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Analysis of Maize (*Zea mays*) Kernel Density and Volume Using Microcomputed Tomography and Single-Kernel Near-Infrared Spectroscopy

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Supporting Information

ABSTRACT: Maize kernel density affects milling quality of the grain. Kernel density of bulk samples can be predicted by near-infrared reflectance (NIR) spectroscopy, but no accurate method to measure individual kernel density has been reported. This study demonstrates that individual kernel density and volume are accurately measured using X-ray microcomputed tomography (μCT). Kernel density was significantly correlated with kernel volume, air space within the kernel, and protein content. Embryo density and volume did not influence overall kernel density. Partial least-squares (PLS) regression of μCT traits with single-kernel NIR spectra gave stable predictive models for kernel density (R^2 = 0.78, SEP = 0.034 g/cm^3) and volume (R^2 = 0.86, SEP = 2.88 cm^3). Density and volume predictions were accurate for data collected over 10 months based on kernel weights calculated from predicted density and volume (R^2 = 0.83, SEP = 24.78 mg). Kernel density was significantly correlated with bulk test weight (r = 0.80), suggesting that selection of dense kernels can translate to improved agronomic performance.

KEYWORDS: *Zea mays, maize, corn, kernel density, seed density, computed tomography, near-infrared spectroscopy, test weight*

INTRODUCTION

Kernel hardness is an important quality trait that affects several stages of grain handling and processing.[1,2] Harder kernels are correlated with higher test weight and are more resistant to breakage during harvest and transport. Softer kernels are more susceptible to mechanical damage as well as pathogen attack. The negative impact of soft kernels on agronomic performance is exemplified by the maize high-lysine mutant, *opaque2* (*o2*), which increases the proportion of essential amino acids in the kernel but confers a soft endosperm texture.[3] Modified *o2* known as Quality Protein Maize (QPM) contains multiple quantitative trait loci (QTL) that improve kernel hardness while maintaining high lysine.[4,5] Kernel hardness also affects the quantity and quality of milled products. Hard, dense kernels produce greater yield and higher quality of dry milled products such as flours and grits.[6] Softer kernels require less steeping time for wet milling and are more amenable to separating seed storage proteins from starch. The starch in soft kernels is also more efficiently digested into simple sugars for applications in animal feeds, ethanol production, and glucose/fructose production.

The quality of hardness is primarily determined by the physical characteristics and chemical composition of the kernel endosperm.[1] The endosperm accounts for 80% of the volume of a typical kernel and contains two types of starch accumulating tissues.[7] The harder vitreous endosperm is composed of densely packed starch granules embedded within a complex protein matrix, whereas the softer, floury endosperm contains larger, loosely packed starch granules. Due to the differences in starch packing between vitreous and floury endosperm, kernel density is a proximate measure of hardness. Kernel density is highly correlated with other measures of hardness including milling characteristics and the vitreous/floury endosperm ratio.[8−11]

Kernel density can be measured in multiple ways. Test weight, typically expressed in pounds per bushel, is a bulk density value used in the assessment of corn lot quality. Finer scale measures of kernel density are the floater test and pycnometer displacement test. The floater test measures the percentage of floating kernels in a salt solution with a known specific gravity.[12,13] Pycnometers measure the water or gas displaced by bulk samples of kernels, and density is subsequently calculated by dividing the mass of the sample by the volume displaced. Both floater and pycnometer density measurements are low throughput, capable of processing only a few samples per hour.

Near-Infrared (NIR) spectroscopy has been used to predict bulk kernel hardness traits for more than 20 years.[1,14] Light in the NIR region of the electromagnetic spectrum (750−2500 nm) is absorbed by overtone and combination vibrations of X−H bonds such as C−H, O−H, S−H, and N−H, which are abundant in organic molecules.[15] Therefore, NIR transmittance and

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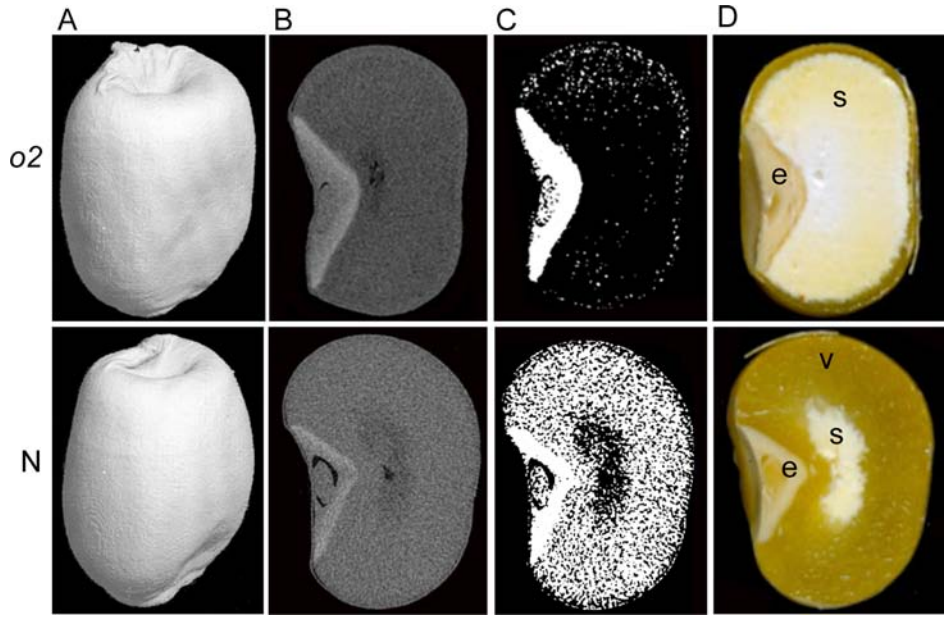

Figure 1. μCT analysis of an *o2* mutant and normal (N) kernel from a segregating ear: (A) 3D reconstruction; (B) single μCT section (gray scale shows relative density, with white indicating the highest density); (C) same section as in panel B with a lower threshold attenuation cutoff to remove signal from soft, starchy endosperm; (D) visible images of transverse hand sections through the same kernels showing embryo (e), vitreous (v), and starchy (s) endosperm at approximately the same location as the μCT section in panels B and C.

reflectance profiles of biological materials are used to derive information about the quality and quantity of organic material within the sample. Predictive models are built using calibration data sets created from analytical reference measurements and associated NIR spectral profiles of diverse sets of samples. To predict kernel density, Siska and Harbaugh[16] developed a partial least-squares (PLS) regression model that estimated density of a bulk sample of kernels. Although bulk seed density measurements are useful to analyze seed lots, finer scale density assessments are needed for research and breeding, where individual kernel density would enable analysis of segregating populations and substantially reduce breeding cycles.

Recently, Armstrong et al.[17] reported a single-seed NIR calibration that discriminates low-, medium-, and high-density kernels from hybrid plants. The PLS model used gas pycnometer density measurements from 10 to12 g of kernels and averaged NIR spectra from individual kernels within the bulk samples. This composite method yielded a model that accounted for 76% of the variance between actual and predicted bulk density measurements. However, the density of individual kernels was not measured, and the accuracy of single-kernel predictions could not be tested. Prior single-kernel NIR calibrations for starch, protein, and oil suggested an improved single-kernel density model can be developed with analytical measurements of single seeds associated with individual spectra.[18,19]

X-ray microcomputed tomography (μCT) is a feasible approach to measure the density of individual kernels.[20] μCT utilizes X-ray attenuation from multiple radiograph "slices" of a sample to reconstruct a three-dimensional (3D) representation of the structure. X-ray absorption is directly related to density, and attenuation of the X-ray signal can be converted into density for any portion of the 3D structure. X-ray radiography has been used in maize kernels to characterize size and shape, stress cracks, moisture dynamics, pathogen infection, and density.[21−24] Moreover, μCT measurements of wood mechanical properties such as density and stiffness were accurately predicted by NIR spectroscopy.[25,26] The objectives of this study were to assess μCT imaging of individual kernels as a method to determine kernel density and volume as well as to develop single-kernel NIR calibrations to predict kernel density and volume based on μCT measurements.

MATERIALS AND METHODS

Kernel Samples. For μCT evaluation and PLS calibration, a set of 312 kernels was collected from 93 ears composed of 64 diverse genotypes. The set overlaps with the genotypes used by Spielbauer et al.[18] Supplementary Table 1 in the Supporting Information gives the specific maize lines, which encompass diverse inbred lines, grain-fill and seed composition mutants, the Illinois long-term selections for oil and protein, QPM, and four grain-fill mutants from the UniformMu transposon-tagging population.[27−30] Many genotypes were sampled from ears produced at the University of Florida (Citra, FL, USA) as well as in the USDA-ARS North Central Regional Plant Introduction Station (Ames, IA, USA). Three to four kernels were selected from each ear except in the case of the UniformMu mutants, for which nine kernels were selected to represent the gradient of grain-fill defects in these lines. For test weight correlations, 40 kernels were sampled from bulk harvests of each of 30 hybrid lines grown in two 15.5 ft rows planted with 30 seeds per row at Iowa State University. Hybrids were selected from a population of testcrosses based on variation in test weight and were derived from LH51, LH82, DJ7, LH119, Oh43, and W64A germplasm. Test weight values were collected at harvest and not normalized to moisture content.

Analytical Methods. The oil content of single kernels was measured with an NMR instrument (Minispec mq 20, Bruker BioSpin, The Woodlands, TX, USA) using the manufacturer's protocol for oil. NMR instrument gain was adjusted by measuring a subset of kernels to obtain approximately 75% of the maximum signal. The instrument was then calibrated with a standard curve of four pure-oil reference masses encompassing the signal range of the initial subsample of kernels. Individual seed mass was then recorded, and seeds were placed in sampling tubes and then preheated to 40 °C prior to NMR oil measurement.

Kernel weights and NIR spectra were then collected from each kernel using a semiautomated single-kernel NIR grain analyzer as described in Spielbauer et al.[18] except that the NIR spectrum integration time was

dropped from 40 to 20 ms. Four weights and spectra were recorded from each kernel and averaged.[18] Outlier measurements among the four seed weights for each kernel were identified with Grubb's tests using the R statistical package "outliers".[31] A total of 312 tests were performed and 12 weights were removed as outliers after Bonferroni correction for multiple tests at a significance value of 0.05. No more than one weight was removed for any individual kernel, leaving three to four weight measurements for all 312 kernels in the study.

Kernel density and volume were determined using a ScanCo Medical μCT 35 (SCANCO Medical AG, Brüttisellen, Switzerland). Each kernel was set in an upright position in a foam block within a 20 mm μCT sample tube and scanned in 20 μm steps and an intensity of 40 kV. The resulting radiographs were analyzed using the manufacturer's software for contouring and 3D reconstruction (e.g., Figure 1A). Contours were completed for each section using the semiautomated contouring software with manual editing when needed. 3D reconstruction used a lower threshold of 105 to discriminate air spaces from kernel material. Standard deviation of repeatability for μCT measurements was calculated by taking the square root of the mean square error of the residuals from a one-way ANOVA of three independent measurements of 20 randomly chosen kernels, where each kernel was used as a separate group. Thirty-six kernels were randomly selected for hand-contouring of the embryo to determine embryo density and volume. Standard deviation of repeatability for embryo traits was calculated from three independent measurements of 10 randomly chosen embryos.

After scanning, kernels were processed for protein analysis. Single kernels were transferred to 2 mL microcentrifuge tubes and milled for 4−8 min in a MiniBeadbeater-96 (BioSpec Products, Bartlesville, OK, USA) using 8 mm steel ball bearings. Maize meal was dried at 50 °C for 3 days and lyophilized for 1 day to remove remaining moisture. Moisture content was determined on a subsample of 95 kernels by weighing the meal before and after drying with 95% of samples containing 9−11.5% moisture prior to drying. Dry meal was stored under vacuum until ready for analysis. Total N content was measured from 1 mg of dry maize meal by combustion analysis on a Carlo Erba NA1500 CNS elemental analyzer (CE Instruments, Milan, Italy). Protein content was calculated from the average of two to three independent measurements as N × 6.25 on a fresh weight basis assuming an average 10% moisture content.

PLS Regression. Each spectrum was mean-centered and transformed with multiplicative scatter correction (MSC) and second-derivative (2der) as indicated.[32] Density and volume values were centered and scaled. The kernels were split into calibration and validation data sets by removing one-third of the kernels from each ear for a total of 104 external validation samples. The remaining 208 kernels were used for PLS regression, which was implemented in the R statistical package "pls".[33] The PLS model was fit using the NIPALS algorithm with cross-validation of eight randomly chosen segments. The optimum number of factors was chosen on the basis of minimization of the standard error of prediction (SEP) for the cross-validation set. Best fit models were used to predict the external validation set.

RESULTS AND DISCUSSION

μCT Measures Single-Kernel Density and Volume. To test the utility of the μCT scanner in measuring individual kernel density, we scanned an *o2* mutant and a normal dent kernel of the same inbred background. Figure 1A shows 3D reconstructions of the kernels. Figure 1B,C shows individual slices from the kernel scans. These kernels were then sectioned at approximately the same plane and region of the kernel for optical imaging (Figure 1D). The *o2* mutant alters endosperm protein composition, resulting in loosely packed starch granules and soft, starchy endosperm.[3] The μCT scans distinguish tissues within the kernel on the basis of density, which is represented on a grayscale with dense material being white and air being black. The embryo has the highest density, with the scutellum being slightly denser than the embryo proper (Figure 1B). The difference in density between vitreous (v) and starchy (s) endosperm can be seen in the μCT scan as a darker endosperm in the *o2* kernel and a darker central portion in the normal endosperm (compare panels B and D of Figure 1). Applying a low-density threshold retains signal for the vitreous endosperm and embryo while removing the signal for soft, starchy endosperm (Figure 1C). Applying this same threshold to the *o2* kernel removes most endosperm tissue, illustrating the lower density of *o2* endosperm as compared to the normal kernel endosperm as well as the spatial distribution of lower density material within the kernels. Total densities for these kernels were 1.22 and 1.47 g/cm^3 for the *o2* and normal kernels, respectively. The μCT kernel densities correlate with the known reduction of kernel hardness in *o2* mutants.[4]

Total density measurements include all volume inside the contour lines drawn around the kernel and should be equivalent to pycnometer kernel density. However, both pycnometer and μCT total densities include air space inside the kernel and do not report the density of the kernel material, per se. Void spaces can account for as much as 13% of the volume of some hybrid kernels,[34] suggesting that total density may not be as informative of kernel hardness as material density. Material density can be measured from the μCT scans by setting a threshold to remove air space from the density calculation. The material densities of the *o2* and normal kernels are 5% greater than total density at 1.290 and 1.543 g/cm^3, respectively. The ability of μCT to partition the kernel into biological material and air for measurements of density and volume provides higher resolution information about kernel characteristics than either pycnometer or floater tests.

μCT Measurement of Diverse Maize Germplasm. On the basis of pycnometer or floater tests, typical kernel densities for U.S. corn belt varieties ranged between 1.20 and 1.36 g/cm^3.[13,16,17,35] We characterized a wider range of genotypic and phenotypic diversity to determine the extent of variation in maize kernel density. Table 1 shows descriptive statistics for μCT traits measured for 312 kernels from 93 ears representing 64 genotypes (see Supplemental Table 1 in the Supporting Information for the sample list). The range of total density is 5.8-fold greater than those in previous studies. However, the 0.13 g/cm^3 difference between mean total and material density is similar to the 0.17 g/cm^3 difference between "apparent" and "true" densities of hybrid maize kernels as reported by Chang et al.[34] The standard deviation and range of material density is approximately half that of total density, indicating the volume of internal air space has a significant impact on kernel density. Variation in kernel volume was greater than density with a 7-fold difference in total volume and a 10-fold difference in material volume between the smallest and largest kernels. Air space as a percent of total kernel volume showed a large range with the largest air spaces observed in grain-fill mutant genotypes. If only field corn lines are considered, the mean void space dropped to 6.22% and ranged from 4.0% in W22

Table 1. μCT Kernel Measurements of Diverse Maize Genotypes

kernel trait	mean	SD[a]	CV[b]	range	SDR[c]
total density (g/cm^3)	1.37	0.16	0.12	0.62−1.56	0.03
material density (g/cm^3)	1.50	0.08	0.05	1.18−1.65	0.03
total volume (mm^3)	173.4	53.1	0.31	53.7−366.1	1.19
material volume (mm^3)	158.0	53.7	0.34	36.1−348.4	1.09
% air space	10.04	10.45	1.04	3.18−58.09	

[a]SD, standard deviation. [b]CV, coefficient of variation. [c]SDR, standard deviation of repeatability.

to 9.3% in Oh43 inbred kernels. The standard deviation of repeatability (SDR) for all μCT measurements was well below population standard deviations, showing that the μCT is capable of precisely measuring densities even within the relatively narrow range of material density phenotypes.

The accuracy of μCT density and volume measurements was tested by calculating an estimated kernel weight from the product of density and volume and comparing this value to measured weights from the analytical microbalance of the NIR grain analyzer. Figure 2 shows these μCT-calculated kernel weights are

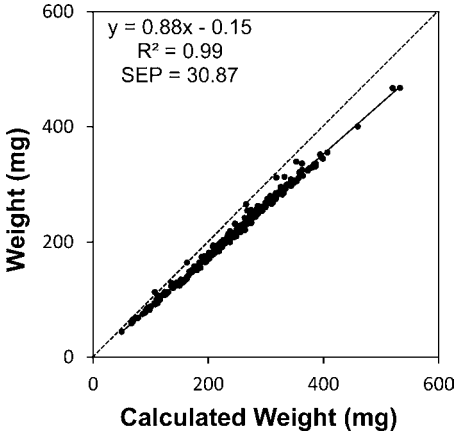

Figure 2. Scatter plot and linear regression trend line of individual kernel weight determined by a microbalance and calculated weight from μCT density and volume measurements (total density × total volume).

closely correlated to measured kernel weight ($R^2 = 0.99$). There is a slight deviation to the linear regression slope with larger, heavier kernels being overestimated in volume or density. This bias could be due to error from X-ray beam hardening in the μCT or a systematic bias in the microbalance. However, the very close correspondence between calculated and measured weight demonstrates that μCT measurements of kernel density and volume are both accurate and precise.

Starch biosynthetic mutants had the least dense kernels in regard to total density. These low densities are due to large air spaces present within the kernels. Figure 3A shows total (gray) and material (black) densities for endosperm mutants affected in kernel hardness (*fl2*, *o2*), starch biosynthesis (*bt1*, *sh2*, *bt2*, *su1*), or starch quality (*ae1*, *wx1*). All mutants analyzed were introgressed into the W64A inbred. The *bt1*, *sh2*, and *bt2* mutants have low total density, whereas their material density is close to the W64A inbred. Figure 3B shows μCT slices from a subset of these mutants to illustrate how mature kernels affecting starch synthesis collapse during drying to leave large air spaces between the seed and pericarp tissues.

To understand the relationships between density and grain fill, we examined the correlations between seed weight and μCT traits (Table 2). Because inclusion of the composition and seed developmental mutant kernels had a strong influence on the distribution of some traits, such as air space, Table 2 reports correlations for the entire population as well as correlations for a subset with the grain-fill mutants removed. Seed weight is most highly correlated with kernel volume. Given the mathematical relationship between mass, volume, and density ($m = vd$), the correlations show that variation in kernel weight (m) in this diverse sample of germplasm is primarily influenced by changes in kernel volume (v) rather than density (d). Air space within the kernel also had significant correlations to seed weight and to all

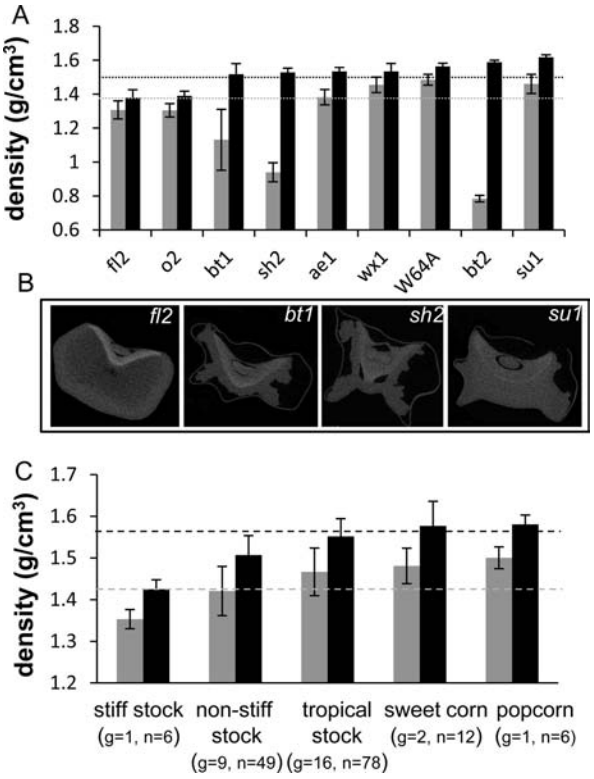

Figure 3. Genetic effects on kernel density: (A) average total (gray) and material (black) density of kernel composition mutants in the W64A genetic background; (B) μCT sections of selected mutants; (C) average total (gray) and material (black) density of maize inbred lines grouped by origin. Error bars indicate standard deviation. The numbers of maize genotypes (g) and kernels (n) used to calculate the mean are given. Dotted lines show population means for total (gray) and material (black) density.

μCT traits for the entire population of diverse germplasm. When the grain-fill mutants were removed from the sample population, air space within the kernel was correlated to only total and material density. This correlation is likely driven by the central region of the starchy endosperm, which can fail to fill completely in field corn varieties, leaving air and lower density endosperm. The air space in the grain-fill mutants also drives a positive correlation between total density and material volume. This relationship becomes negative when the mutants are removed. This change in sign suggests there is an optimum kernel volume that maximizes density.

The density of the primary seed storage molecules is expected to directly influence kernel density. To explore the relationships between the μCT traits and kernel composition, we determined protein and oil levels in the kernels. Again, grain-fill mutants influenced the correlations observed (Table 2). Oil levels had a weak positive correlation with material density when grain-fill mutants were included in the analysis. Most of the kernel oil accumulates in the embryo, and μCT scans show the embryo has higher density than endosperm. Mutants that develop normal embryos and reduced endosperm are expected to have a greater proportion of embryo tissue, resulting in both higher relative oil content and material density. The relationship between oil and material density is lost when grain-fill mutants are excluded. Instead, oil content is correlated to kernel volume, suggesting that larger kernels trend toward lower oil levels and a greater proportion of endosperm tissue.

Table 2. Significant Pearson's Correlation Coefficients (r) between Analytically Determined Kernel Traits[a]

kernel trait	weight	density$^{(t)b}$	density$^{(m)}$	volume$^{(t)}$	volume$^{(m)}$	% air	% oil	% protein
weight		–	–	0.97 a	0.99 a	–	−0.25 c	–
density$^{(t)}$	0.46 a		0.89 a	−0.36 a	−0.27 b	−0.75 a	–	0.27 b
density$^{(m)}$	–	0.61 a		−0.37 a	−0.33 a	−0.38 a	–	0.33 a
volume$^{(t)}$	0.95 a	–	–		0.99 a	–	−0.26 c	−0.25 c
volume$^{(m)}$	0.99 a	0.41 a	–	0.97 a		–	−0.24 c	−0.24 c
% air	−0.51 a	−0.94 a	−0.30 a	−0.26 a	−0.49 a		–	–
% oil	–	–	0.21 c	–	–	–		–
% protein	−0.24 b	–	0.31 a	−0.24 b	−0.27 a	0.22 b	0.36 a	

[a]Coefficients below the diagonal represent all maize lines from Table 1. Coefficients above the diagonal have composition and grain-fill mutants removed. Bonferroni corrected p values of the correlation are indicated as follows: a, $p < 0.0001$; b, $p < 0.001$; and c, $p < 0.01$. Dashes indicate nonsignificant coefficients. [b](t)total; (m)material.

Protein content showed many significant relationships to density and volume. Regardless of whether grain-fill mutants are included, protein levels show a positive relationship with material density and a negative relationship to kernel volume. This is consistent with the role of seed storage proteins in developing the hard, vitreous endosperm. Positive correlations between kernel protein and density have been observed in prior studies with a range of correlation coefficients and significance,[17,36] suggesting that seed protein explains only part of the variance in maize kernel density. Protein content is negatively correlated with kernel volume, indicating an increase in relative amounts of starch in larger kernels. Seed protein levels may be the cause for the negative relationship between kernel volume and density in field corn varieties.

Genotypic variance can also explain some differences in kernel density. Grouping the kernel samples into tropical, stiff stock, nonstiff stock, popcorn, and sweet corn shows varietal origin differences in kernel density. Popcorn and sweet corn lines had the highest density, whereas temperate stiff stock and nonstiff stalk lines had densities lower than those of tropical lines (Figure 3C). These relative rankings were consistent when the genotypes were propagated in either Florida or Iowa.

Relationship of Isolated Embryo with Whole-Kernel μCT Traits. Kernel hardness is primarily determined by the physical properties of the endosperm.[1] If significant enough, variation in embryo density or volume could affect whole-kernel density and confound assessment of kernel hardness. The relationships of embryo density and volume to overall kernel traits were tested by hand-contouring embryos in μCT scans of a subset of 36 kernels (Figure 4A). 3D reconstructions of the embryos were not as smooth as whole kernels due to the relatively low contrast in attenuation between endosperm, embryo, and pericarp tissues (Figure 4B). However, the SDRs for embryo density and volume were <1% of the population means (Table 3), indicating hand-contouring is highly reproducible. Embryo density was as high as 2.38 g/cm^3, which is 63% higher than the average whole-kernel density. Embryo volume spanned a 3-fold range of variation and, on average, accounted for 14% of the kernel volume. Air space in the embryos was minimal, resulting in minor differences between total and material measurements.

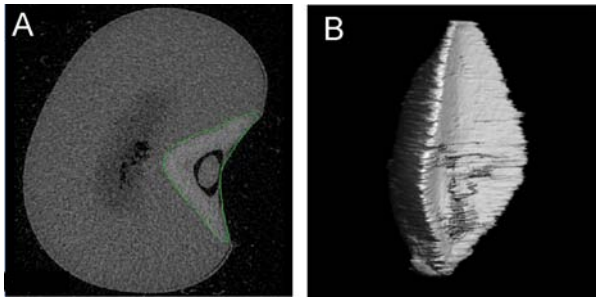

Figure 4. μCT analysis of a W22 embryo: (A) embryo contour (green) of a single μCT section; (B) 3D reconstruction of the embryo.

Table 3. μCT Embryo Measurements of Diverse Maize Genotypes

kernel trait	mean	SD[a]	CV[b]	range	SDR[c]
total density (g/cm^3)	1.67	0.15	0.10	1.33−1.99	0.007
material density (g/cm^3)	1.82	0.17	0.10	1.39−2.38	0.003
total volume (mm^3)	25.11	7.16	0.30	13.98−42.64	0.33
material volume (mm^3)	23.24	6.4	0.29	12.43−39.20	0.30

[a]SD, standard deviation. [b]CV, coefficient of variation. [c]SDR, standard deviation of repeatability.

Table 4 shows that embryo density and volume do not affect whole-kernel density measurements. Embryo density has a significant negative correlation with percent oil. Corn oil has a density of <1 g/cm^3 and accumulates predominantly in the embryo. Increasing the percentage of oil in the seed would be expected to translate to increased oil in the embryo and a reduced

Table 4. Pearson's Correlation Coefficients (r) between Embryo and Kernel Traits

	embryo traits			
trait	density$^{(t)a}$	density$^{(m)}$	volume$^{(t)}$	volume$^{(m)}$
	Embryo			
density$^{(m)}$	**0.88 a**[b]			
volume$^{(t)}$	−0.37	−0.28		
volume$^{(m)}$	−0.34	−0.32	0.98 a	
	Kernel			
weight	0.15	0.26	**0.71 a**	**0.69 a**
density$^{(t)}$	0.34	0.19	0.01	0.06
density$^{(m)}$	0.44	0.34	−0.09	−0.07
volume$^{(t)}$	0.09	0.25	**0.72 a**	**0.68 a**
volume$^{(m)}$	0.12	0.24	**0.72 a**	**0.69 a**
% air	−0.11	0.14	0.25	0.16
% oil	**−0.61 a**	**−0.75 a**	0.18	0.24
% protein	0.12	0.06	0.02	0.03

[a](t)total; (m)material [b]a, Bonferroni corrected $p < 0.0001$. All other coefficients were nonsignificant.

Table 5. Statistics for μCT Traits and PLS Regression for Optimal Prediction Models

			cross-validation (n = 208)[a]				external validation (n = 104)[b]				
kernel trait	NIR pretreatment[c]	PLS factors	mean	SD	R^2	SEP	mean	SD	R^2	SEP[a]	RPD
total density	MC; MSC	8	1.37	0.17	0.80	0.085	1.37	0.17	0.82	0.067	2.53
material density	MC; MSC	10	1.50	0.08	0.81	0.044	1.51	0.07	0.78	0.034	2.06
total volume	MC	8	177.5	54.2	0.89	20.8	172.8	56.9	0.86	19.7	2.88
material volume	MC	8	161.2	55.0	0.92	17.9	156.9	57.6	0.89	18.4	3.13
% oil	MC; MSC	7	3.74	2.08	0.91	0.59	3.81	2.22	0.90	0.70	3.17
% protein	MC; 2der, MSC	6	13.12	2.57	0.92	0.69	13.06	2.56	0.86	0.89	2.88

[a]SD, standard deviation; R^2, coefficient of multiple determination; SEP, standard error of prediction. [b]RPD, SD/SEP. [c]MC, mean-centered; MSC, multiplicative scatter correction.

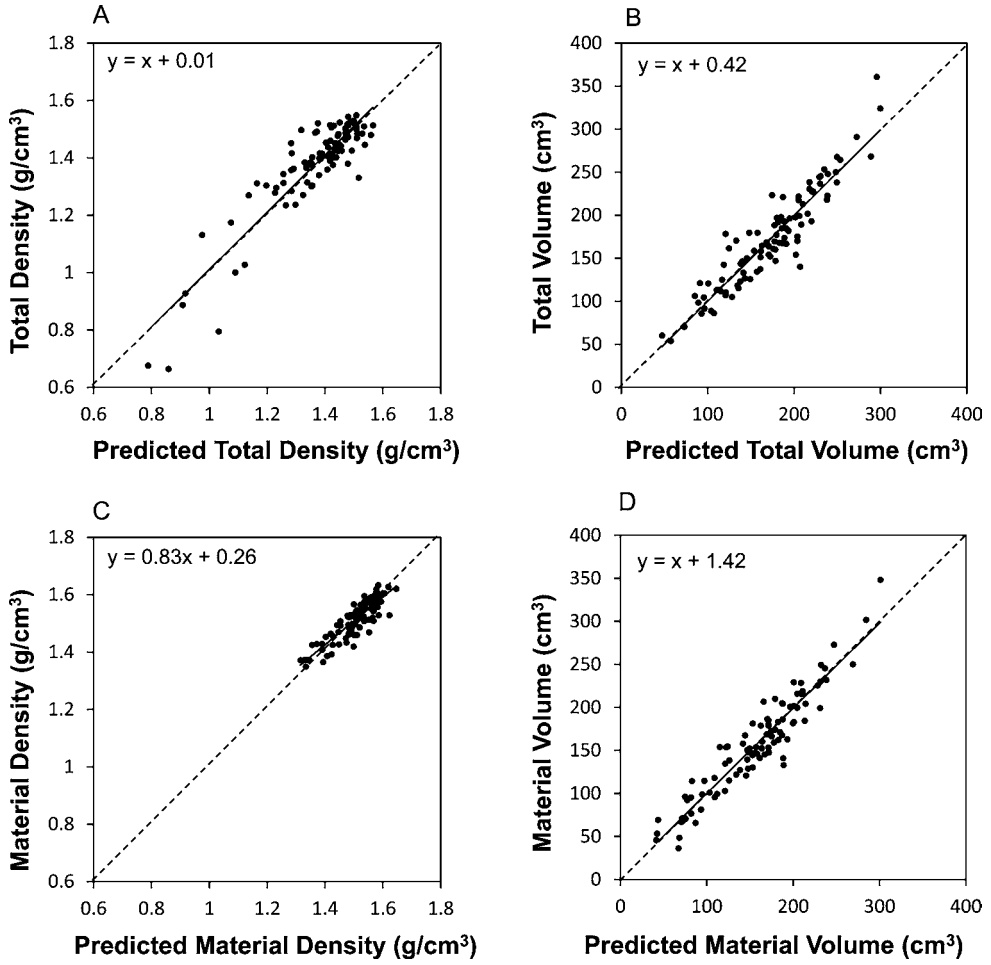

Figure 5. Scatter plots of NIR-predicted and μCT measured kernel traits: (A) total kernel density; (B) total kernel volume; (C) material density excluding air space; (D) material volume excluding air space. Each plot shows values for the external validation and a linear regression trend line. R^2 and SEP for the external validations are reported in Table 5.

density. This association is illustrated in the Illinois long-term low-oil selection. These kernels contain very low relative oil levels, and the embryos have the highest material density (Supporting Information Table S1). Embryo volume is significantly, positively correlated with seed weight and volume. This relationship helps explain why embryo volume and density are not correlated to kernel density. Increases in dense embryo tissue are balanced with a corresponding increase in lower density endosperm tissue. A nonsignificant, positive correlation existed between kernel and embryo density, which could be due to the relatively low number of embryos sampled. If this weak correlation is significant in larger samples, embryo density could have a small impact on kernel density. Overall, these embryo data suggest kernel density measurements primarily reflect endosperm density and therefore should be useful measures of kernel hardness.

Single-Kernel NIR Prediction of Density and Volume. Although μCT scans provide accurate measurements of kernel density and volume, a μCT scan takes about 1 h per kernel, requires an experienced technician to guide contouring for 3D reconstruction, and gives high doses of X-ray irradiation to the kernel. These features of μCT are not ideal to screen or select for altered kernel density. We collected NIR spectra of the 312 kernels prior to μCT scanning and used these spectra for PLS prediction of the μCT traits. Table 5 shows descriptive statistics of the calibration and external validation data sets as well as

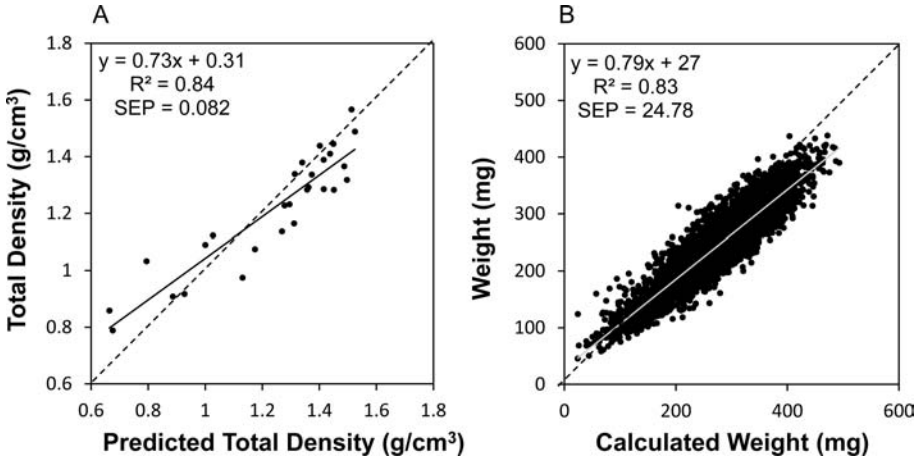

Figure 6. Additional evaluation of NIR predictions of density and volume: (A) scatter plot of NIR-predicted and μCT measured total kernel density for grain-fill mutants; (B) scatter plot of calculated kernel weight and measured weight for 14000 kernels from the Wisconsin Association Mapping Panel. Weight was calculated from NIR-predicted density and volume (material density × material volume). Measured weight was determined by a microbalance. Both plots show linear regression trend lines.

Table 6. Significant Pearson's Correlation Coefficients (r) between Principal Components (pc) of NIR Spectra and Analytically Determined Kernel Traits[a]

pc	density$^{(t)}$[b]	density$^{(m)}$	weight	volume$^{(t)}$	volume$^{(m)}$	% oil	% protein	% starch
1	−0.43 a	−0.35 a	−0.79 a	−0.73 a	−0.76 a	–[c]	–	–
2	0.64 a	0.54 a	–	–	–	–	–	−0.24 a
3	–	0.33 a	−0.38 a	−0.41 a	−0.41 a	0.77 a	0.53 a	−0.56 a
4	–	–	0.24 c	0.27 b	0.27 b	0.49 a	–	–
5	–	–	–	–	–	–	0.23 c	–
6	–	–	–	–	–	–	−0.27 b	–
7	–	–	–	–	–	–	0.49 a	–
8	–	–	–	–	–	–	−0.27 b	–
9	–	–	–	–	–	–	0.26 b	–
10	–	–	–	–	–	–	0.24 c	–
11	–	–	–	–	–	–	–	0.25 a

[a]Bonferroni corrected p values of the correlation are indicated as follows: a, $p < 0.0001$; b, $p < 0.001$; and c, $p < 0.01$. [b](t)total; (m)material. [c]Dashes indicate nonsignificant coefficients. NIR and analytical data for % starch are from Spielbauer et al.[18]

optimal model statistics for each trait. Optimum models had 8−10 factors and accounted for 78−92% of the phenotypic variation observed in the calibration population. The mean and standard deviation for the calibration and external validation populations were very similar, which ensured accurate error estimation by external validation. When the models were used to predict the external validation set of kernels, R^2 and the standard error of the prediction (SEP) for each trait were very similar to those of the calibration set, indicating that the models were not overfit. Plotting predicted versus observed values for the external validation set showed that the regressions were not overly biased and predicted extreme values of the traits well (Figure 5). The standard deviation of the population was more than twice as large as the standard error of the prediction (RPD > 2) for all models, suggesting that, although NIR may not be a true proxy for μCT-determined traits, single-kernel NIR is an effective tool to screen individual kernels for density and volume.[37] Figure 6A shows total density predictions specifically for kernels from grain-fill mutants showing R^2 and SEP statistics nearly identical to those of the overall model, indicating predictions are equally accurate for mutant and normal kernels. We also completed oil and protein PLS regressions with the analytical data collected from the 312 kernels and found prediction statistics very similar to those of Spielbauer et al. (Table 5).[18]

Stability of NIR prediction over time is not typically reported, because collection of analytical measurements is expensive and time-consuming. The NIR grain analyzer used in this study collects a precise kernel weight with the NIR spectrum. We reasoned the stability of density and volume predictions can be inferred by comparing the measured kernel weight to the kernel weight calculated from the product of predicted density and predicted volume. Figure 6B shows a scatter plot of measured versus calculated kernel weights for approximately 14000 kernels from the Wisconsin maize diversity panel[38] collected over the course of 10 months. Similar to μCT kernel weight calculations shown in Figure 2, there is a bias toward overpredicting the weight of heavier kernels, suggesting NIR predictions are a stable fit to μCT traits.

Comparison of Kernel Traits and NIR Latent Factors. PLS regressions of NIR data select latent factors that are most strongly correlated with the analytical trait of interest. The spectral latent factors can be directly related to the trait of interest or from correlated traits. It is not intuitively obvious how density and volume are predicted. We completed correlation analysis between the principal components (pc) of NIR spectra with analytically determined μCT, kernel composition, and seed weight traits (Table 6). Kernel density is most highly correlated with pc2 of the NIR spectra. Kernel volume, weight, protein, and

oil do not have a significant correlation with pc2, whereas starch content has a weak correlation. The strong correlation between density and pc2 along with the corresponding lack of correlation of pc2 to other traits suggests density directly influences NIR spectra in a manner independent of the other measured traits. This correlation also provides a biological explanation for the distinct spectra of kernel composition mutants when compared to their normal siblings.[18] Mutants that influence kernel density, such as *o2*, *fl1*, and *fl2*, are best discriminated with pc2, whereas mutants influencing weight and total density, such as *sh2*, *bt2*, *bt1*, and *su1*, are separated with a combination of pc1 and pc2.[18]

Kernel weight and volume show similar correlations with pc1, pc3, and pc4 (Table 6). This is not surprising because kernel weight and volume are highly covariant traits (Table 2). However, the high correlation to pc1 supports the hypothesis that kernel size influences the spectral path length,[18] which would account for most of the variation in the amount of reflected light received by the spectrometer. It is important to note that the spectral information about the relative composition of protein, starch, and oil is found in pc3 through pc11, which explain much smaller amounts of spectral variation than pc1 and pc2. Although starch, protein, and oil are correlated to pc3, these seed storage molecules have distinct relationships to the other components of the NIR spectra.

Single-Kernel Density Correlation with Test Weight. Test weight is an indirect measure of corn density quantified in mass per unit volume (lb/bushel), which is of primary interest for commodity corn production. Prior studies found bulk kernel density was correlated with test weight.[10,39] To test this relationship using individual kernels, NIR profiles were collected from 40 kernels from 30 elite hybrid lines, and mean predicted density values were compared with corresponding test weight data. Predicted density was strongly correlated with test weight (Figure 7). This regression suggests that for every 0.05 g/cm^3 increase in kernel material density, the weight of one bushel increases by 5 lb. The mean material density for the hybrid lines was 1.45 g/cm^3, which was lower than the mean material density of the calibration population. Test weight, however, had no relationship with kernel weight or volume, which was consistent with prior studies.[8,13,39] The strong relationship between kernel density and test weight and the relatively low material density of many of the hybrid lines suggest breeding efforts to increase kernel density would have positive impacts on agronomic quality.

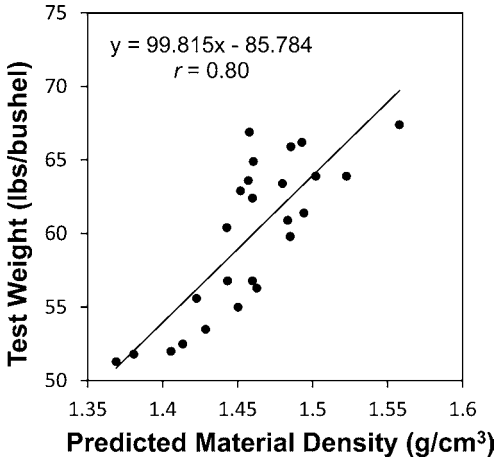

Figure 7. Scatter plot and linear regression of test weight with average NIR-predicted material density for 30 hybrid lines.

This study demonstrates that the μCT scanner can accurately and precisely measure the density and volume of individual maize kernels. The high resolution of 3D reconstructions provides insight into how grain-fill and protein content positively influence kernel material density, although there may be an optimal kernel size for maximum density. We also found that the embryo has relatively little impact on overall kernel density. Data collection and analysis for μCT are slow and expose kernels to radiation damage, making it impractical as a method to screen and select for increased kernel density. However, density and volume can be predicted with a high-throughput single-kernel NIR grain analyzer. These prediction models are stable over extended time with diverse germplasm. Importantly, single-kernel density is directly related to hybrid field test weight. This relationship was significant even though the hybrid kernels had been equilibrated to approximately 10% moisture prior to NIR data collection. Our results predict that selections for increased density would improve test weight through breeding, and these selections can be made at a single-kernel level.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental Table 1 lists the maize genotypes used in this study along with average analytical values for each genotype. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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