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Objective quantification of acetylcholine receptor aggregation using fast Fourier transforms

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ABSTRACT

A new approach for objectively analyzing the aggregation of acetylcholine receptors (AChRs) through power spectrum analysis derived from fast Fourier transform (FFT) of images has been developed. Presently, detection of AChR aggregates at neuromuscular junctions is not easily accomplished. Though the formation of AChR clusters results in periodic gray-level variations that differ with time, no study reporting their correlation with frequency information in the Fourier domain for aggregates' detection purposes exists. To this end, we processed time-lapse images of AChR aggregates' formation on murine myotubes to extract peak values of power spectra. To validate interpretation of the Fourier spectra analysis, a computer routine was developed to semi-automatically count AChR aggregates. We found: (1) logarithmic maxima of Fourier spectra correlated significantly with experimentation time; (2) cluster count correlated significantly with time only after clusters were discernable from images, signifying that this method heavily depended on definitive growth data and thresholding values; (3) exponents of Fourier maxima versus time and cluster count versus time profiles during this phase compared favorably, indicating that both methods were analyzing identical cluster growth rates. Our observations suggest that analysis via FFT power spectrum is sensitive and robust enough to automatically quantify AChR aggregates.

1. Introduction

The neuromuscular junction (NMJ) is a specialized region in muscle tissue, at which a motor neuron makes a functional synapse with the muscle fiber [1–5]. During development, contact between a motor neuron axon tip and the muscle cell results in the rapid recruitment of acetylcholine receptors (AChRs) to the site of contact, which is necessary for efficient signaling from the neuron to the muscle to elicit muscle contraction [6–8]. Importantly, the NMJ shares many functional characteristics with central nervous system (CNS) synapses, so the NMJ is often used as a general model for synaptogenesis due to its accessibility and large size compared to those in the CNS. The discovery of agrin, a protein released by the axon and associated with the extracellular matrix at the junction between neuron and muscle [9–12], led to the development of the agrin hypothesis: release of agrin from the axon determines where the synapse forms. Prior to axon tip contact, AChRs are expressed over the whole surface of the muscle fiber; upon release of agrin by the nerve tip, AChRs migrate to the site of agrin accumulation and form dense complexes of AChRs clusters that are the precursors of the adult NMJ [13,14]. In vitro, bath application of agrin to differentiated myotubes results in the formation of multiple AChR clusters on any given single myotube [15–18]. Determining how cluster number and size correlate with cluster stability are important in establishing numerical parameters that can be used to model AChR clustering and synapse maintenance. Thus,

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objective quantification of AChR aggregates at NMJs – yielding spatiotemporal AChR cluster distribution data – could contribute to our current understanding of the role of agrin in NMJ differentiation.

To quantitate AChR aggregation, various methods have been developed. One of the earliest methods employs correlation of radioactivity with cluster number [19]. Although the radioactivity of suitably radiolabeled AChRs can be determined with a gamma counter, this method offers limited spatial resolution, producing only a single measurement for regions where there are many disparate AChR clusters. This method is also expensive and the investigator runs the risk of being exposed to radioactive compounds. Numerous image analysis methods have been tried. A widely used method for quantification is based on sampling of myotubes whereby outlines of distinct fluorescent patches containing AChR clusters are manually traced and their areas measured using a computer program [10,14]. This is a time-consuming procedure (the cluster number can be on the order of hundreds or thousands per image) with limited throughput, so manual delineation of a large enough number of AChR clusters to characterize changes in quantity and distribution over time is impractical. Moreover, the task of accurately identifying actual AChR clusters is complicated by the fact that their size, densities and distribution vary throughout the image and in time; hence counting is susceptible of bias by the human in charge of the analysis. Automated image analysis methods provide an attractive alternative to manual counting techniques. However, their primary shortcoming lies in the fact that the critical thresholding parameter, which is required for background fluorescence and noise removal, is calculated using a few adjacent images from an entire temporal sequence [20–22]. This can lead to a significant degradation of information because the dynamic process of formation and redistribution of AChR clusters, available from the entire image sequence, is not factored in.

Many image analysis algorithms achieve background noise elimination by performing a 'subtraction' operation on raw images by a background image. The background image could be an image obtained either before the AChR clusters form or after they disappear. To overcome technical difficulties involving background fluorescence and inhomogeneity on images, total internal reflection fluorescence (TIRF) has been utilized to quantify clusters [23]. As TIRF detects fluorescence signal coming only from cell-substrate contact regions where almost all of the AChR clusters are located, background noise from fluorophores either in the bulk solution or inside the cells is suppressed. Though this method for background subtraction serves the purpose, it is costly and not straightforward to set up. Here we report a digital method for background subtraction that is insensitive to observer bias and that can be applied to micrographs taken using conventional fluorescence microscopes.

We apply our image analysis technique to objectively and efficiently quantify the nucleation and growth processes of AChR clusters in time-lapse fluorescence micrograph sequences of cultured myotubes after exposure to agrin. The myotubes were formed by fusion of single myoblasts from the muscle cell line C2C12 using well-established methods (see Section 2). Specifically, we analyzed the clustering patterns by employing the fast Fourier transform (FFT) on the time-lapse images. The FFT is an algorithm that can be used to decompose an image into various sinusoids of different frequencies that collectively sum to produce the original signals present on the images [24]. We hypothesized that by calculating orientations and spacing in an image, characteristics of AChR clusters, such as their periodicity and distribution, can be represented as a power plot of its FFT counterpart. What is more, observer bias is virtually removed in this technique by the incorporation of an unsupervised frequency analysis component via FFT. Another advantage of this approach is that interpretation of the power spectra of all images to determine AChR clusters count is much easier, faster and more accurate than trying to extract the same information from the original image. This is because all the spatial patterns and orientations are effectively averaged in a frequency domain [25]. As a way of verifying the interpretation and performance of the Fourier spectra analysis at quantifying cluster nucleation kinetics, we also developed a semi-automated computer routine to count AChR aggregates present on images pre-processed in the frequency domain. Our results confirm previous reports [14,19,23] that the number of AChR clusters increases exponentially with time.

2. Materials, rat muscle culture and microscopy

Murine-derived muscle precursor line, C2C12, was purchased from American type culture collection (ATCC, Manassas, VA). The cells were maintained in an appropriate growth media, a mixture of Dulbecco's modified Eagle's medium (DMEM, GibcoBRL; high glucose, glutamine, no sodium pyruvate) supplemented with 20% fetal bovine serum (FBS, Hyclone, Logan, UT) and 1% penicillin/streptomycin (Pen/Strep, GibcoBRL) until they were deemed ready for differentiation. The cell media was then changed to a differentiation media (DMEM, GibcoBRL; 2% horse serum (HS, ATCC, Manassas, VA), 1% Pen/Strep) to facilitate differentiation of the myoblasts into myotubes. The differentiation media was renewed every 2 days. AChRs were visualized with α-bungarotoxin conjugated to Alexa 488 (Molecular Probes, Eugene, OR). The cells were labeled with BTX for 30 min at a concentration of 100 ng/ml. Next, the cells were washed three times with the differentiation media prior to image capture. The myotubes were exposed to agrin (R&D Systems, Minneapolis, MN) at a concentration of 20 ng/ml throughout the entire procedure.

Images of the myotubes were taken with Metamorph (Universal Imaging, West Chester, PA) using a Nikon Eclipse TE2000-U inverted stage microscope (Nikon Instech Co., Kanagawa, Japan) equipped with a Hamamatsu ORCA ER CCD camera (Meyer Instruments, Houston, TX). Time-lapse images were taken every 10 min, with a 2.5 s exposure time.

3. Computational method

3.1. *Image processing*

Image processing comprised three main steps: (1) conversion of the original image to 8-bit grayscale; (2) application of a