Video Article

Denver Papillae Protocol for Objective Analysis of Fungiform Papillae

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Abstract

The goal of the Denver Papillae Protocol is to use a dichotomous key to define and prioritize the characteristics of fungiform papillae (FP) to ensure consistent scoring between scorers. This protocol builds off of a need that has arisen from the last two decades of taste research using FP as a proxy for taste pore density. FP density has historically been analyzed using Miller & Reedy's 1990 characterizations of their morphology: round, stained lighter, large, and elevated. In this work, the authors forewarned that stricter definitions of FP morphology needed to be outlined. Despite this call to action, follow up literature has been scarce, with most studies continuing to cite Miller & Reedy's original work. Consequently, FP density reports have been highly variable and, combined with small sample sizes, may contribute to the discrepant conclusions on the role of FP in taste sensitivity. The Genetics of Taste Lab explored this apparent inconsistency in counting and found that scorers were individually prioritizing the importance of these characteristics differently and had no guidance for when a papilla had some, but not all, of the reported qualities of FP. The result of this subjectivity is highly variable FP counts of the same tongue image. The Denver Papillae Protocol has been developed to remedy this consequence through use of a dichotomous key that further defines and prioritizes the importance of the characteristics put forth by Miller & Reedy. The proposed method could help create a standard way to quantify FP for researchers in the field of taste and nutritional studies.

Video Link

The video component of this article can be found at https://www.jove.com/video/52860/

Introduction

Papillae are the visible bumps on the tongue's surface. There are four types of papillae: fungiform (FP), foliate, circumvallate, and filiform. While filiform papillae are spread over the surface of the tongue, circumvallate are localized only on the back of the tongue, and foliate are found on the sides. In contrast, FP are located toward the front of the tongue. FP, foliate, and circumvallate papillae are considered gustatory because they have the potential to contain taste buds while filiform do not. Taste buds are clusters of cells responsible for detecting chemical tastants and transducing the stimuli into a gustatory signal in the brain. Saliva and food molecules enter into the taste buds through openings called taste pores where taste molecules then have the potential of activating taste cells.

Interestingly, there are large individual differences in taste bud density, as first observed in a histological study of tongues from 18 cadavers (Miller, 1988)[1]. Taste buds themselves are not visible on the tongue's surface, however, taste pores are visible when using proper magnification, lighting, and staining techniques which led Miller & Reedy (1990) to develop a method to identify taste pores and fungiform papillae using videomicroscopy in living human beings[2]. A number of labs have modified their original videomicroscopy technique, using still photography and little or no magnification. However, since fungiform papillae are much easier to visualize than are pores using these latter techniques, labs turned to counting fungiform papillae in place of taste pores. In doing so, it is assumed that fungiform papilla density is a reasonable proxy measure for taste pore density, even though many fungiform papillae do not contain taste pores or taste buds[3]; not only are FP more visible and accessible than circumvallate and foliate papillae, but their image capture is less time consuming than taste pore analysis through the alternative method of videomicroscopy[2,4]. In 1990, Miller & Reedy used this technique to find that taste pore densities are correlated with FP densities[3] which was further corroborated in Bartoshuk *et al.*[5] In addition to this anatomical relationship, Miller & Reedy also found that the 8 individuals in the upper half of the taste pore density distribution also rated sucrose, NaCl, and propylthiouracil (PROP) as significantly more intense than did the other 8 individuals[3]. Subsequent work by Bartoshuk *et al.* (1994) observed a correlation between suprathreshold PROP taste intensity and fungiform papilla density, as well as taste pore density, in 42 subjects[5]. This convenient method led to influential studies reporting that subjects whose tongues have many papillae elicit a stronger reaction to many tastants, including the bitter tastants phenylthiocarbamide (PTC) and PROP[6,7].

PTC and PROP are well-researched tastants oft used due to the clear link between a person's taste sensitivity phenotype and their genotype. Population studies have shown that people who have the homozygous recessive diplotype of the gene, *TAS2R38*, have a significantly lower sensitivity to PROP than carriers of the dominant or heterozygous variant of the gene[8-10]. This heritable phenomenon was first reported in 1932 when Arthur L. Fox announced his discovery of "taste blindness" to PTC[11]. Among people able to taste these bitter compounds, the intensity

of taste reported can vary anywhere from slightly unpleasant to excruciatingly bitter[6]. To account for variance that cannot be explained by *TAS2R38*, the theory was put forth that it could be attributed to the density of FP on the tongue[4].

As taste research progressed, the field was divided into two schools of thought around this theory concerning the role of FP and bitter taste sensitivity. Although many studies corroborated the original claim that FP density is a factor in PROP sensitivities[4,6,12,13], there has been a small, but important contingency that has found evidence to refute it, reporting an inability to replicate the role[10,14,15,16]. Delwiche *et al.* (2001) warned that the trend is highly variable; FP density does not uniformly account for differences in bitterness sensitivity across subjects, and was only demonstrable for subjects with at least a moderate sensitivity to PROP[7]. In addition to the use of the less rigorously defined Miller & Reedy method on how to quantify FP, it is important to note that with exception of the Beaver Dam Offspring Study[15] conducted by Fischer *et al.*, most of the above studies had small sample sizes which may also contribute to inconsistent findings on the role of FP in taste.

Briefly, in Miller & Reedy's seminal methodology paper (1990), the authors stained tongues blue to quantify FP because FP remain pink while filiform papillae absorb the dye and turn blue. They classified FP as "rounded pink structures about 0.5 mm in diameter"[2]. While the authors advised that before using this method for further analytical work, FP characteristics should be more strictly defined and concluded that the variation already noted in the literature "might be attributable to different subject populations, different methods, and different investigators"[2], their research has led to the commonly accepted characteristics that FP are round[17,18], large[2], pink or stained lighter[2], and elevated[4]. Taken at face value, these characteristics appear very straight forward. In the Genetics of Taste Lab (GoT Lab or Lab), the use of the method was complicated by the scorers' individual attempts at qualifying and prioritizing the importance of the above characteristics when some, but not all, of the characteristics were present. These complications included small papillae that appeared pink in the midst of much larger papillae; the entire tongue absorbing the blue dye thus eliminating classification based on color variation; oblong papillae instead of round; and tongues having no variation in elevation, all of which contributed to scorers reporting widely-varied FP counts of the same image.

These variations in analysis led us to review the literature in order to pinpoint the subjectivity, identify any exceptions to the commonly accepted FP characteristics and ultimately devise a protocol to accurately and objectively define and score FP.

First, FP shape is described in the literature as a rounded, mushroom-like structure[17,18]. However, notable exceptions were also mentioned. Miller emphasized that the term "fungiform" is a misnomer and that many FP are not mushroom-shaped but can vary widely in size and morphology[18]. Melis *et al.* (2013) also described several papillae that were considered "distorted," where the FP diameter in one direction was at least two standard deviations longer than in another direction[19]. Furthermore, Cheng & Robinson found that when indicating FP by staining, they ranged from flat-topped to elongated in appearance[20]. Noting the above exceptions, the characterization of an FP being round and mushroom-shaped appears too narrow a definition.

Second, FP color is described as "pink circles against a blue background"[7] after staining the tongue with blue dye. Though staining lighter was the most consistent criterion of FP throughout the literature it was still not a uniform qualifier. Cheng & Robinson observed that FP were not always lightly stained, making identification difficult and uncertain[20]. It appears then that tongues absorb dye differently and while the contrast is apparent on many tongues, some become entirely blue with no distinction while others immediately lose any trace of blue dye[21].

The third characteristic, FP size, was fairly consistent among papers, ranging from 0.5 mm to 0.97 mm[6,18]. In the literature Miller noted that there were two size ranges of papillae on the dorsal anterior of the tongue. The papillae with larger diameters were primarily fungiform and conical papillae, while the papillae with a smaller diameter were mostly filiform[18]. However, with the exception of the Beaver Dam Offspring Study[21], size did not appear to determine if a structure is a FP in the literature, but was provided for papillae already classified as "fungiform".

Finally, elevation is listed as the fourth FP characteristic[4] though Miller pointed out that distinction between filiform and fungiform papillae is difficult when using this criteria near the margin of the tongue. He gave two examples of FP that varied greatly in elevation, one being 0.8 mm in height while another was less than 0.1 mm in height, but still had two taste pores that were clearly observed[18]. This observation was also followed up and confirmed by Shahbake *et al.*[4]

These definitions and their reported exceptions show that the taste field has long recognized the inconsistency in characterizing FP and the shortcomings of the commonly-used protocol from Miller & Reedy. Indeed, Miller & Reedy exhorted the field to further define the features of FP[2]. We hypothesized that scorers were giving different weight to the characteristics and prioritizing their importance differently when papillae failed to meet every criterion, such as when a papilla was large and mushroom shaped, but stained blue. It was reasoned that investigators of the previously published studies likely did the same which, when combined with small sample sizes, may account for the varied results coming from use of this method.

The GoT Lab addressed this gap in FP methodology by developing a guideline that defines each characteristic using objective measurements and prioritizes the characteristics to indicate which takes precedence in analysis. This method is the Denver Papillae Protocol (DPP), a dichotomous key with clear and distinct characteristics of FP to ensure accurate and repeatable counts from digital photographs of the tongue. DPP is a proposed method to standardize the previously used Miller & Reedy method, thus removing individual interpretation and ensuring more consistent results when analyzing FP density. By doing so, DPP can be used to more confidently determine the role of FP in taste.

The tongue images taken of the subjects were analyzed by the citizen scientists (scorers) of the Lab who are the true subjects of this study. The Lab's core of citizen scientists is comprised of members from the community ranging in age (years) from 16 to mid-80s. A summative evaluation report commissioned by the Museum in 2012 describes citizen scientists as having a strong interest in science and with two-thirds having earned a college degree in a scientific discipline[22]. Candidate citizen scientists are currently recruited via word of mouth, must successfully complete an interview process with veteran volunteers at the Museum, and undergo a trial period in the Museum's permanent health exhibition, Expedition Health, before they have the opportunity to apply for a position in the Lab. Once accepted into the Lab, citizen scientists undergo a 12-week training to become certified to enroll human subjects. Following this certification, they are able to take training modules on various techniques in the Lab (*e.g.* papillae counting, DNA extraction) and following additional certifications and regular quality control measures, these citizen scientists have the opportunity to actively participate in data analysis. Citizen scientists volunteer their time and are not compensated for their contributions to the Museum.

Protocol

The images scored in this study were collected as part of a larger taste study conducted in the Genetics of Taste Lab at the Denver Museum of Nature & Science (the Museum)[10]. Subjects of the larger study were museum visitors (n = 1195) ranging in age from 18-93. While subjects came from six of the seven continents, participants were primarily of European descent. Each subject's data was collected in one 30 min lab session. The Western Institutional Review Board approved the protocol, informed written consent was given, and subjects volunteered their time and were not compensated for their participation in the study.

1. Data Collection

1. Image Capture
   1. Show subjects a photograph of how to pose themselves (**Figure 1**) and what the captured image will look like.

Figure 1:

Image Capture Pose. This is the photograph shown to subjects in the GoT Lab in order to correctly pose for their tongue photo.
   2. Direct subjects to dry their tongue with a paper hand towel and leave tongue protruding from their mouths.
   3. Apply approximately 3 ml of blue food dye at a 1:36 concentration to the apex of the tongue using a sterile, rayon applicator with a 1-inch tip. Have subjects return tongue to their mouth and swallow to remove excess dye.
   4. Have subjects pose themselves as shown in **Figure 1**, with their chin on their hands and elbows propped on a table for stability. Direct subjects to extend their tongue a comfortable distance and secure it gently between their teeth.
   5. Adhere a 2.5 cm piece of filter paper with a 10 mm diameter circular cutout punched in it to the tip of the anterior of the left side of the tongue next to midline (see **Figure 1**).
   6. Take at least three close-up images of the tongue, capturing the entire 10 mm circular cutout to ensure visualization of all FP within that area. Use the macro setting of a high-quality digital point and shoot camera attached to a tripod for stability.
      1. Zoom in to the extent that the entire cutout is photographed while still within the camera's recommended zoom range. Ensure that the plane of the camera lens is parallel to the plane of the tongue and that the cutout appears circular in the photo instead of oblong. Review photos to ensure they are high quality and useful for counting.

2. Image Selection and Preparation
   1. Upload the photos to the computer.
   2. Of the images taken of the same subject's tongue, select only one for further analysis based on clear definition between structures on the tongue at the highest zoom and the least distorted angle so the tongue is broad and flat toward the camera.
   3. Open the selected raw image in the open source software, ImageJ. Click on "Plugins" and in the dropdown menu scroll to "Analyze" and click "Cell Counter." When the cell counter opens, click "Initialize" to link the image to the cell counter.
      Note: Here, use ImageJ 1.45 sec with 32-bit Java 1.6.0_10 version.
      1. Use a standard magnification of 50% for consistency between scorers and for use in subsequent steps to score FP. Move this image to the left side of the screen next to the cell counter. This image will be referred to as Copy A (See **Figure 2**, left side of screen).

3. Open a second copy of the raw image (copy B) for measuring the diameters of the papillae being quantified. Zoom in and out as needed to the preference of the individual scorer throughout the scoring process. Move this copy to the right side of the screen so the two copies are not accidentally confused. (See **Figure 2**, right side of screen)

4. Click on the line tool. Using copy B, zoom in as much as necessary to accurately draw a diameter across the 10 mm inner circle of the filter paper at any angle and click "Analyze" and "Set Scale." Fill in the "Known Distance" to be "10." Verify the scale is correct by measuring an alternate angle and ensure the diameter range is between 9.8-10.2 mm. If it is not, repeat this step.
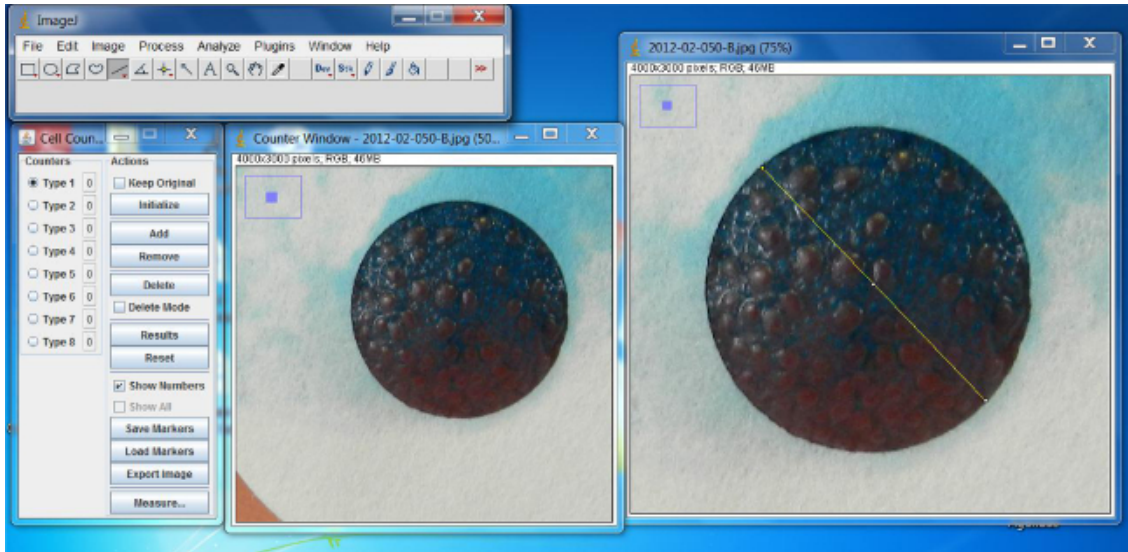
Figure 2: Copy A and Copy B. Copy A (left) is linked to the cell counter window and remains at 50% magnification for consistency from scorer to scorer while copy B (right) may be zoomed to the preference of the individual.

2. Scoring FP Using the Denver Papillae Protocol Dichotomous Key

1. For each candidate papilla, use the following dichotomous key (step 2.2-2.5) detailing the criteria to determine if it is a FP. Refer to **Figure 3** for a visual of papillae that are classified as FP and for ones that are rejected at each step in the process.
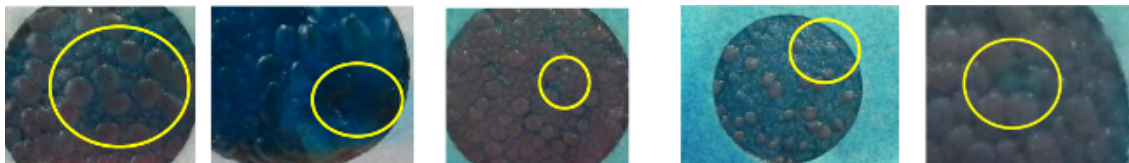

Figure 3: FP vs. Rejected Papillae. Figure 3a is a tongue with several qualifying FP while **3b-3e** have circled areas that violate each rule. (**3b**) area is amorphous; (**3c**) papillae are too small; 3d) papilla is blue compared to those surrounding it, (**3e**) papilla is recessed compared to those around it.

2. Shape
   1. Determine if the candidate papilla is amorphous (shapeless). At 50% zoom see if there is a commonly-recognized geometric shape (oval, cuboidal, round).
   2. If there is a geometric shape (see **Figure 3a**), move to step 2.3.
   3. If there is no geometric shape (see **Figure 3b**), go to the cell counter window and click "Type 1." On copy A (50% zoom), click the candidate papilla to mark it as amorphous and not a FP. Clicking on types 1-4 in this and subsequent steps allows scorers to know they have addressed all candidate papillae and categorizes which rule was violated when rejecting a structure which will aid in the discussion in step 2.7.2.

3. Color
   1. Refer to copy A at 50% zoom and determine if there is any color differentiation over the surface of the tongue. If so, continue to the next 2.3.2. If not, do not use color as a determining factor; move to step 2.4.
   2. Determine if the candidate papilla is lighter than the tissue or papillae surrounding it (see **Figure 3a**). If any part of the candidate papilla has remained pink or stained lighter, move to step 2.4.
   3. If the candidate papilla is blue and the surrounding papillae are lighter (see **Figure 3c**), go to the cell counter window and click "Type 2" on the cell counter. On copy A, click the candidate to mark as too blue and not a FP.

4. Size
   1. Using copy B, zoom in to the distance where one can comfortably see the outline of the candidate papilla.

2. Click on the line tool and measure across the longest dimension of the candidate papilla. Click "Analyze" and then "measure." If the measured length is 0.5 mm or greater (see **Figure 3a**), move to step 2.5.

3. If the measured length is 0.499 mm or less, measure once more to ensure accuracy. If it is still 0.499 mm or less (see F**igure 3d**), click "Type 3" on the cell counter window and on copy A, click the candidate papilla to mark it as too small and not a FP.

5. Recession
   1. Using copy A, assess if the candidate papilla is either uniform height with the rest of the tongue or elevated. If the papilla is in a crevice, determine its height compared to the other structures in the crevice, not the surface of the tongue. If the papilla is lower than the papillae surrounding it (see **Figure 3e**), use "Type 4" and on copy A, click the candidate papilla to mark it as recessed and not a FP.
   2. If the candidate papilla is either of uniform height with the rest of the tongue or elevated (see **Figure 3a**), use "Type 5" to click it and mark that this is a FP. This type 5 total is the raw FP score. Do not close the copies at this point as ImageJ does not save the scores on the cell counter nor the colored marks from types 1-5 on copy A.

6. Saving Copy A
   1. Write down in notebook raw FP score.
   2. On the cell counter window, click "Export Image." Save with the desired naming system of the lab. This will allow the opening of copy A in the future to retain the colored marks from types 1-5. It will not save the raw FP score.

7. Quality Control
   1. Score each photo by two individual scorers and compare[7].
   2. If the higher FP raw score is within 10% of the lower FP raw score, average the two scores together for a consensus FP score. If the two FP raw scores differ by more than 10%, pull both scored photos onto the screen.
      1. Use counter types 1-4 to aid in discussion of discrepancies and confer on a final consensus score. If no consensus can be reached during the discussion, take a break from scoring. At a later point, rescore the image and discuss. If a consensus still cannot be reached, pull in a third scorer. Ask them to score the image and dialogue with the other two.

3. Training Scorers to Use DPP

1. Background on Training
   1. Have two scorers count a variety of images, scoring individually and conferring regularly to ensure that they were using the same criteria. Only when individual counts were consistently within 10% of each other for each the images when scored was the method (finalized above) considered acceptable and termed DPP.
   2. Choose 15 official images and 15 training images (Supplemental **Figures 2-17**) in sequential order to score. If an image was deemed uncountable during selection, skip the image and choose next sequential image.
      Note: These images vary in quality and size as they were chosen while the Lab was still mastering the correct photography technique. One of the selected images was deemed uncountable through this primary counting process but was left in to ensure that scorers were able to make that call when needed. Therefore there are 16 images given to trainees though only 15 scored images.
   3. Using DPP, have the above-mentioned scorers count the 15 training images and create a consensus score for each.
   4. Give trainees the 15 official images and ask to score using Miller & Reedy to create a baseline. Record scores. Conduct a group training which followed step 3.2. Once step 3.2 was completed, have the scorers score the 15 official images again and record their scores.

2. Training Subsequent Scorers on DPP
   1. Give training scorers the first training image and demonstrate DPP for a few papillae on this image. Discuss reasoning for selecting or rejecting each papilla as an FP. Allow trainees to continue practicing scoring for the rest of that photo and confer with the consensus number (for the GoT Lab's consensus number and Type 1-5 markers, see supplementary **Figure 1**). For this photo discuss any discrepancies regardless of the 10% to ensure understanding of DPP.
   2. Give subsequent scorers the second training image and have them score using DPP.
   3. If the trainee raw FP score is within ten percent of the established consensus count from section 3.1.3., allow trainee to move onto the next image and repeat steps.
   4. If the score is outside the ten percent range, use the trainee's colored marks representing types 1-5 to understand and identify the inconsistency and then address discrepant understanding of DPP.
   5. Give the trainee the opportunity to rescore the image and verify the new score once more. If successful, trainee moves onto the next image until all images are completed.

Representative Results

Here is a representative photograph scored by two individual scorers when using the commonly-used Miller & Reedy methodology for FP identification based on the field standards of round, large, pink or stained lighter, and elevated, and then the same photograph scored by two scorers using DPP. The counts shown are representative of the high variance observed using the original method compared to the lower variance using DPP.
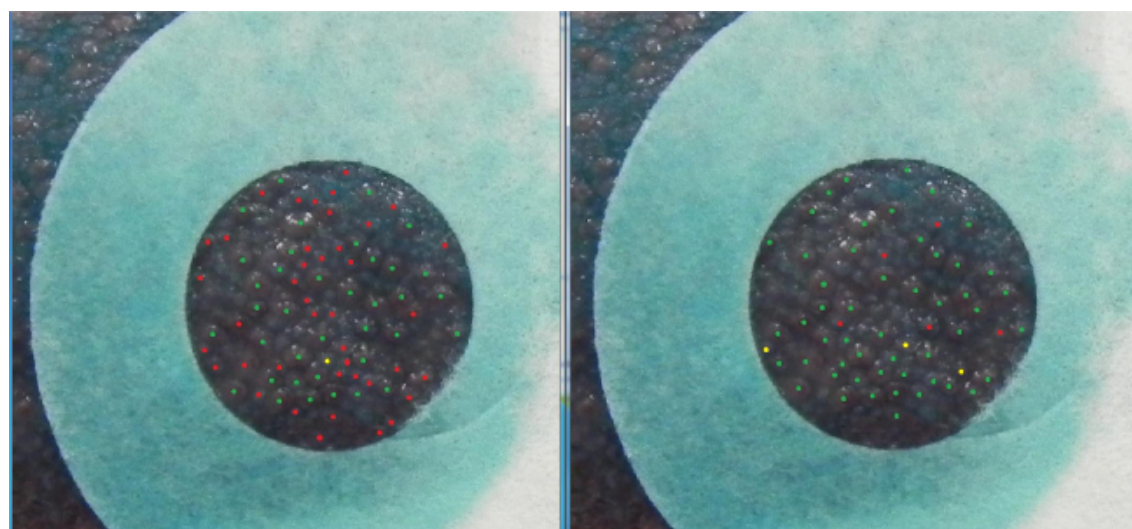
Miller & Reedy   DPP

Figure 4: Miller & Reedy Quantification vs DPP Quantification. The image on the left is representative of two scorers' counts using the Miller & Reedy Method. The image on the right represents two scorers' counts using DPP. Red is scorer 1, Yellow is scorer 2, Green is scorers 1 & 2.

Statistical reliability of DPP was previously studied using a mixed linear model to assess variability[10]. Briefly, to generate data to assess the role DPP plays in consistency of scores, fifteen images were scored twice. In the first batch of scores, DPP naïve scorers used the Miller& Reedy method[2]. The scorers were then trained to use DPP and the images were individually rescored without the final consensus step described in section 2.7 detailing Quality Control (**Figure 5**). The FP score data set was then used in the mixed model to assess the combined differences due to protocol (**Figure 6**), within-scorer variability and the interaction between the two. This model showed a significant difference in scoring when using the Miller & Reedy method compared to DPP (p-value $<1 \times 10^{-6}$) with DPP leading to a higher count by 6.99 (SE = 0.99); 5.2% of the variability in the score was due to training, 25.9% due to scorer, and the remaining due to variability specific to the image[10]. Next, to generate data to determine within-scorer variability, a series of thirty images in random order were scored by 11 individuals. Unbeknownst to scorers, this series of images included three images that were repeated. We observed a drastic difference in score variability comparing distinct images versusrepeated images.
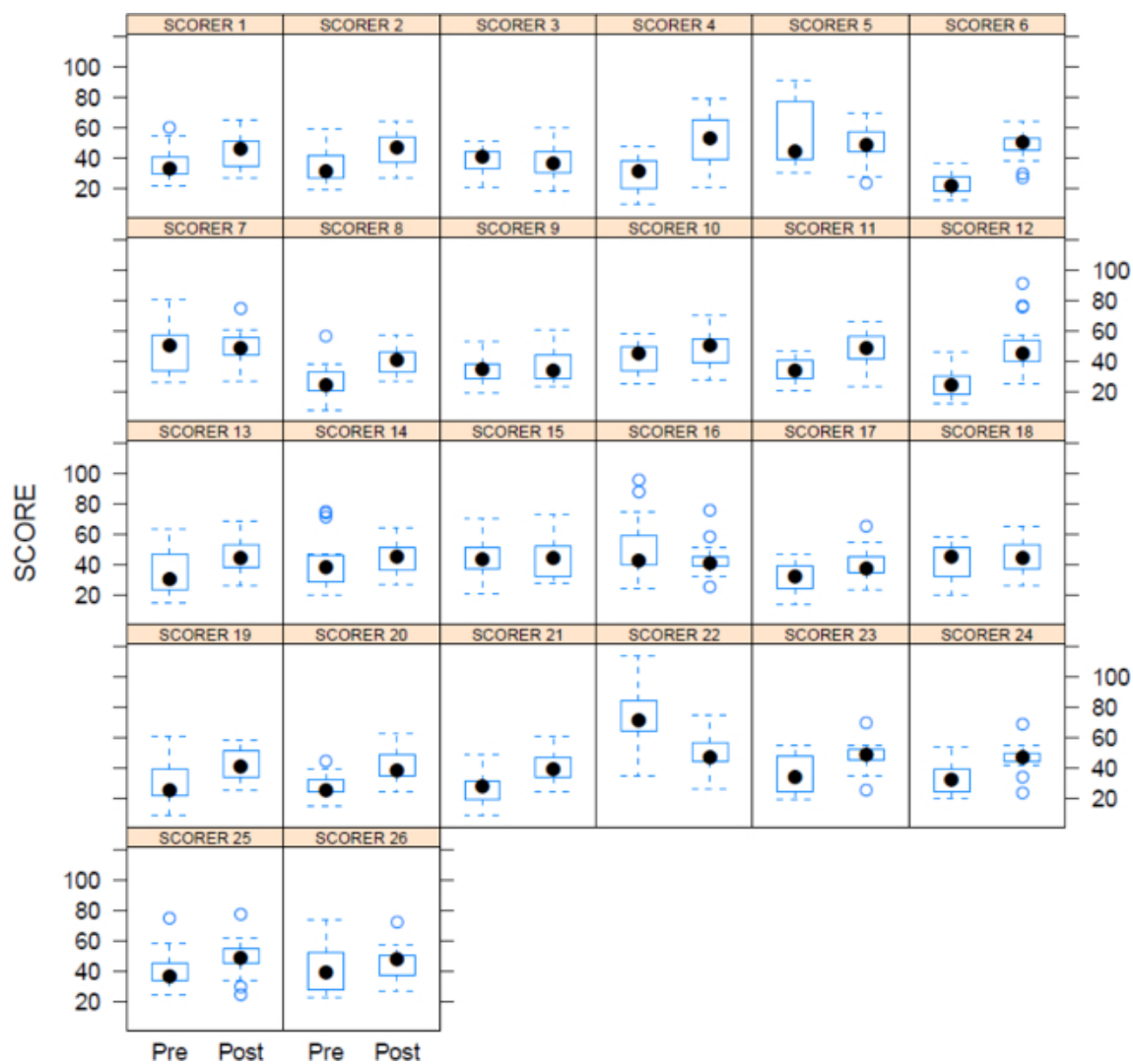
Figure 5: DPP-Naïve vs Post DPP Training. Panels provide boxplots of reviewer scores for 15 images pre and post Denver Papillae Protocol training. For each boxplot, a filled in circle indicates the mean whereas an open circle indicate an outlier.
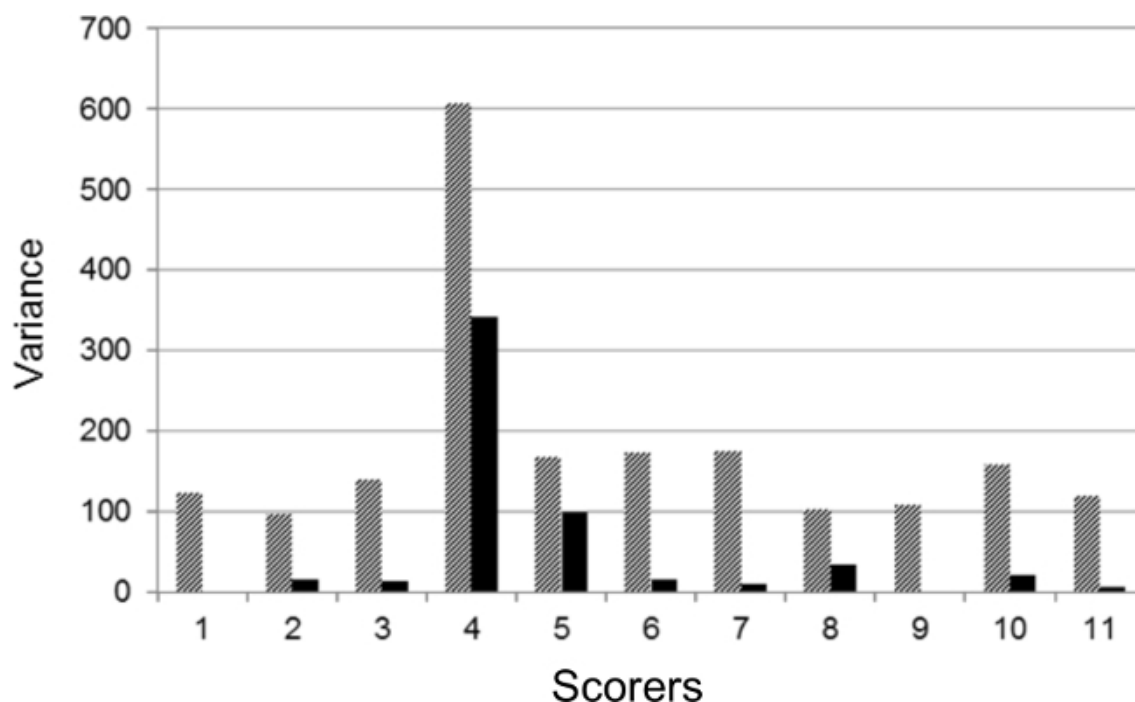
Figure 6: Change in Scorer Variance. The gray represents the variance for individual scorers prior to DPP training. Black is the same scorer post DPP training.

Discussion

Using DPP, the variance within image counts across independent scorers and within-scorer's counts decreased significantly. While this method makes visual FP density analysis without videomicroscopy less subjective, it should be noted that this method only scores FP. DPP cannot guarantee that structures classified as FP have taste pores and are therefore gustatory papillae nor that the counts are reflective of real taste bud density[20,23].The nature of this method is that it establishes reliability through consistency and precision; however, it may be biasing results in a consistent direction. To test accuracy relative to other methods using a true measure of taste bud density (*e.g.*, videomicroscopy) would extend beyond the scope of this study.However, by characterizing the morphology of FP more stringently and creating a dichotomous key, this method addresses Miller & Reedy's charge to the field that more characterization of FP needs to be done in order to have consensus on their morphology and quantification[2]. Additionally, they indicated that this characterization might "clarify some of the differences among subjects and explain some of the disparate results among investigators[2]." By providing a uniform method of quantification, many of the discrepancies on the role of FP in taste and nutrition-related research may be rectified.

The dichotomous key may be adjusted for the different image capture techniques. The known distance for the measurement scale can easily be set to the diameter used in each lab and recession can be discounted if saran wrap or glass were placed over the tongue for image capture. In the GoT Lab, it was found that the neutral tongue provided the best definition of papillae. This may be due to the amount of light in the Lab which caused saran wrap or glass to reflect light back into the camera creating a glare that made assessment difficult. Some labs have said they find the dye more challenging to count, but the GoT Lab found a diluted blue food dye at a concentration of 1:36 provided the ideal contrast between fungiform and filiform papillae on the majority of tongues.

The creation of a dichotomous key for FP characterization will allow for researchers in different labs, using a variety of image capture methods, to consistently analyze structures on the tongue and have confidence in the reporting of their results. Finally, use of a dichotomous key may have purposes in other areas of scientific methodology where qualities of structures or objects are providing inconsistent numbers from study to study.

Disclosures

The authors declare that they have no competing financial interests.

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