity:**

Look over the final set of experimental data provided by Cisar *et al.* and displayed on the [Work Sheet for Part V](#). What conclusions can you make?

Decide if these experiments explain the observations of

1. the formation of the biofilm,
2. the ability of gamma radiation to prevent the formation of the biofilm,  
and
3. the transferability of biofilm formation.



## Work Sheet for Part V

Experiment	Results	Cisar Lab Conclusion	Alternate Conclusion
Energy use by nanobacteria	Cultures of nanobacteria were exposed to 0.1% <i>sodium azide</i> - a powerful inhibitor of cellular respiration. The formation of a biofilm continued even in the presence of this poison.		
"Growth" of dilute cultures	Cultures of nanobacteria were diluted to a higher degree than that used by Kajander. Dilutions of 1:100 or 1:1000 were cultured as before. At these high dilutions there was no evidence of biofilm formation even after 8 weeks.		
Biofilm formation in the absence of nanobacteria	<p>Sterile DMEM culture media will not form a biofilm on its own. When purified phosphatidyl inositol (a phospholipid common to biological membranes) was added to the culture, biofilm formation occurred within two weeks. The appearance of the particles was very similar to those found in nanobacterial cultures.</p> <p>This ability for a phospholipid to induce biofilm formation was prevented when the phospholipid was exposed to gamma radiation.</p>		

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### Conclusion—The Debate

Scientific theories are based on our best understanding of the evidence. These theories must either be modified or abandoned when new evidence is made available that challenges our understanding. In this case study you have been asked to consider experimental results from two competing labs. The contradictory data reported by the two groups resulted in the publication of an independent news item entitled "Researchers fail to find signs of life in 'living' particles" by Allison Abbott (*Nature* Vol 408:394, 2000). In this article Cisar is quoted as saying, "There is a need for hard molecular evidence" to support a claim of life, while Ciftcioglu is quoted as saying, "We have evidence that the particles are living. We are not fanatics, we are scientists." Who is right?

#### **Activity:**

Discuss which set of evidence (Kajander and Ciftcioglu or Cisar *et al.*) you find most convincing.

Decide whether you believe nanobacteria are alive or not!

We will be debating the status of nanobacteria as a living organism during the first week of classes.

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