Versatility of Electrophoresis as a Tool for Inquiry Based Collaboration in Developmental Biology and Comparative Physiology

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INTRODUCTION

The genetic revolution has infiltrated many fields in the natural sciences from molecular biology to ecology. Classroom exercises promoting an understanding of the fundamentals of nucleic acid and protein biochemistry have relied on the use of electrophoresis as an analytical tool. These exercises are implemented at the high school level through the use of commercial kits. Many of these kits highlight the use of DNA fingerprinting and protein electrophoresis to help students understand their implications in forensics and evolution. At the college level, introductory biology courses include some version of these kits, although other novel approaches reflect the background of the teaching faculty who are performing their own research. The focus of the experimental kits includes criminal forensics, paternity determination, bacterial transformation and evolutionary relationships among fish. Plant or animal developmental studies are rarely used. In addition available kits use a limited pedagogical approach to experimentation relying mainly on prescribed hypotheses. As a result the exercises become cookbook recipes with predictable outcomes.

We are suggesting a more open-ended approach to developing lab exercises in the early stages of undergraduate education. In the 1990's, the use of inquiry-based collaborative learning in the classroom had already engendered much excitement in college laboratories. The National Science Foundation (NSF) emphasized that rapid accumulation of scientific knowledge, changes in the workforce requirements, and the lack of science literacy in our general population are reasons to reconstruct science curricula. It proposed inquiry-based experiences in which students perform steps that scientists execute as a way to answer research questions. The students must come to "own" the research (NSF, 1996). According to Drayton and Falk (2001),inquiry based learning is characterized by various elements: learners are engaged by scientifically oriented questions, they give priority to data in order to address and develop tenable hypotheses, in light of alternative explanations and they

use cogent arguments to convince others of their explanations. This approach gives students the opportunity to explore avenues of study, which promote curiosity and creativity. In one model of this approach
(Hoegler, 2005), there are two phases- a prescribed phase and an implementation phase. In the first phase, students learn an experimental technique by using dry or simple wet lab experimentation. Then in phase two, small teams of students research databases to find available literature about a topic of interest and proceed to develop a project tailored to their interests. This gives them an opportunity to pursue a personally rewarding idea. A learning pyramid shows how skills in the earlier phase provide a foundation for developing the research proposal(Fig 1). One advantage of this approach is that it engenders much enthusiasm and encourages wavering students who shun the didactic approach, to seriously re-consider science. In addition students who are turned off by dissection might welcome exploring the development of plant embryos (seeds) or animal embryos (tadpoles). By studying such developing systems, students might be motivated to investigate the regulation of protein expression over time .

Seed study provides a clever method of exploring evolutionary relationships. Thirty years ago, Ladizinsky and Hymowitz (1979) reviewed a number of different studies about the role of evolution in underpinning the taxonomic relationships among different families of plants. These authors provided evidence that electrophoretic banding of seed proteins might presage relationships within plant families. Traditional taxonomy of plants has used morphological characteristics to classify botanical specimens. There are over 15 different families of plants whose members are countless. Based on the current trends in molecular biology, it would be interesting to determine whether the long standing criteria for classifying plants into families is corroborated by such protein expression studies.

Seeds are universally available and reasonably priced so that students would have a wide choice of selections to test their hypotheses. Seed preparation is rather routine and is unlikely to trigger any student protest. Yet this approach will test the students' expertise in making extracts, protein equilibration, electrophoresis and analyses of banding patterns to the same extent as the use of animal tissues.

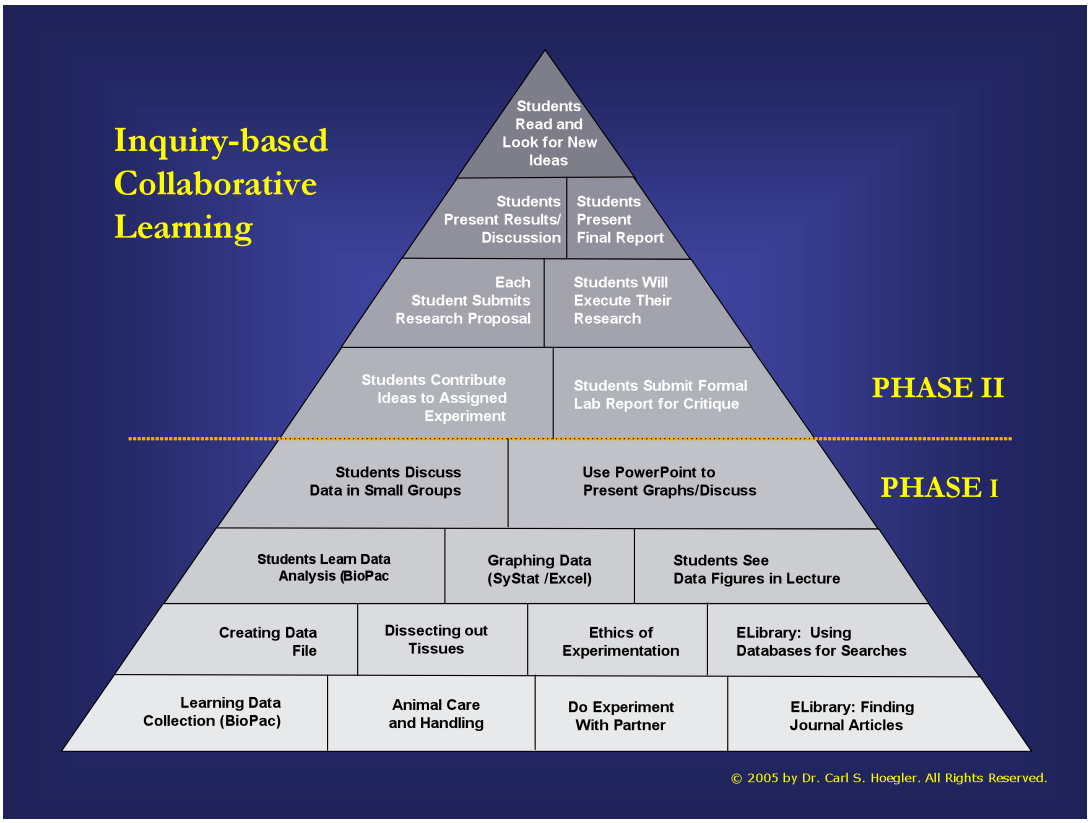


Figure 1. Learning pyramid showing student activities during Phase I and Phase II of inquiry-based collaborative learning (Hoegler, 2005) Students first perform activities at the base of the pyramid before reaching higher levels.

MATERIALS AND METHODS

A. Preparation of Seeds: Plant seeds from different plant families are commercially available. The following seed selections were used: Chinese cabbage (Michili), *Brassica rapa* (New England Seed Company); cabbage (Ferry's Round Dutch) *Brassica oleraceo capitata* (Ferry Moss Seed Company; garden bean (Tenderpick) *Phaseolus vulgaria* (Burpee Seed Company); lima bean (Baby Fordhook) *Phaseolus lunatus* (Burpee Seed Company); Swiss chard (Fordhook Giant) *Beta vulgaris var. cicla* (Burpee Seed Company); turnip (Purple Top- White Globe) *Brassica rapa* (Burpee Seed Company); pea (Sugar Snap) *Pisum sativum var. macrocarpum* (Burpee Seed Company); lettuce (Burpee Bibb) *Lactuca sativa* (Burpee Seed Company); cucumber (Straight Eight) *Cucumis sativas* (Burpee Seed Company).

After rinsing three times with distilled water, seeds were weighed (0.5g) and ground with hammer, mortar and pestle. The mash was mixed with 5 ml distilled water for 2 minutes over ice. The slurry was vortexed briefly and centrifuged at (10,000 rpm) (*Serofuge)* for 5 min. Supernatant was aliquoted into separate labeled microfuge tubes and frozen at -20° C.

B. Protein Calibration of seed homogenates: The total protein of each sample was determined to equilibrate protein levels within each of the wells of the PAGE gel. Either of two methods was used. 1. *Bio-Rad protein assay*: This required the use of visible light spectroscopy (595 nm). A calibration curve is first developed using a known protein (*Sigma-Aldrich* BSA- bovine serum albumin). Unknown optical densities (OD) were compared along the curve to determine quantity of protein. Reliable OD readings are usually found along the lower range of concentrations of BSA (2-6 μg). Use of 1 ml cuvettes minimized the quantity of extract.
2. Spot plate protein analysis: this is less accurate, but provides general estimates of the protein content. BSA is done first to determine range of color (tan to azure-blue). Unknowns must be done quickly, since the dye complexes change in a few minutes.

C. PAGE (Polyacrylamide Gel Electrophoresis): The technique of electrophoresis is generally known to most biologists and the protocols will differ slightly from one lab to another. Two published protocols can be found in papers from a previous ABLE Conference (Racusen and Thompson, 1996; Frame, K. 2000). Samples in microfuge tubes (See A.) were thawed in tap water and re-pulverized over ice with a thin glass pestle (engineered from a Pasteur pipet). The samples were then vortexed and centrifuged for 5 minutes in a *Serofuge* centrifuge. Remember that if you used lysis buffer, you must consider the additional volume before loading. Aliquots of the tissue extract (25μL more or less, depending on the protein content) were diluted 1:1 with Bio-Rad sample buffer (mixture of 950 μL Laemmli buffer and 50 μL of mercaptoethanol). The mixture was re-vortexed and re-centrifuged for five minutes. Supernatants were then placed in a 65° C hot bath for 5 minutes and then placed in an ice bath.

Precast Tris-Glycine SDS-PAGE gels (15%) were inserted in a *Bio-Rad* gel cassette sandwich of the Protean-3 electrophoresis system, which slides into the electrode assembly. Pre-mixed Tris-glycine buffer (Bio-Rad) is poured into upper and lower chambers. Then the wells are loaded. The first lane has a protein ladder with *Bio-Rad* Kaleidoscope markers (8) (6.5- 205 kD). The remaining wells are loaded with various unknown prepared extracts. N.B. Each well can be loaded with 5-25 μg volumes depending on the amount of protein in the original sample. Remember to ascertain the total protein in your sample before dilution with the sample buffers. The volume in each well also depends on the thickness of your precast gel.

Once loaded, the gels are run at 180 V for approximately 40 minutes using a *Biorad* Power supply. Check the tracking dye! After the run, the gel is removed and notched at one end. After several washes in distilled water, it is stained in commercially available *Bio-Rad* Coomassie blue solution for 1 hour (this stain can be re-used). Destaining with distilled water over the next 2-3 days is absolutely essential for clarifying the bands.

D. Data Analysis: After scanning and pre-processing the image using the *NucleoVision* imaging system, the position and intensity of the bands in each lane could be tracked. The intensity of the bands is displayed as a graph over the length of the lane. *Sony* Digital Color Printer (D2500) was used to create hard copies of the gel and the graphs. There are other similar systems, simpler and more complex, available commercially. After marking the bands, it was possible to determine whether there was coincidence between bands of different plant extracts. A similarity index (SI) was

employed (Vaughan and Denford, 1968). Similarity index = the number of coincidental band sets between two extracts / number of coincidental band sets + number of other bands counted in all two comparison lanes.

An alternative approach to the analysis would be to digitally photograph the stained gels after a run and save the images as .jpg or .tif files. Ideally, before doing this analysis, one should preprocess the gel image using photo-editor software (from a photograph as a .tif or .jpg file or from a scan) in order to make the protein bands more clear. The photos can then be annotated to describe the lanes and bands and measurements can be made. Direct comparisons of bands using R_f values relative to the origin of run are a useful starting point. The presence of a molecular weight ladder of known protein standards allows students to estimate band molecular weights.

RESULTS

Our results clearly show that members (turnip, Chinese cabbage and cabbage) of the *Brassicaceae* family have distinctive protein banding characteristics that support the classical taxonomy based on morphology (Figure 2.) This profile was demonstrated across 4 different electrophoretic runs using 2 different sample preparations. A display analysis of peak band intensities shows much coincidence among the members of this family (Figure 3). The seed preparation were easily performed and the preparations appeared to sustain their protein banding patterns months and even years after initial preparation provided that the tissues are stored under frozen (-20° C)temperatures. This latter feature might be useful when preparing extracts for large groups of students or multiple sections.

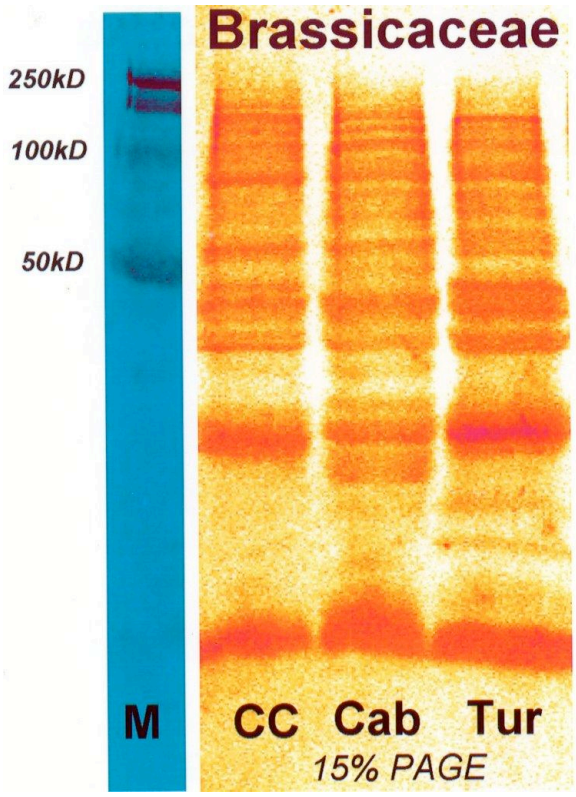
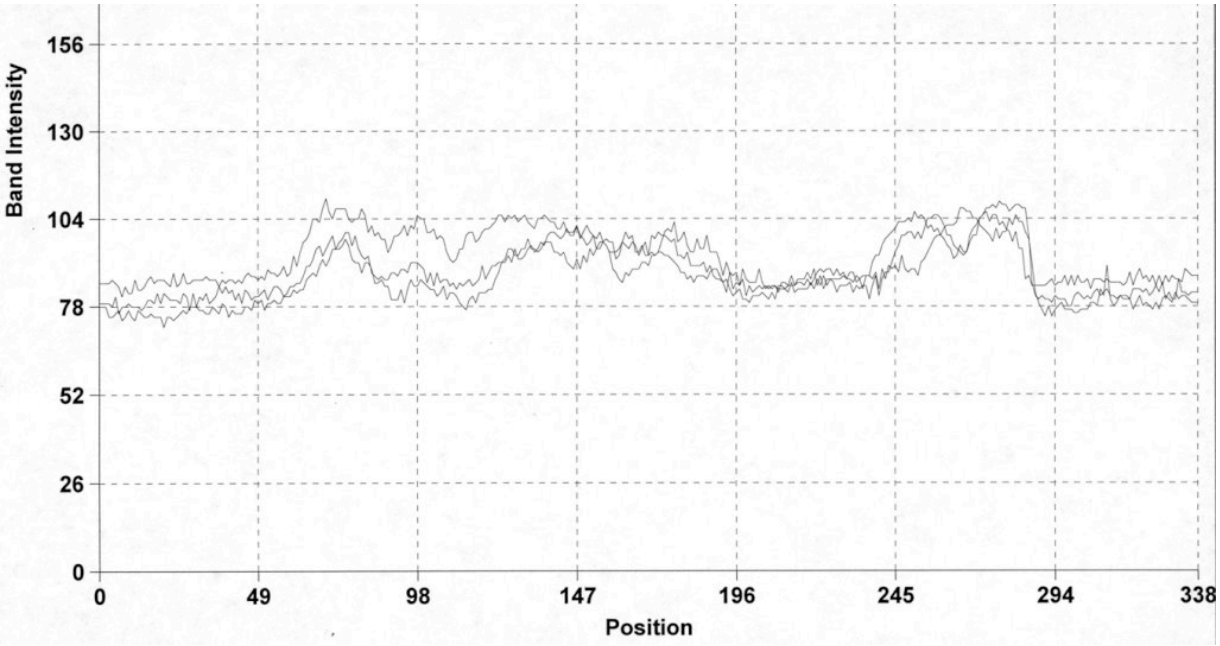


Figure 2. PAGE gel of seed extracts of three members of the ***Brassicaceae*** family [CC (Chinese cabbage), Cab (Cabbage) and Tur (Turnip)]. There appears to be coincidence among banding patterns of these members at low and high molecular weights.

This obvious coincidence of the banding patterns between the members of the *Brassicaceae* family was corroborated by quantitative SI (Vaughan and Denford, 1968), a method highlighted in the review by Ladizinsky and Hymowitz (1979). Table 1 shows the calculated index between the members of 5 different families of plants. The SI among members of the *Brassicaceae* family is high (0.44-0.65), suggesting kinship between such species as turnip, cabbage and Chinese cabbage.



Legend: from top to bottom in the region of 0-68 are Chinese cabbage, Cabbage and Turnip.

Figure 3. Densitometric graphic scan of 3 lanes showing protein bands of 3 members of the ***Brassicaceae*** family. Large peaks represent prominent bands. Bands of all three members are coincidental at several positions: including 76,102,136 and the region of 272.

Table 1. Similarity Indices between Seed Protein Profiles

	Pea	**Bean**	**Lima**	**ChCab**	**Cabbage**	**Turnip**	**SwChard**	**Lettuce**	**Cucum**
Pea		0.29	0.23	0.25	0.20	0.26	0.19	0.23	0.11
Bean	0.29		0.24	0.10	0.16	0.26	0.20	0.25	0.12
Lima	0.23	0.24		0.12	0.04	0.18	0.15	0.24	0.14
ChCab	0.25	0.10	0.12		0.63	0.44	0.29	0.24	0.16
Cab	0.20	0.16	0.04	0.63		0.50	0.31	0.19	0.11
Turnip	0.26	0.26	0.11	0.44	0.50		0.36	0.18	0.31
SwChard	0.19	0.20	0.15	0.29	0.31	0.36		0.17	0.06
Lettuce	0.23	0.25	0.24	0.24	0.19	0.18	0.17		0.04
Cucum	0.11	0.12	0.14	0.16	0.11	0.31	0.06	0.04	

The consensus between representative members (beans, lima beans and peas) of the *Fabaceae* was less convincing than those of the *Brassicaceae* (Figure 4). The protein pattern in the members of the *Fabaceae* family showed SI indices, which resembled indices between members of non-related families (*Asteraceae, Amarthaceae and Curcubitaceae*) (Figure 4 and Table 1).

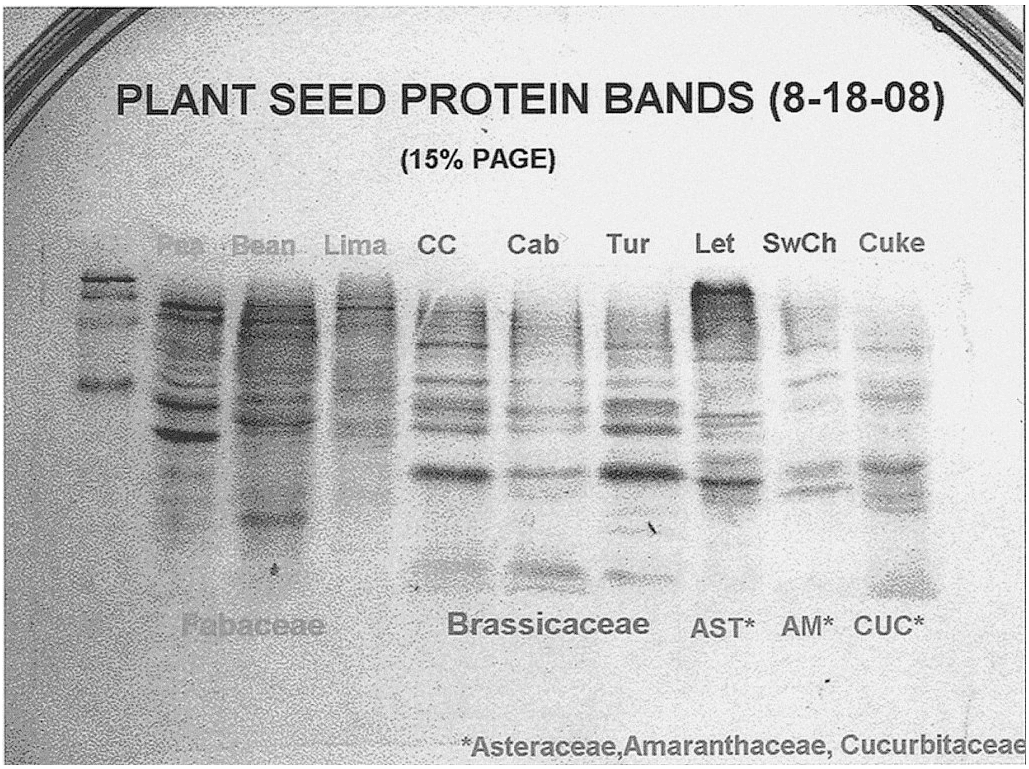


Figure 4. PAGE gel of seed extracts of 9 members of 5 different families. Marker lane (left) are 250kD, 150kD, 100kD and 50kD from top to bottom. Pea, Bean, Lima (lima bean), CC (Chinese cabbage),Cab (cabbage) and Tur (turnip),Let(lettuce), SwCh (Swiss chard) and Cuke (cucumber).

The protein banding patterns of each representative from 2 of 3 other family representatives (*Asteraceae, Amarthaceae and Curcubitaceae*) could be readily distinguishable from those of the *Brassicaceae and Fabaceae*. Interestingly Swiss chard, a member of the *Amarthaceae,* showed an SI (0.29-0.36), which marks it, a possible candidate for close relationship with the *Brassicaceae.*

DISCUSSION

This exercise could provide an opportunity for students to develop their skills in molecular biology, including extract preparation, electrophoresis and data analysis. The collected data could foster an understanding of relationships among members of a particular plant family. These results appear to provide additional evidence for the subtle evolutionary relationships.

For example, it would appear that the similarity indexes (SI) between members of the Brassicaceae family are relatively high suggesting biochemical corroboration of the traditional classification between such species as turnip, cabbage and Chinese cabbage. This relationship stood up in spite of the hundreds of different seeds of each representative member that were sampled to make the seed extracts. This would suggest stability of the protein bands within a large population.

On the other hand, with members of the Fabaceae family, only one or two seeds were used to make the seed extracts, suggesting less variability. Yet the SI of representative members of this family was relatively low (0.23-0.29). This suggested that for specific members of these *Fabaceae*, the tested electrophoretic seed protein patterns did not corroborate traditional phylogenetic kinship.

It remains to be seen whether other members of the *Fabaceae* family exhibited more obvious band comparisons.

There are probably some limitations of this SI method. Since numbers of bands of both light and dark are given equal value, quantification of the protein is ignored. In addition, the thickness of the band is not qualified. One possible strategy to resolve these limitations would be to measure the distribution of banding intensity at different molecular ranges. For example, how many dark bands were found in the low molecular weight or the high molecular weight regions?

Though this particular project is restricted to exploring the basis for taxonomic relationships among members of different plant families, follow-up exercises are possible. Students who are in an honors program or students in a Botany course could compare different varieties of one seed family member, such as seeds of different characteristics (textures-wrinkled, smooth, or colored- green, white) or from plants of different types (normal vs. dwarf). Gottlieb and de Vienne (1988) found that isotypes of pea (*Pisum sativum)* seeds and seedlings (by virtue of texture of the seed coats) showed differences in proteins reflecting pleiotropic changes**.**

In courses such as Developmental Biology or Genetics, extended exercises could go beyond germination of seeds of a particular type, by comparing changes in the protein profile in parts of the seedling (epicotyls vs., hypocotyls) over time. Gottlieb and de Vienne (1988) have suggested that between phenotypically different strains of pea seedlings such protein differences were slight. This reduction in protein differences might be explained by decreases in storage proteins, such as cruciferin and napin in *Brassicaceae* (rape seed, *Brassica napus)* due to enzymatic hydrolysis during germination (Hoeglund *et al*., 1992). Another extended exercise could test the effects of different shock procedures on mature plants to determine whether such interventions would cause epigenetic effects on the seeds produced by these plants.

For future study, we will be exploring the use of Western blotting to determine the identity of particular bands. We would expect that the legumin in the broad bean to be one of the large storage protein bands, similar to that recovered by Millerd *et al*. (1971) in *Vicia faba.* Several other conspicuous candidate bands belong to another group of storage proteins.

Though we have yet to test this particular exercise in a lab class, a comparable approach at North Carolina State using of corn seed extracts in many sections of General Biology has engendered much positive feedback (Niedzlek-Feaver *et al*. 2008). Unfortunately, they have discontinued using the wet labs because of the arduous nature of lab preparation for large numbers of students. Nevertheless, for colleges with smaller student bodies, these kinds of experiments could be more easily implemented.

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