

DESIGN, FABRICATION, AND VALIDATION OF A TWO-DYE FLUORESCENCE PHANTOM FOR EXCITATION–EMISSION MATRIX HYPERSPECTRAL IMAGING (EEM-HSI) BENCHMARKING

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ABSTRACT

Excitation–emission matrix hyperspectral imaging (EEM-HSI) promises richer chemical discrimination than single- or multi-excitation hyperspectral imaging (3D-HSI, ME-HSI) by resolving a full excitation profile at every pixel. Benchmarking and validating unmixing algorithms for EEM-HSI requires a phantom with a known, spatially resolved ground truth in which the constituent fluorophores are chosen to stress the excitation dimension specifically. This paper describes the design, fabrication, and validation of a two-dye fluorescence phantom built from harmaline and harmine — a pair of structurally related β -carboline alkaloids whose emission bands overlap substantially while their excitation profiles remain distinguishable. We detail the rationale for this dye pair, the preparation of a seven-class mixture series spanning pure-dye endpoints to intermediate ratios, the protocol used to fix the mixtures in a spatially addressable array, and their usage in the EEM-HSI acquisition. The resulting phantom and its ground-truth mask are intended as a reusable benchmarking target for chemometric unmixing methods (e.g. MCR-ALS, PARAFAC) applied to EEM-HSI data.

Index Terms— fluorescence phantom, hyperspectral imaging, excitation–emission matrix, harmaline, harmine, chemometric unmixing, ground-truth dataset

1. INTRODUCTION

Multi-Excitation and Excitation–Emission Matrix Hyperspectral Imaging (ME-HSI and EEM-HSI) extend conventional single-excitation fluorescence HSI by acquiring image cubes across several excitation wavelengths (λ_{exc}), in principle allowing fluorophores that overlap in emission to be separated by their distinct excitation signatures. This approach has raised attention in recent years, and it was applied to different fields as biomedical, biological samples determination and chemical compositions determinations.[1], [2], [3], [4] However, demonstrating and benchmarking this capability requires a standard sample in which the ground-truth chemical composition is known at every pixel, and in which the constituent fluorophores are deliberately chosen so that emission wavelength (λ_{em}) dimension alone cannot

separate them — otherwise the λ_{exc} dimension is never actually tested. Despite this, published EEM-HSI and ME-HSI studies frequently use samples whose composition, preparation, and validation are reported only briefly, or not at all, which limits reproducibility and makes it difficult to compare unmixing algorithms across studies. The creation of a well-characterized phantom to be used to test EEM-HSI acquisition and analysis methods remains, to our knowledge, a gap in the literature.

This paper documents, as a self-contained methods contribution, the design and fabrication of a two-dye fluorescence phantom built specifically to instantiate this “ λ_{exc} -discriminating, λ_{em} -overlapping” regime, together with the steps used to validate that the phantom’s actual composition matches its nominal design. The phantom described here will be used as the physical substrate for examining how illumination beam-shape deformation across excitation wavelengths distorts per-pixel excitation profiles recovered by EEM-HSI.

2. DYE SELECTION

A phantom intended to test λ_{exc} -based unmixing must be built from fluorophores that occupy a specific position in spectral-overlap space. Three regimes are possible for a two-component system: (*Scenario A*) the components differ in both λ_{exc} and λ_{em} , in which case even single-excitation imaging can partially separate them; (*Scenario B*) the components differ mainly in λ_{em} , with overlapping λ_{exc} , which conventional single- or multi-excitation HSI already handles via the λ_{em} dimension; and (*Scenario C*) the components differ mainly in λ_{exc} , with overlapping, unimodal emission bands — the case in which single- λ_{exc} HSI cannot separate the components at all, multi- λ_{exc} HSI (ME-HSI) is misled into treating the mixture as a single fluorophore, and only a full excitation–emission acquisition (EEM-HSI) with per-pixel excitation resolution offers a route to unmixing. A benchmarking phantom should target *Scenario C* specifically, since it is the case that most directly exercises the added value EEM-HSI claims over ME-HSI and 3D-HSI.

Harmaline (Hlina) and harmine (Ha) were selected to instantiate *Scenario C*. Both are naturally occurring β -carboline alkaloids with closely related molecular structure

(Ha is the aromatic, fully unsaturated analogue; Hlina is the 3,4-dihydro derivative), which gives them fluorescence emission bands that overlap substantially and are both effectively unimodal, while their excitation behavior differs enough to be discriminable by a well-resolved excitation scan. Reference emission maxima in aqueous solution are approximately 417 nm for Ha and 486 nm for Hlina, placing both well within the λ_{em} window (420–720 nm) and the λ_{exc} window (310–415 nm) used for the phantom imaging described below.[5] Although the maximum λ_{exc} for the Ha will be truncated by the usage of a 425 nm long-pass filter coupled to the HSI camera, the resolution of the pair is expected to be possible with the available signal in the camera range.

Beyond their spectral suitability, Hlina and Ha were chosen for practical reasons:

1. Commercial availability at known purity, avoiding in-house synthesis or purification;
2. Sufficient fluorescence quantum yield across the tested excitation range to give usable signal at practical camera exposure times;
3. Hlina reported solubility is 1.07 g/L in phosphate buffer pH 7.4 at 310.15 K [Volkova et al., 2026]. For Ha, the only available experimental aqueous solubility data are reported at pH 7 (3.31×10^{-5} M $\approx$ 7.0 mg/L, 20°C) [Varela et al., 1995].[6], [7] Also, these compounds have reported to have reasonable photostability and fluorescence quantum yields in aqueous solution.[8], [9]
4. It was previously shown that aqueous solutions of mixed Ha and Hlina can be resolved by chemometric analysis, obtaining each component concentration besides their spectral overlap.[8]

3. PHANTOM FABRICATION

3.1. Stock solutions and mixture series

2 ml of stock solutions of Hlina 2.54 μ M and Ha 2.34 μ M were prepared in phosphate buffer (pH 7.4). Concentrations were determined by measuring the absorbance of each solution and considering the previously reported absorbance coefficients.[8] Also, the concentrations were calculated to match the signal height of each dye at their respective $\lambda_{exc}/\lambda_{em}$ maxima, considering their absorption coefficients and fluorescence quantum yields. From those solutions, 200 μ l of each Hlina/Ha mixture were prepared by combining the two stocks in the volumetric ratios summarized in **Fig 1**. After, the obtained solutions (pure dyes and mixtures) were diluted 50:50 with 10 % polyvinyl alcohol (to a final 5% PVA concentration).

3.2. Phantom creation

To create the phantom, 5 μ l aliquots of the above-mentioned mixtures (Hlina/Ha in phosphate/PVA 5%, aqueous

solutions) were placed in a microscope slide to create a dot pattern (8.5 mm between one drop and the neighboring one) as the one depicted in **Fig 1**. Three repetitions of each solution were placed at different locations in the glass and were allowed to dry in the dark overnight. This phantom is intended to fix the mixtures spatially and permit repeated, non-destructive imaging across multiple light sources and sessions. The final obtained phantom consists of a 3 x 8 (rows x columns) array of solid dots of approximately 3.5 mm diameter, with each region large enough to contain a well-populated pixel area for per-class averaging after registration to the ground-truth mask. Region boundaries were kept apart to avoid optical or diffusive cross-contamination between adjacent classes. The usage of PVA as solid matrix yielded transparent solid dots, which avoids non-linear inner filter effects from highly absorptive matrices.

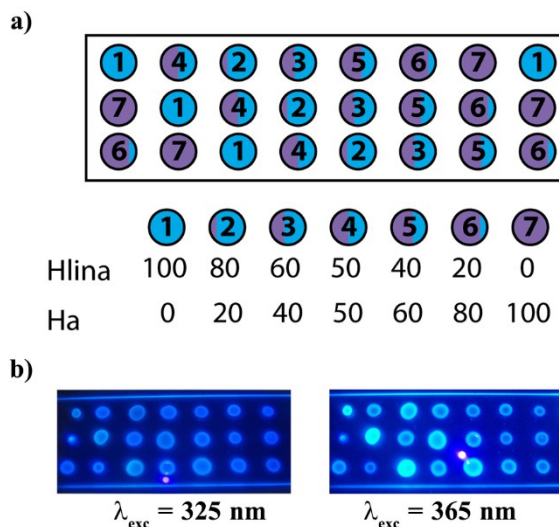


Fig. 1. (a) Schematic representation of the created phantom, exposing each dot position and their Hlina/Ha relative concentration. (b) phantom photos under $\lambda_{exc} = 325 \text{ nm}$ and $\lambda_{exc} = 365 \text{ nm}$ (left and right panel, respectively).

3.3. EEM-HSI data acquisition and correction

The prepared phantom was imaged using a fluorescence HSI setup consisting of: (i) a multi-LED source (WHeeLED, Mightex; 310, 325, 340, 365, 385, 400, 415 nm, 10 nm FWHM) coupled to a liquid light guide (Newport) and a 425 nm short-pass filter (Edmund Optics); (ii) a NUANCE FX hyperspectral camera (CRi) coupled to a 425 nm long-pass filter (Edmund Optics), which acquires images in the 420 to 720 nm emission spectral range (10 nm step). Seven 3D HSI datasets were acquired under illumination with the above-mentioned excitation wavelengths, without changing the geometry of the HSI system. Then, the obtained datasets were

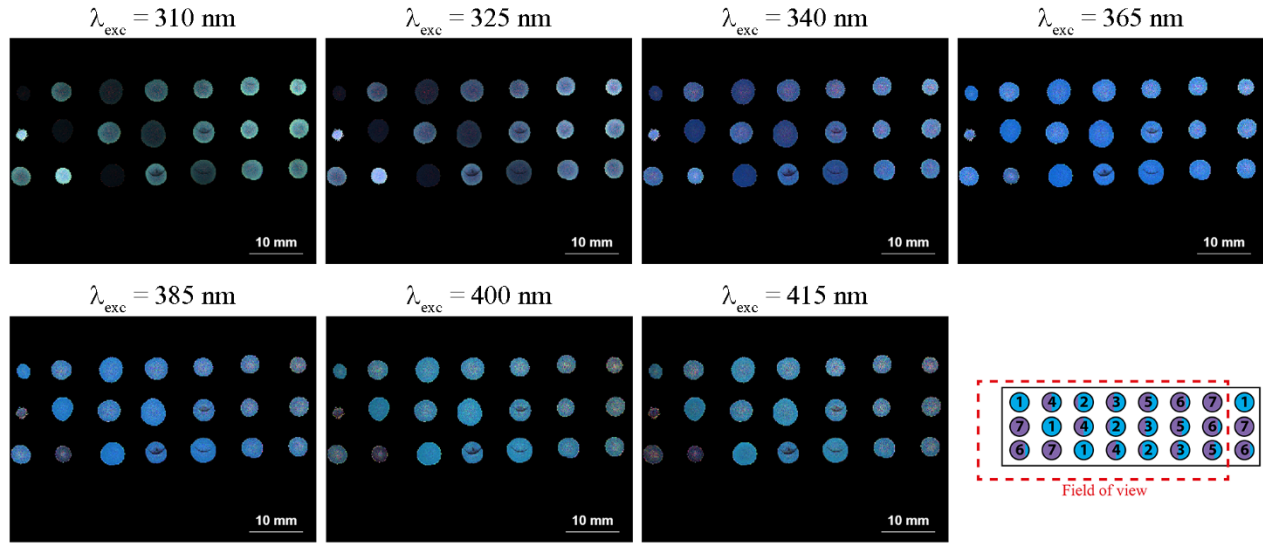


Figure 2. RGB representation of the HSI 3D cubes obtained at each different λ_{exc} . The scheme shows the HSI dataset field of view.

re-arranged in a 4D-dataset with dimensions (x , y , λ_{exc} , λ_{em}), where each pixel has its own fluorescence EEM. Because our system's field of view was too narrow to capture all dots in a single frame, the last column of the phantom was omitted from the image, as shown in **Fig. 2**.

After the EEM-HSI data acquisition, the obtained datasets were corrected both by the lamp power at each λ_{exc} and the exposure time used in each 3D cube acquisition. Then, a min-max normalization was applied to each pixel, so every pixel's EEMs are in the 0-1 range of values. The RGB representations of the obtained HSI dataset, at each different λ_{exc} , are exposed in **Fig 2**. It can be observed that the dots with higher Hlina relative concentration have a higher signal in the higher λ_{exc} , while the dots with higher Ha concentrations have a higher signal at lower λ_{exc} .

3.4. Ground truth mask construction

The background pixels from the acquired HSI dataset were filtered by setting a signal threshold. A pixel-wise ground-truth mask (GTMask) was then manually constructed by registering the known array layout to the imaged field of view, assigning each pixel to one of the seven mixture classes or to background. This mask is intended to serve as a reference for any further instrumental or unmixing algorithms performance evaluation (e.g. Pearson correlation and R^2 of recovered vs. nominal Hlina fraction) reported in the companion analyses of this dataset.

4. COMPOSITIONAL VALIDATION

From the obtained HSI dataset, the pure-dye endpoints (classes **(1)** 100:0 and **(7)** 0:100 Hlina:Ha relative concentration) serve as internal references: any unmixing algorithm applied to the full dataset should recover pure-component spectra qualitatively consistent with expected spectral behavior. This provides a data-driven, algorithm-independent sanity check that complements the direct compositional validation described above. The recovered EEMs from one example pixel from each class are exposed in the **Fig 3**. It can be observed that the EEMs corresponding to the pure-dye classes show characteristic spectral profiles which are overlapped in the emission but can be differentiated in their excitation. The mixture classes, on the other hand, show this overlapped spectral characteristic at higher or lower degree.

5. DESIGN CONSIDERATIONS AND LIMITATIONS

During the engineering and construction of this phantom, several possible issues were found which are worth to mention:

- **Dye selection:** for our case, the EEM-HSI datasets were planned to be acquired in the 310-415 nm and 420-720 nm wavelength range for λ_{exc} and λ_{em} , respectively. When creating phantoms to be used in a different wavelengths range, other dye pairs can be selected, such as riboflavin, fluorescein, etc. The usage of inorganic fluorescent references can be considered to improve the stability over time.

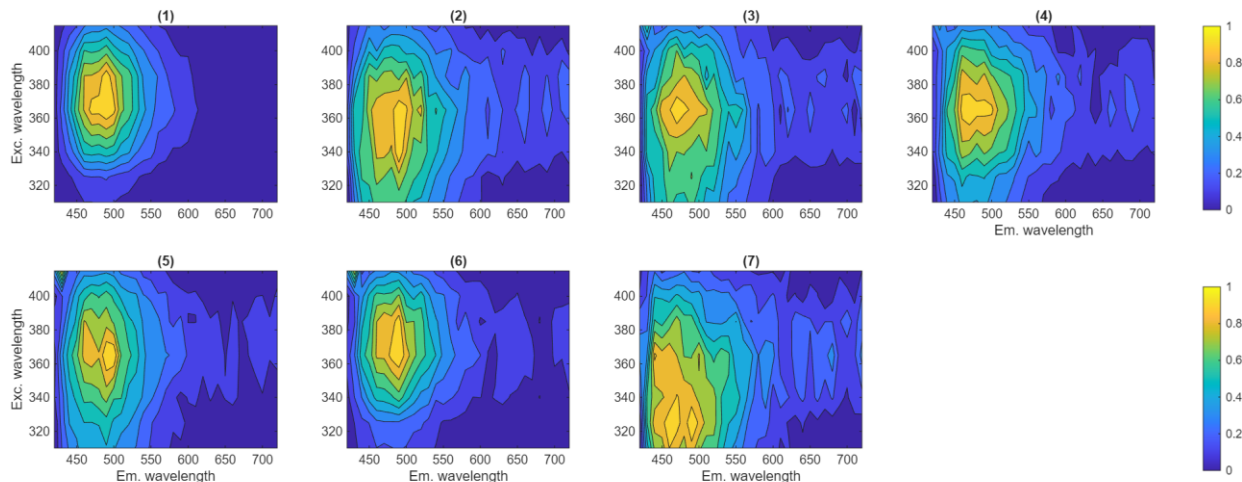


Figure 3. EEMs obtained from representative pixels belonging to each class in the phantom. Numbers indicate the dot class as denoted in Fig. 1.

- Solvent selection: the selection of the solvent for the solutions preparation should assure the compound stability, significant fluorescence quantum yield and low light absorption in the $\lambda_{\text{exc}}/\lambda_{\text{em}}$ wavelengths range. Previous trials were assayed at different pH values only to find the dyes were non-fluorescent or even worse, degraded. Thorough research on the selected dyes stability should be done across different pH values before phantom construction.
- Solid matrix selection: the usage of PVA for the obtention of solid dots was selected over the liquid sample’s alternative. Many shortcomings are related with the maintenance of liquid solutions over time, such as dye degradation, microbial contamination, the need of storage in cold temperature, etc. The solid matrix needs to be also carefully selected taking into account the following possible issues: the “coffee ring” effect during the drying process, the absorption coefficient of the solid matrix in the $\lambda_{\text{exc}}/\lambda_{\text{em}}$ wavelengths range and the interaction of the selected dyes with it (which may lead to shifted or diminished fluorescence signal). Previous trials were attempted using polyvinyl acetate, finding that the obtained solid dots were highly absorptive (white to the naked eye) which creates artifacts in the fluorescence measurement.

6. DISCUSSION

The phantom described here is deliberately narrow in scope — two dyes, seven mixture classes, a single spatial layout — by design: its purpose is to provide an unambiguous, validated ground truth for the specific spectral regime (Scenario C) that EEM-HSI is uniquely positioned to resolve, rather than to serve as a general-purpose multi-analyte target.

This narrowness is what makes the validation in Section 4 tractable and the resulting benchmark trustworthy. Extensions of this design — additional dye pairs spanning Scenarios A and B, finer mixture-ratio sampling, or three-component mixtures — are natural directions for follow-up phantoms but are outside the scope of the present work.

We note one limitation openly: a recurring, mild non-monotonicity in recovered composition near the 50:50 class has been observed downstream, across multiple unmixing algorithms and illumination conditions, in the companion analyses of this phantom. Whether this reflects a genuine local irregularity in the physical phantom (e.g. at a class boundary or seam) or an algorithmic effect specific to near-equal mixtures remains open and is flagged here for transparency, since it is a property of this specific phantom instance rather than of the design methodology in general.

7. CONCLUSION

We presented the design, fabrication, and compositional validation of a two-dye fluorescence phantom purpose-built to benchmark EEM-HSI unmixing in the excitation-discriminating, emission-overlapping regime (*Scenario C*). The phantom’s seven-class mixture series, their disposition on glass, and validated ground truth are intended as a reusable resource for evaluating instrumental artifacts and analytical processing as chemometric unmixing methods (MCR-ALS, PARAFAC, and others) on EEM-HSI data, independent of any single analysis performed on it. The same phantom underlies a companion study of illumination-induced excitation-profile distortion in EEM-HSI [1]. Future work will focus on the detailed description and validation of each dot composition by standard analytical chemistry techniques, as solid-state EEMs using a benchtop spectrofluorometer.

This complete characterization will allow us to release the EEM-HSI datasets together with the characterization data in an open-source repository such as *Zenodo*, so it can be used as a reference for further work on computational analysis methods development.

8. ACKNOWLEDGEMENTS

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