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Beyond Single-Stain Controls in Plant-Based Cheese Microstructure Analysis: A tri-phase approach for validating multiplex food microscopy

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Abstract

The development of plant-based cheese alternatives requires robust analytical methods for microstructural characterisation of starch-containing systems. This study established a validated confocal laser scanning microscopy protocol for simultaneous visualisation of protein, fat, and starch components through systematic evaluation of fluorescent staining methodologies. A systematic tri-phase experimental approach, progressing from single-component gels through binary to ternary systems, validated staining specificity across all fluorescent probes. Calcofluor White (CFW), established for visualising β-1,3 and β-1,4 linked polysaccharides (cellulose, chitin, β-glucans), were evaluated for binding α-linked starch polysaccharides (amylose and amylopectin) in cheese matrices. However, commercial CFW preparations containing Evans Blue demonstrated significant cross-reactivity with proteins, compromising analytical accuracy. Fluorescent Brightener 28 was identified as an effective alternative, providing selective starch labelling without protein interference. This research provides a robust methodology for simultaneous three-component visualisation in plant-based cheese systems, enabling accurate microstructural analysis essential for product development in this rapidly expanding market segment.

Keywords: Calcofluor white; Fluorescent brightener 28; Starch; Plant-based cheese; Confocal Microscope; Microstructure.

1. Introduction

The global shift towards plant-based alternatives is disrupting the dairy industry (Aschemann-Witzel et al., 2021), with plant-based cheese representing one of the most rapidly expanding segments within this market is poised to achieve a 12.1% CAGR from 2025 to 2033, with the market reaching 8.63 billion GBP by 2033 (Grandview Research, 2024). Consumer demand for these products stems not only from ethical and environmental considerations but also from their potential health benefits, including reduced saturated fat content and absence of cholesterol (Craig et al., 2021; Jeske et al., 2018). However, the technological challenge of replicating the complex microstructural properties of traditional dairy cheese while maintaining desirable sensory attributes remains formidable.

Traditional cheese manufacture relies heavily on the complex interactions between casein proteins, milk fat, and water to create the characteristic texture, mouthfeel, and functional properties that consumers expect (Grossmann & McClements, 2021; Lamichhane et al., 2018). In plant-based formulations, this intricate protein network is mostly substituted with alternative ingredients, often necessitating the complete removal or substantial reduction of animal proteins in favour of plant-based alternatives. When protein content is minimised or eliminated entirely, starch emerges as a critical functional ingredient, providing structural integrity through its ability to form gels, bind water, and contribute to the desired rheological properties of the final product (Liu et al., 2019). Modified potato starch, in particular, exhibits excellent emulsification properties and thermal stability, making it suitable for cheese analogue applications (Ørskov et al., 2024). The gelatinisation and retrogradation behaviour of starch can be manipulated through processing conditions and chemical modifications to optimise texture and functionality.

The microstructural analysis of food systems has long been recognised as fundamental to understanding the relationship between composition, processing conditions, and final product quality (El-Bakry et al., 2018; Ong et al., 2013). Confocal laser scanning microscopy (CLSM) has established itself as the gold standard technique for investigating the three-dimensional microstructure of cheese systems, the techniques ability to provide simultaneous multi-component visualisation through fluorescent labelling makes it particularly valuable for understanding the intricate

relationships between different structural elements in food systems. Extensive research has documented well-established methodologies for imaging conventional dairy cheese, where the primary components of interest protein and fat can be readily visualised using established fluorescent dyes such as Fast Green FCF for proteins and Nile Red for lipids (Ali et al., 2025; Grasso et al., 2021; Ong et al., 2022).

However, the incorporation of starch as a major structural component in plant-based cheese formulations presents novel challenges for microstructural analysis. The successful development of these products requires robust analytical methods capable of simultaneously visualising three distinct phases: protein, fat, and starch. This necessitates the development of reliable staining protocols that can selectively label each component without cross-reactivity, thereby enabling accurate microstructural characterisation through confocal microscopy.

The fluorescent labelling of starch presents particular difficulties. Fluorescein isothiocyanate (FITC) has been employed in some research applications for polysaccharide detection (van de Velde et al., 2002; Ørskov et al., 2024); however, its mechanism of covalent binding to primary amine groups present in proteins render it unsuitable for complex food applications. FITC forms stable thiourea linkages with amino groups through its isothiocyanate reactive group (Barbero et al., 2016), resulting in inevitable cross-reactivity with protein components in complex food matrices and compromising analytical specificity. Alternative polysaccharide stains including Aniline Blue (which primarily targets β-1,3 glucans such as callose in plant cell walls;(Algae, 2016)) have been reported. However, neither has been reported to demonstrate validated specificity for α-linked starch polysaccharides in protein-rich food matrices. Calcofluor White (CFW) represents a potentially viable alternative, with several studies documenting its use in polysaccharide research.

CFW is traditionally understood to bind β-1,3 and β-1,4 glycosidic linkages present in cellulose, chitin, and β-glucan structures (Florczuk et al., 2023; McLauchlan et al., 2025). Systematic evaluation of CFW and related fluorescent brighteners for visualising α-linked starch polysaccharides (amylose: α-1,4 linkages; amylopectin: α-1,4 and α-1,6 linkages) in complex food matrices, to the authors knowledge, has not been reported. While CFW has found application in food systems for the detection of

β-glucans, its specificity and reliability for starch visualisation in cheese systems requires validation.

Furthermore, many commercial CFW preparations contain Evans Blue as a counter stain (Sigma Aldrich, 2021), which exhibits documented affinity for proteins (Jacobson et al., 2016; Saunders et al., 2015) and could therefore introduce unwanted cross-reactivity in multiplex staining protocols.

While some research has explored triple-component staining (Florczuk et al., 2023; Ørskov et al., 2024) in plant-based systems, the specific challenges posed by starch-containing cheese matrices including the identification of suitable starch-specific dyes and the mitigation of cross-reactivity issues remain inadequately addressed in current analytical methodologies. Without reliable methods to visualise the spatial distribution and interactions between protein, fat, and starch phases, the development of plant-based cheese products with optimised microstructure and functionality remains severely constrained. Furthermore, the lack of understanding regarding CFW's binding specificity beyond its established targets of cellulose and chitin limits its potential application in food microscopy.

This study sought to address these methodological limitations by developing alternative approaches for starch visualisation in confocal microscopy applications and establishing a robust protocol for simultaneous imaging of all three major components (protein, fat and starch) in plant-based cheese systems. Through systematic evaluation of fluorescent dyes and their binding specificities, this research aimed to provide the food science community with validated tools for microstructural analysis of next-generation plant-based dairy alternatives, thereby facilitating the continued development of products that can meet both consumer expectations and industrial requirements for functionality and quality.

2. Materials and Methods

2.1 Materials

Whey protein isolate (WPI, >90% protein content) was obtained from Myprotein (THG company, UK). WPI was selected for protocol development due to its high purity with complete absence of starch contamination, Whereas commercial plant protein isolates can contain residual carbohydrates from extraction processes (Etzbach et al., 2024), potentially leading to ambiguous signal from cross-staining. Plant proteins also typically form weaker gels requiring starch-based gelling agents (Moniharapon et al., 2026), which would confound single-component validation, while WPI has well-characterised gelation properties (Zhang et al., 2023). While WPI and plant proteins differ in amino acid composition, fluorescent protein stains such as Fast Green FCF bind through electrostatic interactions and van der Waals forces with ionizable groups common to all proteins (Tas et al., 1980),rather than through amino acid composition-specific interactions. Cheese maker CF77 (Modified Potato Starch, Starch Sodium Octenyl Succinate) was obtained from KMC (Brande, Denmark). REDUSAT C35 vegetable fat (shea & sunflower oil) was purchased from Fuji Oil Europe (Gent, Belgium), Commercial vegan cheese was obtained from local store. CFW-M2R was obtained from Sigma-Aldrich (Merck, Gillingham, Dorset UK.) CFW-FB28, Nile Red and Fast Green FCF were from Fisher scientific (Leicestershire, UK.). All other chemicals were of analytical grade and obtained from standard laboratory suppliers.

2.2 Methods

2.2.1 Gel Preparation

2.2.1.1 Single-Phase Gels

Protein gels were prepared with the protocol from Badjona et al. (2025) with modifications. Whey protein isolate (WPI) (40% w/w) and salt (1% w/w) were hydrated overnight at 6°C under continuous agitation at 150 rpm (Infors HT Ecotron incubator, Basel, Switzerland). The resulting suspension was brought to room temperature before thermal treatment in a water bath (P Series TC120, Grant instruments, Cambridge, UK.) with gradual heating to 95°C. The solution was maintained at this temperature for 45 minutes to ensure complete protein denaturation and gel network formation, then rapidly cooled to 4°C (Nortech Plus 10, Normann, Italy) where gelation

occurred, conforming to container geometry. Samples were not kept for more than 2 weeks to avoid spoilage. Starch gels were formed by dispersing CF77 modified potato starch (40% w/w) in warm water (60°C) to create a uniform slurry. The mixture was processed in a Thermomix food processor (Model TM6, Vorwerk, Germany) for 5 minutes at 75°C and speed setting 3 to achieve complete starch gelatinisation. The gelatinised material was transferred into mould and rapidly cooled to 4°C, then stored under refrigeration for minimum 24 hours to allow gel maturation samples were not kept for more than 2 weeks to avoid spoilage. Fat gels were created through emulsion structuring. Hard fat (75% w/w) was melted at 70°C until fully liquefied, then combined with water (20% w/w) and Tween 20 emulsifier (5% w/w). The components were homogenised at 5000 rpm for 3 minutes using a high-speed homogeniser (Silverson L5M-A, Silverson Machine Limited, Bucks, UK.) to form a stable emulsion. The mixture was immediately poured into mould and refrigerated at 4°C, where fat crystallisation occurred.

2.2.1.2 Binary Gel Systems

For Fat-Protein gels, Hard fat (20% w/w) was melted until fully liquefied. WPI (20% w/w) and salt (1% w/w) were dispersed in the remaining water (59% w/w) and hydrated overnight at 6°C under continuous agitation at 150 rpm. The protein solution was brought to room temperature before the melted fat was gradually incorporated. The hydrated protein and melted fat mixture was shortly homogenised at 5000 rpm for 1 minute before being transferred to a water bath and heated gradually to 95°C, maintained at this temperature for 45 minutes to ensure complete protein denaturation while maintaining fat emulsification. The gel was rapidly cooled to 4°C then transferred into storage to aid curing of the gel. For Fat-Starch gels, CF77 (25% w/w) was dispersed in warm water (55% w/w) at 60°C to form a slurry. The starch slurry was then transferred to the Thermomix and hard fat (20% w/w), previously melted was added to the starch slurry. The speed was increased to 5 for 1 minute to aid homogenisation, then reduced to 3 and cooked for 5 minutes at 75°C to achieve complete starch gelatinisation. The mixture was immediately transferred into moulds and rapidly cooled to 4°C, then transferred into storage to aid curing of the gel. For Starch-Protein gels, 20% (w/w) CF77 and 20% (w/w) WPI were dispersed together in water (60% w/w) at room temperature to create a homogeneous suspension. The mixture was transferred to the Thermomix and processed at 85°C for 10 minutes at

speed setting 3 to simultaneously achieve starch gelatinisation and protein hydration and gelation. The composite system was immediately transferred into moulds and rapidly cooled to 4°C where the final gel structure developed through synergistic interactions between the starch and protein networks.

2.2.1.3 Ternary Gel System (Protein-Fat-Starch Composite Gels)

CF77 (15% w/w) and WPI (15% w/w) were dispersed together in water (60% w/w) at room temperature to create a homogeneous suspension. The mixture was transferred to the Thermomix and hard fat (10% w/w), previously melted, was added to the suspension. The speed was increased to 5 for 1 minute to aid homogenisation, then reduced to 3 and processed for 10 minutes at 85°C to simultaneously achieve starch gelatinisation, protein hydration and gelation. The complete gel was immediately transferred into a mould and rapidly cooled to 4°C then transferred into storage to complete gel curing.

Each gel type was prepared once (n=1 biological replicate). The gel was then divided into three portions, with each portion serving as a technical replicate (n=3) and subjected independently to all subsequent analyses.

2.2.1.4 Plant-Based Cheese Samples

Commercial plant-based cheese samples was obtained from retail outlets and stored at 4°C while another was prepared in the lab. Sample 1 (coconut oil-based) contained water, coconut oil (24%), modified starch, starch, lentil protein (1.3%), and other minor ingredients according to the manufacturer's declaration. Sample 2 (protein-free formulation AFIC) contained modified starch and vegetable fat as primary structural components.

2.2.2 Fluorescent Staining Protocols

2.2.2.1 Stain Preparation

Fluorescent stains were prepared following Florczuk et al. (2023) with modifications. Nile Red (lipid-specific dye) stock solution 1mg/ml was prepared by dissolving the dye in dimethyl sulphoxide (DMSO), then diluted to a working concentration of 0.1 mg/mL using MilliQ water. Fast Green (protein-specific dye) was dissolved directly in MilliQ water to achieve a final concentration of 0.1 mg/mL. Calcofluor White (FB28), starch-

specific dye) without Evans Blue was prepared using MilliQ water and diluted to 0.05 mg/mL. CFW with Evans Blue was prepared from a pre-mixed stock solution containing 1 g/L CFW and 0.5 g/L Evans Blue, which was further diluted to 0.1 mg/mL working concentration using MilliQ water. These dye concentrations and incubation protocols have been validated for confocal microscopy of dairy products and were applied without modification to enable direct comparison with existing literature (Florczuk et al., 2023) and ensure inter-laboratory reproducibility. Fluorescent Brightener 28 is chemically equivalent to Calcofluor White M2R and represents the pure fluorescent brightener compound without Evans Blue addition (Sigma Aldrich, 2026). This comparison enabled direct assessment of Evans Blue's contribution to observed protein cross-reactivity, as FB28 provides the fluorescent brightener component alone whilst the commercial CFW preparation contains both the brightener and Evans Blue as a counter-stain. All stain solutions were prepared fresh daily and stored in dark conditions at 4°C to prevent photo-degradation.

2.2.2.2 Staining Procedure

All staining procedures were performed under controlled lighting conditions to minimise photobleaching. Gel samples were cut into small thin sections using a scalpel blade. Samples were incubated with staining solutions for 15 minutes at room temperature, as per established protocols for fluorescent labelling of cheese systems (Florczuk et al., 2023), allowing complete dye equilibration without oversaturation. Following staining, samples were washed three times with MilliQ water to remove unbound dye whilst maintaining target-bound fluorescence. Following staining, samples were mounted with gold anti-fade mounting medium (Fisher Scientific, UK) to prevent photobleaching and fluorescence quenching while preserving fluorescent signal intensity and maintaining sample integrity throughout the imaging process.

To assess stain specificity and confirm absence of spectral cross-talk, all gel systems (single-component, binary, and ternary) were stained individually with each of the three fluorescent dyes (Nile Red, Fast Green, and CFW FB28). Each stained sample was imaged across all three detection channels (405 nm, 488 nm, and 633 nm excitation) to quantify potential bleed-through between channels. Unstained gel samples were imaged across all three detection channels to assess baseline autofluorescence and confirm imaging parameters produced negligible background signal.

Protein-containing gel systems (protein only, protein-fat, protein-starch, and protein-fat-starch) were stained with both CFW variants (CFW+EB and CFW FB28) to assess cross-reactivity. All gel systems (single-component, binary, and ternary; n=4 gel types) were stained individually with each of the three fluorescent dyes (Nile Red, Fast Green, and CFW FB28), with each stained sample imaged across all three detection channels. This systematic matrix approach (4 gel types × 3 dyes × 3 imaging channels) enabled comprehensive evaluation of stain specificity, binding selectivity, and spectral separation without multi-channel interference.

2.2.3 Confocal Laser Scanning Microscopy

CLSM imaging was conducted on a LSM 800 confocal microscope (Carl Zeiss, Jena, Germany) using a 20× objective, with image acquisition and processing performed using Zeiss Zen 2.3 (blue edition) software. Nile Red fluorescence was captured using 488 nm excitation (0.09% laser power) with emission collected at 410-617nm, Fast Green using 633 nm excitation (0.2% laser power) with emission at 645-700nm, and Calcofluor White using 405 nm excitation (0.2% laser power) with emission at 400-495nm. All images were acquired at 1024 × 1024 pixel resolution and saved in TIFF format for subsequent analysis. Imaging parameters including laser power and gain were kept constant across all samples to ensure quantitative comparability and all imaging was completed within 2 hours of staining to ensure signal stability.

2.2.3.1 Image Analysis and Quantification

All image analysis was performed using ImageJ/Fiji software version 2.15.1 (Schindelin et al., 2012) equipped with the JACOP (Just Another Colocalisation Plugin) package (BOLTE & CORDELIÈRES, 2006). Each gel composition was prepared as a single batch and divided into three technical replicates, with multiple fields of view acquired per replicate. Images were acquired with identical settings (laser power, gain, offset, pinhole) across all samples to ensure comparative analysis. Background correction was applied to remove uneven illumination and ensure accurate fluorescence quantification. Automatic thresholding was applied using Costes' method (Costes et al., 2004) embedded within the JACOP plugin. The Costes automatic thresholding approach was essential for these systems as component concentrations varied across gel types (e.g., 40% protein in single-component gels vs 15% in ternary systems), resulting in different fluorescence intensities and spatial distributions. This

method calculates optimal threshold values based on pixel intensity correlation within each image, ensuring accurate segmentation regardless of component concentration or distribution pattern (Costes et al., 2004).

2.2.4 Cross-reactivity Analysis

To assess stain cross-reactivity of CFW, co-localisation analysis was performed using methods described by (Dunn et al., 2011) and the JACOP plugin specifically on protein containing samples to quantify spatial relationships between protein and starch components in multi-component gel systems. Analysis employed Pearson's correlation coefficient (PCC) to assess the degree of spatial overlap between fluorescent channels. PCC quantifies spatial colocalisation by measuring fluorescent intensity relationships between signals, ranging from +1 (identical intensity distributions) through 0 (independent intensities) to -1 (inversely related intensities). In addition to colocalisation analysis, percentage overlap was conducted to quantify the degree to which one fluorescent channel overlaps with another. The percentage overlap was calculated to determine the overlapping region as a proportion of the total composite coverage area, providing a measure of spatial correspondence between the two fluorescent signals. The overlap percentage was calculated using the following formula

$$Percentage\ Overlap\ (\%) = \frac{(AreaCh1 + AreaCh2) - AreaComposite}{AreaComposite}\ X\ 100$$

Where:

- AreaCh1 = coverage area of channel 1
- AreaCh2 = coverage area of channel 2
- AreaComposite = total coverage area of the merged image

2.2.5 Stain Specificity Analysis

Fluorescent stain specificity was assessed using Mean Fluorescence Intensity (MFI) measurement as described by (Shihan et al., 2021) with modifications. For each stained gel sample, target regions (areas containing the component of interest) were identified and quantified. Background fluorescence was quantified by measuring 10 randomly selected regions of interest (ROIs) in areas lacking the target component

(black/dark regions) within each stained gel sample. ROI size was adjusted to accommodate spatial constraints based on the heterogeneous distribution of components within each gel composition, while maintaining representative sampling of background signal. This within-image background correction approach accounts for sample-specific optical and chemical variations, providing more rigorous quantification than using a single reference background. Mean background intensity was calculated for all background measurements and subtracted from signal measurements to obtain corrected MFI values. Unstained control samples were imaged under identical parameters to assess baseline autofluorescence and optimise imaging parameters but were not used for background correction of stained samples, as this approach ensures MFI measurements accurately reflect specific staining while accounting for optical and chemical changes occurring during the staining process.

2.2.6 Statistical Analysis

Data were analysed using GraphPad Prism software version 10 (GraphPad Software, San Diego, USA). Data was subjected to the Shapiro-Wilk normality test. If parametric, data was analysed by one-way ANOVA test for comparison of three or more groups followed by Tukey's correction post-hoc test. If non-parametric, data was analysed by Kruskal-Wallis test for comparison of three or more groups followed by Dunn's correction post-hoc test. Statistical significance was set at $p<0.05$. Results are presented as mean ± standard deviation; statistically significant differences are denoted using superscripts and graphical annotations.

3. Results and Discussion

3.1 Applicability of Calcofluor White (CFW) for Cheese Microstructure

Confocal microscopy analysis revealed that Calcofluor White successfully bound to and fluoresced the starch in the gel matrices, demonstrating clear blue fluorescence characteristic of CFW (Fig. 1). This finding represents a significant departure from the established understanding of CFW's binding specificity, which has traditionally been limited to β-1,3 and β-1,4 linked glucans such as those found in cellulose and chitin (Florczuk et al., 2023; McLauchlan et al., 2025).

The starch components exhibited large fluorescent areas distributed across all gel phases, indicating successful binding and visualisation of these polysaccharide structures throughout the cheese matrix systems (Fig.1).

The observed binding of CFW to starch components in these gel systems is reproducible and analytically useful, though the molecular mechanism underlying this interaction has not been established. Both amylose and amylopectin contain α-1,4 glycosidic linkages as their primary structural feature, with amylopectin additionally possessing α-1,6 branch points. These linkages differ in stereochemistry from the β-linkages in cellulose and chitin where CFW binding is well characterised.

Several hypothetical mechanisms could explain CFW-starch binding. The linear amylose chains with regular α-1,4 linked glucose units (Seung, 2020) might provide binding sites geometrically compatible with CFW attachment, or the planar aromatic CFW structure (Weng et al., 2019) could intercalate between glucose residues or hydrogen bond with hydroxyl groups along the polymer chain. However, these proposed mechanisms remain speculative. Definitive characterisation of the binding mechanism would require techniques such as X-ray crystallography, nuclear magnetic resonance spectroscopy, or molecular dynamics simulations, which were beyond the scope of this methodological study. For the purposes of microstructural analysis in plant-based cheese systems, the empirically validated staining specificity demonstrated here provides an analytical foundation regardless of the underlying molecular interaction.

3.2 Cross-reactivity Analysis

While the application of Calcofluor White (CFW) staining proved successful in binding and visualising starch within various gel matrices, certain limitations were observed. Notably, there was evidence of cross-reactivity, particularly between the protein and starch channels, where overlapping fluorescence signals were detected (Fig. 2). This raised concerns regarding potential spectral bleed-through or non-specific binding, especially in protein-containing systems. As a result, a cross-reactivity assessment was necessary to investigate the extent and nature of this interference, allowing for a clearer understanding of signal origin and improving confidence in the interpretation of starch localisation in multicomponent gels.

Confocal microscopy analysis revealed significant cross-reactivity when using commercial CFW preparations containing Evans Blue (CFWEB) across all experimental gel matrices. Phase 1 (protein-only gels) exhibited fluorescent signal in both starch and protein channels despite the absence of starch components, providing direct evidence of CFWEB binding to protein. Similar phenomena were observed in Phase 2 binary systems, particularly in protein-fat and protein-starch gels (Fig. 2). Phase 3 ternary systems continued to show cross-reactivity, though with reduced intensity. In contrast, CFW(FB28) demonstrated no observable cross-reactivity across all phases (Fig. 4), with Phase 1 showing no signal in either channel and Phase 2 protein-starch systems exhibiting signal exclusively in the starch channel as expected.

Quantitative colocalisation analysis substantiated these observations. Phase 1 showed the highest cross-reactivity with PCC values of 0.37 to 0.84 (mean = 0.59 ± 0.23) and percentage overlap of 34.8 ± 0.27%. Phase 2 exhibited PCC values of 0.09 to 0.65 (mean = 0.35 ± 0.19) with 32.3 ± 2.5% overlap. Phase 3 showed predominantly negative colocalisation (PCC = -0.095 ± 0.09) but maintained substantial overlap (21.37 ± 2.94%), indicating spatial segregation with interface contact (Fig. 3).

The observed cross-reactivity can be attributed to Evans Blue's well-documented binding mechanism with proteins (Jacobson et al., 2016; Saunders et al., 2015). In albumin binding, reactions between the sulfonic acid groups of the dye and amino groups on the protein surface are responsible for the dye-protein binding mechanism (Yao et al., 2018). Plant proteins possess similar surface amino groups and basic amino acid residues (particularly lysine and arginine) to animal proteins, making them susceptible to analogous binding interactions with Evans Blue. This structural similarity

between plant and animal protein binding sites provides a plausible explanation for the observed cross-reactivity in plant protein-rich systems and the consistent false-positive protein signals observed across all gel phases. However, these mechanisms remain hypothetical and require validation and more in-depth analytical interrogation definitively establish binding interactions. Similar findings were observed in the research conducted by McLauchlan et al. (2025) where partial colocalisation were reported in protein rich moieties resulting in a suggestion of further investigation.

These findings have implications for cheese microstructure analysis. The positive colocalization between CFW+EB and protein in Phase 1 (PCC = 0.59, 34.8% overlap) indicates genuine cross-reactivity, where fluorescence intensities vary together on a pixel-by-pixel basis. This demonstrates that commercial CFW creates false-positive protein signals through Evans Blue binding to protein components, leading to overestimation of protein content in starch-containing regions. Up to one-third of apparent "protein" signals in CFW+EB-stained samples potentially represent cross-reactive binding rather than genuine protein structures (Fig. 2).

In Phase 3, the negative PCC (-0.09) coupled with 21% overlap requires careful interpretation. Negative PCC values indicate that fluorescence intensities do not co-vary; where one signal is high, the other tends to be low, confirming absence of true cross-reactivity at the molecular level. However, non-zero percentage overlap reflects the physical co-occurrence of labelled regions within the same imaging volume. This apparent contradiction arises because percentage overlap quantifies spatial proximity of phases in heterogeneous gel systems, while PCC specifically assesses whether fluorescence intensities correlate pixel-by-pixel. In multi-phase food systems, distinct components (protein and starch) can occupy adjacent regions within the same field of view, producing overlap values without genuine cross-reactivity. The combination of negative PCC with moderate overlap therefore indicates spatial heterogeneity rather than dye binding interference, the phases are physically near each other but not co-staining.

CFW(FB28) validation as a truly specific starch stain (consistent near-zero PCC values and minimal overlap across all phases) provides a reliable tool that eliminates false-positive protein signals. These results confirm that commercial CFW preparations containing Evans Blue create protein channel artefacts through genuine

cross-reactive binding (evidenced by positive PCC), whilst Evans Blue-free CFW (FB28) maintains starch specificity without generating protein false-positives. This distinction between true cross-reactivity (correlated fluorescence intensities) and apparent overlap (spatial proximity of distinct phases) underscores the importance of using multiple quantitative metrics and Evans Blue-free formulations for accurate microstructural analysis of protein-rich food systems.

3.3 Stain Specificity Analysis

3.3.1 Spectral Separation and Stain Selectivity

Unstained gel samples imaged across all three detection channels showed no detectable autofluorescence, confirming that imaging parameters were optimised to detect dye-specific signals without background interference.

Systematic single-stain analysis across all gel compositions confirmed absence of spectral bleed-through and validated stain selectivity (Supplementary Fig. S1-3). When single-component gels were stained with their target dye and imaged across all channels, fluorescence signal appeared exclusively in the intended channel. Starch gels stained with CFW (FB28) showed signal only in the 405 nm channel with no detectable fluorescence in protein (633 nm) or fat (488 nm) channels. Protein gels stained with Fast Green exhibited signal exclusively in the 633 nm channel. Fat gels stained with Nile Red fluoresced only in the 488 nm channel. Non-target channels showed only background-level signal comparable to unstained controls, confirming genuine stain specificity rather than optical artefacts.

Binary and ternary gels stained with individual dyes further validated selectivity. Protein-starch gels stained solely with CFW showed blue fluorescence in starch regions only, with no signal in protein domains. Similarly, protein-fat gels stained with Fast Green showed protein-specific signal without fat labelling. These single-stain controls across increasingly complex matrices confirmed that each dye selectively binds its target component without non-specific adhesion to other gel phases, providing confidence that signals observed in triple-stained samples represent genuine component distribution rather than staining artefacts.

Changes in MFI across gel systems may reflect multiple factors including structural reorganisation affecting dye accessibility, local concentration differences arising from

phase separation, or potential quenching effects in regions of high protein density. Whilst the systematic trends observed across the tri-phase approach support the interpretation of genuine accessibility changes, these alternative optical and structural factors cannot be entirely excluded without complementary analytical techniques.

3.3.2 Mean Fluorescent Intensity (MFI)

Having established Fluorescent Brightener 28 as an effective alternative to CFWEB, comprehensive stain specificity analysis was conducted to validate the selectivity of all fluorescent probes and confirm the reliability of the proposed triple-staining protocol.

Mean fluorescence intensity (MFI) measurements provide objective assessment of fluorescent stain binding efficiency while validating target specificity. All stains demonstrated specificity across single-component, binary, and ternary gel systems (Figure 5). Nile Red (lipid-targeting) showed progressive increase in fluorescence intensity across gel types: 127.44 ± 15.06 (fat-only), 146.30 ± 57.10 (binary), and 186.41 ± 25.98 (ternary), representing a 46.3% increase from single to ternary systems. Fast Green (protein-specific) exhibited variation across gel types: fluorescence intensity was 142.34 ± 5.48 in protein-only gels, decreased to 89.46 ± 38.73 in binary gels, then returned to comparable levels at 142.27 ± 4.39 in ternary gels. Calcofluor White (CFW, starch-selective) demonstrated consistently high performance: 175.35 ± 10.70 (starch-only), 193.60 ± 22.06 (binary), and 234.42 ± 6.53 (ternary), showing a 33.7% increase from single to ternary systems.

CFW exhibited significantly higher MFI values than both Nile Red and Fast Green across all gel compositions ($p < 0.001$). Statistical analysis confirmed significant differences between all stain combinations, whilst within-group analysis showed significant variation amongst stains across different gel compositions. Background-corrected measurements confirmed target specificity for all fluorescent stains.

The observed MFI variations across gel systems reflect multiple potential factors. Progressive Nile Red increase may indicate enhanced lipid accessibility in multi-component matrices, where binary gels create structured matrices facilitating lipid organisation and ternary systems optimise this through synergistic protein-starch networks creating stable microenvironments that preserve surface area while preventing phase separation (Talló Domínguez, 2021). However, alternative explanations including local concentration differences arising from phase separation,

changes in refractive index affecting photon collection efficiency, or reduced self-quenching in more dispersed fat distributions cannot be excluded without complementary analytical techniques.

Fast Green's biphasic response - decreased MFI in binary gels followed by recovery in ternary systems - suggests complex protein-matrix interactions. The binary reduction may reflect competitive binding where proteins interact with starch through hydrogen bonding reducing amino acid accessibility, or fat through hydrophobic clustering sequestering binding sites (Scott & Awika, 2023), protein aggregation concentrating dye-binding sites into smaller volumes, or optical quenching effects in regions of high protein density. The ternary recovery could indicate balanced microenvironments restoring dye accessibility through starch-mediated protein unfolding or fat-induced network restructuring (Hughes et al., 2021), though the high standard deviation in binary systems suggests substantial spatial heterogeneity that complicates interpretation.

CFW's consistent elevation with increasing matrix complexity demonstrates improved starch accessibility as additional components are incorporated. This trend may arise from binary systems disrupting granule packing and exposing β-glucan sites, whilst ternary systems maximise this through protein-fat interactions optimising chain mobility (Li & Li, 2024). The superior MFI stability in ternary systems (lowest coefficient of variation) indicates polysaccharide networks provide structural consistency regardless of component interactions. While these interpretations are consistent with the systematic trends observed across the tri-phase approach, definitive mechanistic assignment would require more in-depth analysis to distinguish between structural reorganisation, concentration effects, and optical phenomena.

3.4 Triple-Staining Microscopy Demonstration

The optimised triple-staining protocol successfully demonstrated simultaneous visualisation of all three components within a representative matrix (Fig. 6). Confocal micrographs revealed distinct fluorescent signals for each component without interference or cross-reactivity between channels.

Starch components exhibited characteristic blue fluorescence distributed throughout the matrix Starch components as irregular domains forming interconnected clusters and discrete rounded structures confirming effective Fluorescent Brightener 28 binding.

Fat globules appeared as vivid red and small spherical droplets well-distributed throughout the system, indicating successful Nile Red incorporation and retention within lipid phases. Often clustered near or partially overlapping the green protein regions, indicating spatial association between these phases. Several larger red aggregates were also observed, potentially reflecting some degree of coalescence within the lipid phase. Protein networks displayed green fluorescence that clearly delineated protein domains, with protein structures observed to engulf fat globules, demonstrating the expected microstructural organisation typical of emulsified systems.

The clarity and specificity of fluorescent signals across all three channels confirmed the effectiveness of the developed staining protocol and validated its suitability for routine microstructural analysis of complex plant-based cheese formulations. The absence of spectral bleed-through between channels further substantiated the specificity of each fluorescent probe for its intended target component.

3.4.1 Application to Plant-Based Cheese Systems

The validated staining protocol was applied to two commercial plant-based cheese products with contrasting formulations to assess applicability beyond model systems (Fig. 7).

The coconut oil-based sample showed fat as the dominant structural phase, appearing as an extensive red fluorescent matrix throughout the sample. Protein signal was minimal, appearing only as small discrete green regions, consistent with lentil protein being present at low concentration (1.3g/100g) primarily for emulsification. Starch showed limited blue fluorescence, appearing as small scattered domains within the fat-dominated matrix. This fat-continuous, starch-poor structure contrasts directly with the starch-continuous network of the protein-free sample described below, illustrating how the same staining protocol resolves fundamentally different structural arrangements across formulations.

The protein-free sample displayed markedly different microstructure, with starch forming extensive blue fluorescent networks throughout the matrix. Fat appeared as large discrete red globules (ranging 55-75 μm) with clear boundaries. No protein signal was detected, consistent with the product formulation. The continuous starch network observed here is consistent with recent synchrotron micro-CT analysis of commercial plant-based cheese which reported a starch-structured microstructure in which a

continuous starch matrix governs both the cold microstructure and the response to heating (Dobson & Marangoni, 2025). In that work, however, starch was assigned on the basis of X-ray attenuation and density rather than by a starch-specific signal, and could not be chemically distinguished from the surrounding phases. The present method confirms this matrix directly, identifying starch by fluorescent affinity rather than by inferred density.

Both samples were successfully visualised without spectral interference or cross-reactivity, confirming the protocol's applicability to diverse commercial formulations.

The results presented here confirm that staining specificity established using WPI transfers effectively to plant protein systems, validating the model system approach.

4.0 Conclusion

This investigation established a validated confocal microscopy protocol for simultaneous visualisation of protein, fat, and starch components in plant-based cheese systems. Through systematic tri-phase validation, Fluorescent Brightener 28 (CFW without Evans Blue) was identified as a selective starch-labelling agent capable of binding α-linked starch polysaccharides (amylose and amylopectin) without protein cross-reactivity. Commercial CFW preparations containing Evans Blue exhibited significant protein cross-reactivity, rendering them unsuitable for multiplex staining applications in protein-rich matrices.

The validated staining protocol combining Nile Red (fat), Fast Green FCF (protein), and Fluorescent Brightener 28 (starch) enables accurate microstructural characterisation of plant-based cheese formulations. Single-stain validation across single-component, binary, and ternary gel systems confirmed staining specificity and absence of spectral interference. Application to commercial plant-based cheese products demonstrated the protocol's robustness in realistic food matrices.

This methodological contribution addresses a critical analytical gap in plant-based cheese research and provides a robust foundation for microstructural analysis in this sector. Whilst this study focused on cheese systems containing modified potato starch, the fundamental principle, simultaneous three-component visualisation using spectrally separated dyes without cross-reactivity is applicable to other starch-containing food matrices. The validated protocol enables systematic investigation of

component distribution and phase interactions, supporting continued development of plant-based dairy alternatives.

Future studies should apply this validated protocol to quantify spatial relationships (domain size, phase connectivity) and correlate microstructural parameters with functional properties such as texture or meltability, enabling structure-function relationships to be established in plant-based cheese systems. We believe the current work establishes the essential analytical foundation upon which such structure-function studies can be built. Additionally, future work should validate these protocols across diverse plant-based systems and investigate the molecular mechanisms underlying fluorescent brightener binding to various polysaccharide structures through techniques such as NMR spectroscopy or molecular dynamics simulations.

References

Algae, S. G. (2016). Aniline Blue and Calcofluor White Staining of Callose and Cellulose in the.

Ali, F., O'Mahony, J. A., O'Sullivan, M. G., & Kerry, J. P. (2025). Comparative Analysis of Composition, Texture, and Sensory Attributes of Commercial Forms of Plant-Based Cheese Analogue Products Available on the Irish Market. *Foods, 14*(15)https://doi.org/10.3390/foods14152701

Aschemann-Witzel, J., Gantriis, R. F., Fraga, P., & Perez-Cueto, F. J. (2021). Plant-based food and protein trend from a business perspective: Markets, consumers, and the challenges and opportunities in the future. *Critical Reviews in Food Science and Nutrition, 61*(18), 3119–3128.

Badjona, A., Cherono, B., Bradshaw, R., & Dubey, B. (2025). Gelation and rheological properties of ultrasound-extracted faba bean protein: A comparative

study with commercial plant proteins. *Food Hydrocolloids, 162*, 110997. https://doi.org/10.1016/j.foodhyd.2024.110997

Barbero, N., Barolo, C., & Viscardi, G. (2016). Bovine serum albumin bioconjugation with FITC. *World Journal of Chemical Education, 4*(4), 80–85.

BOLTE, S., & CORDELIÈRES, F. P. (2006). A guided tour into subcellular colocalization analysis in light microscopy. *Journal of Microscopy, 224*(3), 213–232. https://doi.org/10.1111/j.1365-2818.2006.01706.x

Costes, S. V., Daelemans, D., Cho, E. H., Dobbin, Z., Pavlakis, G., & Lockett, S. (2004). Automatic and quantitative measurement of protein-protein colocalization in live cells. *Biophysical Journal, 86*(6), 3993–4003.

Craig, W. J., Mangels, A. R., Fresán, U., Marsh, K., Miles, F. L., Saunders, A. V., & Orlich, M. (2021). The safe and effective use of plant-based diets with guidelines for health professionals. *Nutrients, 13*(11), 4144.

Dobson, S., & Marangoni, A. G. (2025). Exploration of structural differences between dairy and plant-based cheese. *Food Structure, 44*, 100424. https://doi.org/10.1016/j.foostr.2025.100424

Dunn, K. W., Kamocka, M. M., & McDonald, J. H. (2011). A practical guide to evaluating colocalization in biological microscopy. *American Journal of Physiology-Cell Physiology, 300*(4), C723–C742.

El-Bakry, M., Sanchez, A., & Mehta, B. M. (2018). *Microstructure of dairy products*. John Wiley & Sons.

638 Etzbach, L., Gola, S., Küllmer, F., Acir, I., Wohlt, D., Ignatzy, L. M., Bader-
639 Mittermaier, S., & Schweiggert-Weisz, U. (2024). Opportunities and challenges
640 of plant proteins as functional ingredients for food production. *Proceedings of the*
641 *National Academy of Sciences, 121*(50), e2319019121.

642 Florczuk, A., Załęcki, M., & Aljewicz, M. (2023). The applicability of Calcofluor White
643 (CWS) and Fluorescent Brightener (CFB) dyes for confocal laser microscopic
644 analysis (CLSM) of various β-glucans in selected dairy products and water. *Food*
645 *Chemistry, 404*, 134508. https://doi.org/10.1016/j.foodchem.2022.134508

646 Grandview Research. (2024). *UK Vegan Cheese Market Size & Outlook.*
647 https://www.grandviewresearch.com/horizon/outlook/vegan-cheese-market/uk

648 Grasso, N., Roos, Y. H., Crowley, S. V., Arendt, E. K., & O'Mahony, J. A. (2021).
649 Composition and physicochemical properties of commercial plant-based block-
650 style products as alternatives to cheese. *Future Foods, 4*, 100048.
651 https://doi.org/10.1016/j.fufo.2021.100048

652 Grossmann, L., & McClements, D. J. (2021). The science of plant-based foods:
653 Approaches to create nutritious and sustainable plant-based cheese analogs.
654 *Trends in Food Science & Technology, 118*, 207–229.

655 Jacobson, O., Kiesewetter, D. O., & Chen, X. (2016). Albumin-binding Evans blue
656 derivatives for diagnostic imaging and production of long-acting therapeutics.
657 *Bioconjugate Chemistry, 27*(10), 2239–2247.

Jeske, S., Zannini, E., & Arendt, E. K. (2018). Past, present and future: The strength of plant-based dairy substitutes based on gluten-free raw materials. *Food Research International, 110*, 42–51.

Lamichhane, P., Kelly, A. L., & Sheehan, J. J. (2018). Symposium review: Structure-function relationships in cheese. *Journal of Dairy Science, 101*(3), 2692–2709.

Li, J., & Li, L. (2024). Preparation of a novel nanoparticle with extruded soy protein isolate-oat β-glucan: Interfacial properties and mechanism of emulsion stability. *Food Hydrocolloids, 150*, 109686.

Liu, S., Yuan, T. Z., Wang, X., Reimer, M., Isaak, C., & Ai, Y. (2019). Behaviors of starches evaluated at high heating temperatures using a new model of Rapid Visco Analyzer – RVA 4800. *Food Hydrocolloids, 94*, 217–228. https://doi.org/10.1016/j.foodhyd.2019.03.015

McLauchlan, J., Tyler, A. I. I., Orfila, C., & Sarkar, A. (2025). Characterisation of an oat protein-beta-glucan co-extract. *Food Hydrocolloids, 171*, 111756. https://doi.org/10.1016/j.foodhyd.2025.111756

Moniharapon, N., Zhu, M., Daborn, L., & Dhital, S. (2026). High-Temperature Gelation and Structural Characterisation of Commercial Yellow Pea, Faba Bean, and Mungbean Protein–Starch Systems. *Gels, 12*(1), 89.

Ong, L., Dagastine, R. R., Kentish, S. E., & Gras, S. L. (2013). Microstructure and composition of full fat Cheddar cheese made with ultrafiltered milk retentate. *Foods, 2*(3), 310–331.

679 Ong, L., Li, X., Ong, A., & Gras, S. L. (2022). New insights into cheese
680 microstructure. *Annual Review of Food Science and Technology, 13*(1), 89–115.

681 Saunders, N. R., Dziegielewska, K. M., Møllgård, K., & Habgood, M. D. (2015).
682 Markers for blood-brain barrier integrity: how appropriate is Evans blue in the
683 twenty-first century and what are the alternatives? *Frontiers in Neuroscience, 9*,
684 385.

685 Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T.,
686 Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J., White, D. J.,
687 Hartenstein, V., Eliceiri, K., Tomancak, P., & Cardona, A. (2012). Fiji: an open-
688 source platform for biological-image analysis. *Nature Methods, 9*(7), 676–682.
689 https://doi.org/10.1038/nmeth.2019

690 Scott, G., & Awika, J. M. (2023). Effect of protein–starch interactions on starch
691 retrogradation and implications for food product quality. *Comprehensive*
692 *Reviews in Food Science and Food Safety, 22*(3), 2081–2111.

693 Shihan, M. H., Novo, S. G., Le Marchand, S. J., Wang, Y., & Duncan, M. K. (2021).
694 A simple method for quantitating confocal fluorescent images. *Biochemistry and*
695 *Biophysics Reports, 25*, 100916.

696 Sigma Aldrich. (2021, Sept). *Calcofluor White Stain.*
697 https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/
698 383/810/18909bul-mk.pdf

699 Sigma Aldrich. (2026). *Fluorescent Brightener 28 disodium salt solution.* Retrieved
700 11 May, 2026 12:41, from

https://www.sigmaaldrich.com/GB/en/product/sial/910090?srsltid=AfmBOoq4LrrqPd_Mv_ZbQGNhuP5898lH9pay5UolkbHyFLeZInIhb5HA

Talló Domínguez, K. (2021). Colloidal lipid gels: A novel approach to structure dispersions of lipids.

Tas, J., Ploeg, M. v. d., Mitchell, J., & Cohn, N. (1980). Protein staining methods in quantitative cytochemistry. *Journal of Microscopy, 119*(3), 295–311.

van de Velde, F., van Riel, J., & Tromp, R. H. (2002). Visualisation of starch granule morphologies using confocal scanning laser microscopy (CSLM). *Journal of the Science of Food and Agriculture, 82*(13), 1528–1536.

Yao, L., Xue, X., Yu, P., Ni, Y., & Chen, F. (2018). Evans blue dye: a revisit of its applications in biomedicine. *Contrast Media & Molecular Imaging, 2018*(1), 7628037.

Zhang, L., Zhang, Z., Euston, S. R., Li, B., Li, E., Fu, C., & Chen, G. (2023). Structural and gelling properties of whey proteins influenced by various acids: Experimental and computational approaches. *Food Hydrocolloids, 144*, 109003. https://doi.org/10.1016/j.foodhyd.2023.109003

Ørskov, K. E., Christensen, L. B., Wiking, L., Hannibal, T., & Hammershøj, M. (2024). Microstructural studies of imitation cheese with a shift in continuous phase. *Food Research International, 184*, 114210. https://doi.org/10.1016/j.foodres.2024.114210