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Investigating Linear Superposition of Multi-Species Neurotransmitter Voltammetric Measurements In-Vitro

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Abstract - **Fast-Scan Cyclic Voltammetry (FSCV) is frequently used to monitor the concentrations of neurotransmitters in real-time. However, few studies have examined the issue of monitoring the concentration of multiple neurotransmitters at the same time, despite their coexistence at brain synapses. This stems from the fact that some neurotransmitters have relatively similar electrochemical profiles. In this work we use Factor Spaces to analyze the current signals obtained using FSCV for both individual and mixed solutions of neurotransmitters. It is shown that the behavior of the current signals during the interaction between the neurotransmitter species approaches the principle of superposition. This potentially results in a significant simplification in the way combined voltammetric data is interpreted. The performance of Principal Component Analysis in extracting suitable Factor Spaces is evaluated.**

I. Introduction

Fast-Scan Cyclic Voltammetry (FSCV) is a bio-analytical technique that offers excellent temporal resolution, allowing monitoring of rapid chemical fluctuations [1]. FSCV measurements can be taken on a sub-second time-scale, and the technique has found applications varying from evaluating electron-transfer dynamics to monitoring biological environments [2],[3]. Its use is often combined with that of microelectrodes, since the latter allow for better discrimination due to bulk diffusion effects, increased spatial resolution and minimal tissue damage when working in-vivo [4]. In particular, this combination has predominantly been used for monitoring the concentration changes of monoamines such as Dopamine (DA), Serotonin (5-HT) and Norepinephrine (NE) [5]-[7] in vivo. The release of these molecules in the Central Nervous System is very brief, with concentration transients spanning the sub second timescale.

The usefulness of this technique is however limited when investigating complex signals consisting of the combined contributions from different species. FSCV only returns a single current signal per electrode, whereas in the physiological scenario multiple neurotransmitters must necessarily coexist [8]-[10]. Besides neurotransmitters, the FSCV signal also contains contribution from a range of different neurochemicals which are always present simultaneously. The measured signal therefore does not explicitly indicate which and how many species contribute to it, nor can it provide information about their relative concentrations. This is therefore what in signal processing fora is termed a classic *cocktail party problem*. Isolating the individual contributions contained within the measured signal makes this an inverse problem, which must be addressed using non-trivial signal processing techniques.

Significant work has been conducted to address the multi-species FSCV problem. Heien *et al.* [11] used FSCV to analyse an in-vitro preparation consisting of a single mixture of DA and 5-HT, both present in concentrations of 0.5 μM in a buffer solution. Principal Component Regression was then utilized to resolve the composite signal and estimate the constituent neurotransmitter concentrations *a-posteriori.* Encouraging results were obtained for both species, with errors in the region of ~6% for the DA and ~22% for the 5-HT. Similarly, Anastassiou *et al.* [8] examined the separation of a single mixture of DA and 5-HT in the presence of Ascorbate. This approach consisted of extracting the complex faradaic signal from AC voltammetric data using the Hilbert Transform and then selecting voltage 'windows' where the effect of one neurotransmitter predominates over the other. Within such windows, the concentration could be related to the amplitude computed from the Hilbert Transform.

Now, in addition to the above studies, it would be fruitful to assess whether there exists an underlying relationship describing the way multiple neurotransmitters contribute to a single composite FSCV signal. In particular, if it can be established that the contributions to an overall voltammetric signal occur through a (mostly) linear additive process, this may potentially simplify the way voltammetric data are interpreted. Unfortunately, this is not immediately obvious in raw data. In this work, Factor Spaces are used to analyse the composite FSCV signals of several pairs of neurotransmitters. Principal Component Analysis (PCA) is studied as a candidate means for projecting the voltammetric data into Factor Space, to identify whether such underlying linear behaviour exists. We first demonstrate the behaviour expected given perfect linearity, through the use of 'synthetic' voltammetric data. This is then compared to the results obtained from measured data. Three different neurotransmitters, DA, 5-HT and NE, are considered in their isolated form, as well as when mixed together in all possible combinations and at various different concentrations.

II. Materials and Methods

A. Chemicals, Electrodes and Equipment

All neurotransmitters, DA, 5-HT and NE, were bought from Sigma Aldrich and used as received. All solutions were prepared in 0.1M phosphate-buffered saline. Stock solutions of the analytes were prepared at 1 mM in PBS buffer having pH 7.4 and these were diluted to desired level on the day of use.

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In all experiments, a three-electrode configuration was used with a general-purpose electrochemical flow-cell. The auxiliary platinum electrode, on which the varying stimulus is applied, was prepared in house. A miniature Ag|AgCl electrode, purchased from In Vivo Metric (California), was used as a reference. The working microelectrodes were made basing on the method described by Millar [12]. A robust nylon encasing was custom-machined for each electrode to enable them to be fitted into their respective ports in the flow cell. Electrode tips were allowed to protrude from the ends of the encasings, such that they would lie centred within the glass channel compartments. A constant flow rate of 3 ml/min was used in all experiments.

B. Data Acquisition and Pre-Processing

The stimulus applied to the auxiliary electrode was a digitally created triangular ramp potential generated at double precision using MATLAB (Mathworks, Natick, MA) and converted to an analogue signal using a PC-based, 24-bit A/D, D/A card (Creative Labs, Singapore). The card was interfaced to a custom-built *potentiostat* circuit, used to apply the stimulus to the auxiliary electrode. The potentiostat was also used to read in the current signal from the working electrode and to convert it to a voltage, whereby it was then read into the PC using the A/D, D/A card at a rate of 96 kSamples/s and at a resolution of 24-bits/sample. For all experiments a scan rate of 400 V/s [1],[11] was used, along with a resting potential of -1 V and an anodic limit of 1.5V. Stimuli were repeated at 30 Hz.

Recorded data consisted initially of just periodic background current waveforms obtained from the PBS buffer. Following neurotransmitter introduction, a smaller faradaic current signal could then be seen superimposed on the background signal. In order to extract the faradaic component, raw signals were first digitally low-pass filtered using an Equiripple filter (pass-band frequency: 2 kHz; stop-band frequency: 3 kHz [7]). Signals were then normalized and aligned by the point where the faradaic contribution was seen to peak. Through a process of digital subtraction, the background signal was then removed from the composite waveform. What remained was the faradaic current signal of interest in each experiment, i.e., the contribution due to the redox reaction of the neurotransmitter itself. Figure 1 shows voltammograms of DA at various concentrations.

To examine the behavior obtained when given perfect linearity, a set of 'synthetic' faradaic current waveforms was constructed by averaging a number of real individual neurotransmitter signals and then scaling their amplitudes to represent different concentrations. The synthetic signals were also added together to form synthetic 'mixtures'. This was done to create an ideal scenario where the principle of superposition is known to hold. The synthetic data was placed in a matrix consisting of 5 signals of DA and 5 signals of 5-HT at increasing concentrations, as well as 5 signals of different mixtures of DA and 5-HT. The mixtures were made to add up to an overall concentration of 3 µM (1.0µM DA + 2.0µM 5-HT, 1.5µM DA + 1.5µM 5-HT, etc). PCA was then applied to this matrix (rank of covariance matrix = 2) in order to project the original multivariate data into a new factor space, where uncorrelated orthogonal axes (principal components - PCs) lie along the direction of maximum variance. The application of PCA thus returned a number of basis waveforms and a set of corresponding scores, indicating the new position of the data in transformed space.

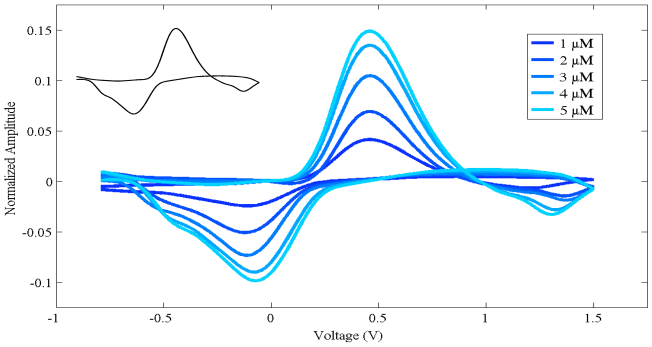

Figure 1 - Voltammograms for Dopamine at concentrations between 1 µM to 5 µM. Inset: a typical cyclic voltammogram, exhibiting a forward positive oxidation peak and backward negative reduction peak.

III. Results and Discussion

Since most of the variance was present in the first two PCs, the first two basis waveforms and corresponding 15 pairs of coefficients were retained. This allowed for a dimensionality reduction from N, the number of time sample points in the original data, to just two. Plotting each of the pair of coefficients as points in the new 2D factor space gave the result shown in Figure 2a. In this figure, points in blue correspond to DA signals, points in red to 5-HT signals, and points in black to varying mixtures of DA and 5-HT, (where the latter always add up to an overall concentration of 3 µM).

The five blue points corresponding to DA signals of increasing concentration are seen to lie regularly spaced on a straight (blue) line. Similarly the next five red points corresponding to the 5-HT signals are also seen to lie regularly spaced on a straight (red) line. This was as expected, given that the individual neurotransmitter signals were linearly scaled versions of a single averaged DA signal and a single averaged 5-HT signal. The last five black points corresponding to mixtures of DA and 5-HT are seen to lie on a straight line, which is itself intersecting the 'individual neurotransmitter lines' through both their third (3 µM) neurotransmitter points. This pattern was seen to repeat itself when synthetic mixtures of DA and 5-HT adding up to a different overall neurotransmitter concentration were analysed using PCA. Mixtures adding up to an overall concentration of 2 µM for example, was seen to lie on a straight line intersecting the second (2 µM) neurotransmitter points on the 'individual neurotransmitter lines'. The regularly spaced points on these transverse intersecting lines represent each of the mixture pairs. In Figure 2a, the mixture point lying closest to the blue DA line is that of the 2.5 µM DA + 0.5 µM 5-HT mixture. Conversely, the point lying closest to the red 5-HT line is that of the 2.5 µM 5-HT + 0.5 µM DA mixture. The point in the middle corresponds to the 1.5 µM DA + 1.5 µM 5-HT mixture. This regular behavior is also not surprising, given that the mixtures were synthetic linear additions of the individual neurotransmitter signals.

Now if the principle of linearity was to hold for the actual test data, then one would expect the latter to have similar behavior as that noted for the synthetic data. In this case then, the following assumptions may be put forward:

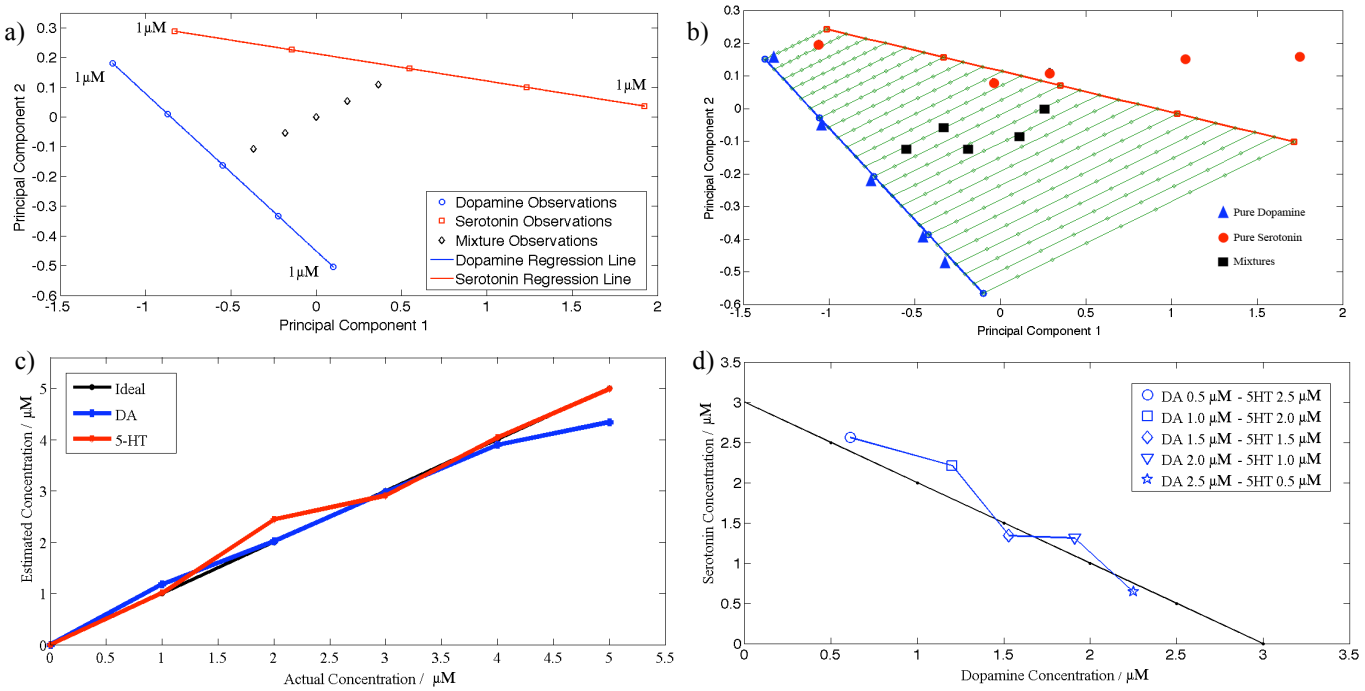

Figure 2: a) Factor space model for synthetic data and with b) superimposed measured data; c) Plots of Actual Vs. Estimated concentrations for DA and 5-HT; d) Results for mixed pairs of DA and 5-HT

Assumption 1: Faradaic signals of individual neurotransmitters with different concentrations differ only in their amplitude, and this strictly in direct proportion to their level of concentration. Thus, the signals of a series of preparations of an individual neurotransmitter with regularly increasing levels of concentration correspond to a series of points in the factor space that are linear and evenly spaced.

Assumption 2: Faradaic signals of mixtures of neurotransmitters are equivalent to the linear superposition of the respective individual neurotransmitter signals. Thus, signals of mixtures whose concentration adds up to an overall concentration of **[x]** µM, correspond to points on a transverse 'mixture line' in the transformed principal component space. This line intersects the corresponding **[x]** µM points on the 'individual neurotransmitter lines' of the neurotransmitters making up the mixture. The position of a point on the mixture line is according to the ratio of the constituent concentrations that make up that mixture.

With these assumptions in place, the faradaic signals of the actual test data were reduced by re-computing PCA on both synthetic and actual test datasets (rank of full covariance matrix = 15). The resulting coefficients for the test data were included with those of the synthetic data as shown in Figure 2b. The resemblance was seen to be remarkably close, suggesting that the principle of linear superposition seems to hold to some reasonably good extent. The actual test data for individual DA neurotransmitters are seen to match their synthetic counter parts very closely, with some minor deviation at the 5 µM point. The test data for the individual 5-HT neurotransmitters are noted to curve off the synthetic regression line beyond 4 µM, even when the 2 µM outlier was omitted. This may indicate that PCA detects a subtle change in shape of the 5-HT voltammogram as its concentration increases. The authors in [8], and [13] report similar deviations from linearity for individual neurotransmitter concentrations exceeding 5 µM, and cite electrode surface fouling and saturation as possible explanations. This implies that the linear model may only be applicable to concentrations < 5 µM - which so happen to be more physiologically relevant [8].

The mixture points are seen to lie about a transverse line which crosses from the individual 3 µM DA point to the individual 3 µM 5-HT point. While their placement is not precise, their relative positions more or less reflects the ratio of constituent neurotransmitter concentrations. Following these observations, the synthetic data was used as a representative measurement gauge, in order to estimate *a-posteriori* the concentration of the actual test signals, and thus to gauge the accuracy of our assumptions. Examples are shown for individual DA and 5-HT, as well their mixtures. In Figure 2b, the test data of individual DA and 5-HT are seen as blue triangles and red circles, lying close to (but not on) the 'individual measuring lines'. In order to obtain the concentration of each signal, each of these points was projected onto the nearest respective 'individual neurotransmitter line'. Once the 'new' projected point was obtained, its position on the line was used to estimate the concentration of that signal. For mixtures of neurotransmitters, the test data (black squares) were projected onto the closest green mixture line. The position of each black square on these lines allowed for the estimation of the overall concentration of the mixture, and the ratio of constituents in which it was split up.

The estimated values of concentration obtained using this method were predicted to within an accuracy of approximately 10% in the case of the individual neurotransmitters. Figure 2c shows a graphical representation of the concentration estimates for DA and 5-HT. The x-axis represents the actual concentration of the species, while the y-axis represents the estimated concentration obtained using

the measuring gauge in our factor space. The results show lines with gradients close to unity. The results shown in Table 1 give the percentage error for each neurotransmitter and the overall average error (graph for NE not shown).

Figure 2d shows the results obtained for concentration estimates of mixed pairs of DA and 5-HT. The x-axis represents increasing DA concentration while the y-axis represents increasing 5-HT concentration. The black line represents the ideal results, since the mixed pairs of neurotransmitters always add up to an overall concentration of 3 µM, with the concentration of DA increasing while that of 5-HT decreasing. It can be noted that the constituent concentration of the actual test data are estimated fairly accurately, with an average percentage error of 14.8%. More importantly, the behavior of the actual test data is seen to be generally consistent with that of the synthetic data, where the principle of superposition is known to hold.

Table 1 – Average % Errors for Individual Neurotransmitters

Individual Neurotransmitter	**% Error Estimated using PCA**
Dopamine	6.94%
Serotonin	5.47%
Norepinephrine	12.05%
Total Average	**8.15%**

Figure 3 shows the results obtained for mixture pairs of DA and Norepinephrine using PCA. The result obtained shows the inability of PCA to distinguish between DA and NE, since all mixture pairs are misclassified as NE signals having a concentration of approximately 3.0 µM. The reason for this is that DA and NE have faradaic signals which are practically impossible to distinguish due to their similar molecular structure which differs only by a single OH group. Average percentage errors in estimating neurotransmitter concentration are not calculated here due to the results being completely incorrect. A far more interesting result however is the fact that the principle of superposition is once again closely adhered to. Given that DA and NE have practically identical faradaic signals, adding two such signals with a cumulative concentration of 3.0 µM should be the same as having a single, individual (DA or NE) signal of a concentration of 3 µM. These results confirm and further indicate that the underlying relationship between mixtures of neurotransmitters approaches the principle of superposition.

Results for mixture pairs of NE and 5-HT (not shown) are similar to those in Fig. 2.1d (as expected since DA and NE

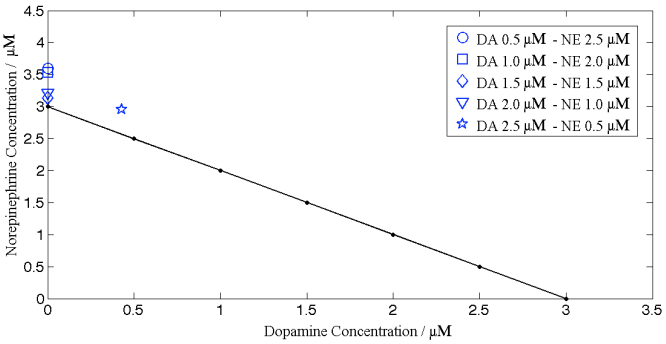

Figure 3 – Result obtained for mixed pairs of DA and NE

are practically identical). However, the constituent concentrations of the mixtures show a nearly systematic error. The technique used to estimate concentrations from the factor space model incorrectly predicts the overall concentration, though it correctly estimates the concentration ratios. This error is mostly attributed to experimental error, and possibly also due to adsorption onto the electrode which in turn causes increased sensitivity [8], [11].

IV. Conclusion

These results show that the voltammetric process used to detect multiple neurotransmitters is mostly additive and seems to follow the principle of superposition over small concentrations. The aggregate voltammetric current contribution at a microelectrode is therefore approximately equal to the sum of the individual contributions if these were measured separately. This novel result may introduce significant simplifications in how combined voltammetric data is interpreted, since it enables further studies employing more complex signal processing to exploit this property.

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