High-resolution quantitative visualization of glutamate and GABA receptors at central synapses

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Glutamate and GABA are the main transmitters in the central nervous system and their effects are mediated by ionotropic and metabotropic receptors. Immunogold electron microscopy has revealed the quantitative localization of these receptors at 20–30 nm resolution. SDS-digested freeze-fracture replica labeling (SDS-FRL), a newly developed immunogold method, provides an accurate estimate of molecule numbers. Here, we summarize the recent advances in quantitative receptor localization, including use of SDS-FRL analyses to determine numbers of AMPA-type glutamate receptors in the cerebellum. The two-dimensional view and high sensitivity of SDS-FRL have revealed small, irregularly shaped AMPA receptor clusters within cerebellar synapses.

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Introduction

Neurotransmitter receptors in the central nervous system (CNS) are localized not only to the postsynaptic but also to the presynaptic, perisynaptic and extrasynaptic membrane compartments at variable densities [1–3]. Together with the functional properties of the receptors, their number and subsynaptic localization contribute to the physiological conditions for activation of receptor-mediated pathways. High-resolution quantitative visualization of receptors is therefore essential for understanding their roles in neurotransmission. Although recent fluorescent imaging methods, including confocal and two-photon laser microscopy, are powerful tools for visualizing real-time dynamics of functional proteins, immunogold electron microscopy [1–3] is indispensable for studying receptor localization at a submicron level within and around synapses. In this review, we provide an overview of such high-resolution microscopy techniques, and then discuss in detail data obtained over the past two years using the highly sensitive SDS-digested freeze-fracture replica labeling (SDS-FRL) method.

Immunogold visualization of receptors using electron microscopy

Figure 1 shows three immunogold electron microscopic methods that can be used to visualize receptor localization: pre-embedding, post-embedding and SDS-FRL. The pre-embedding immunogold method is the most widely used for visualizing subcellular localization of various molecules in the CNS. It is particularly useful for identifying labeled profiles in corresponding images obtained from the same specimens using light and electron microscopy [4]. However, antibodies do not evenly penetrate the pre-embedding materials because immunoreaction occurs in the thinner sections (Figure 1a), rendering quantitative comparison of the signals difficult among the profiles in different regions or in different animals. Furthermore, the pre-embedding method hardly detects the numerous ionotropic receptors that are located within postsynaptic densities because of the penetration problem (Figure 1a, arrows) [3,5,6].

The post-embedding immunogold method overcomes these problems by reacting immunochemicals with the antigens exposed on the surface of the ultrathin sections (Figure 1b) and then detecting postsynaptic receptors with the same sensitivity as that for nonsynaptic receptors. This improves quantitative evaluation of the receptor densities [7]. However, in resin-embedded sections from post-embedding materials, substantial populations of proteins are buried and not accessible by antibodies, limiting the detection sensitivity of this technique (Figure 1b).

SDS-FRL can complement these conventional immunogold approaches (Figure 1c). This method was originally developed by Fujimoto [8] to visualize two-dimensional distribution of membrane molecules on freeze-fracture replicas [8,9,10**,11**]. The tissue is frozen, freeze-fractured, shadowed with platinum–carbon and then replicated using carbon. Subsequently, the tissue beneath the replica is dissolved with SDS, leaving the membrane proteins in the carbon-fixed membrane halves. Antibodies for the intracellular and extracellular domains can be applied to the membrane proteins separated on the protoplasmic face (P-face) and exoplasmic face (E-face), respectively [8]. The postsynaptic membrane areas of the excitatory synapses are clearly indicated by clusters of intramembrane particles (IMPs; Figures 1c, 2, 3) on the E-face [12,13].
Technical considerations for SDS-FRL

The SDS-FRL method has several advantages over conventional immunogold methods. First, its sensitivity in detecting membrane proteins is considerably higher. This is probably because the membrane proteins are exposed on the two-dimensional surface of the replica, so are readily accessible by immunoreagents, and because epitopes are denatured by SDS, so the antibodies used for immunoblot analysis can react similarly with proteins immobilized on the replica membrane. Second, the label-
ing intensity obtained by SDS-FRL is highly reproducible. Third, SDS-FRL is more efficient and rapid with regard to the analysis of a large number of individual synapses than the post-embedding method, which requires laborious and time-consuming reconstruction of serial ultrathin sections.

However, SDS-FRL also has certain potential limitations. First, identification of labeled profiles from the morphological features in the replicas is often difficult, and it is frequently necessary to label marker proteins to facilitate identification of the fractured membranes. Second, fractures occur randomly. This makes it difficult to target specific cells and can also lead to sampling bias in synapse collection, because of differences in the chances of fracturing membranes that have different curvatures. Third, the separation of the membrane proteins to the P-face or E-face is unpredictable: certain proteins are preferentially allocated to either the P-face or the E-face whereas others are allocated to both faces, as demonstrated for GluRδ2 in synapses between parallel fibers (PFs) and Purkinje cell (PCs) (Figure 2). Thus, particularly for quantitative studies, the allocation of molecules should be carefully examined using antibodies that are reactive to intracellular and extracellular domains.

In the conventional freeze-fracture method, the fractured faces are shadowed using platinum–carbon followed by carbon to support the shadowed layer. In SDS-FRL, we often apply a thin (~5 nm) coat of evaporated carbon before shadowing with platinum–carbon, to increase the detection efficiency [11*,14*]. Retention of membrane lipid molecules is also better when the first carbon layer is applied [15]. However, this procedure enlarges the IMPs and blurs morphological details (compare Figure 3a and 3b). Use of a thinner initial carbon precoating might increase both the replica resolution and the labeling intensity [16].

Quantitative analysis of ionotropic glutamate receptors in individual synapses

Although post-embedding immunogold labeling is widely used for high-resolution quantitative analyses of receptor localization in synapse populations [17–22], the low sensitivity of this method can hamper reliable analysis of receptor numbers in individual synapses. Because our replica immunogold labeling for AMPA receptors provided almost one-to-one detection sensitivity for functional AMPA receptors [10*], we applied this method to analyze the distribution of AMPA receptors in individual synapses of three different types in the cerebellar molecular layer [11**]. Using an antibody against the highly conserved extracellular amino acid residues of the AMPA receptor subunits GluR1–GluR4 [17], we found uneven distribution of gold particles within individual IMP clusters (Figure 3a,c), which were identified as PF–PC synapses by labeling for GluRδ2 [23]. However, despite the variable density, virtually all the synapses were positive for AMPA receptors. This result seems to be inconsistent with the idea of ‘silent synapses’ in the cerebellum [24]. In fact, we found no glutamatergic synapses without AMPA receptors, even in hippocampal CA1 pyramidal cells, wherein silent synapses have been well described. This is probably because synapses with a low number of AMPA receptor channels are difficult to detect in the electrophysiological recording obtained from cell bodies. In contrast to the uneven distribution of AMPA receptors in the PF–PC synapses, we detected extremely dense and homogeneous labeling in climbing fiber (CF)–PC synapses that were identified by labeling for the vesicular glutamate transporter VGlut2 [25] on

![Figure 3](image-url)
metabotropic glutamate (mGlu) receptors (mGlu2/3) are

actin cytoskeleton by different scaffold proteins

selective anchoring of these receptors to the subsynaptic

Such microdomains within the synapses might reflect

the P-face of the adjacent terminals (Figure 3d). AMPA

receptor labeling at the PF–interneuron synapses was also
dense and homogeneous. The average density of the

immunogold particles in the CF–PC and PF–interneuron

synapses (1800–2400 μm⁻²) was approximately four to

five times higher than that in the PF–PC synapses (400–

500 μm⁻²). We found that signals in the post-embedding

serial sections were three to four times weaker and more

variable than those in the SDS-FRL materials [11**]. The

average number of gold particles (80) in immature

CF–PC synapses revealed by SDS-FRL [11**] was compar-
able to the minimum number of functional AMPA

receptors (66) activated by the quantal transmitter

packet at this synapse [26]. Distinct AMPA receptor

numbers and densities in the three different types of

synapses in the molecular layer indicate input-dependent

and target-dependent regulation of AMPA-receptor-

mediated synaptic strength. Furthermore, quantification

of AMPA receptors by SDS-FRL has facilitated reliable

comparison between different samples that display only

minor differences (e.g. 30% reduction) [27].

Some studies have reported that AMPA receptors might

not be uniformly distributed over the postsynaptic density

but might instead be concentrated laterally or centrally

[28–30]. These analyses are based mainly on the average
distribution of immunogold particles in the synapse pop-

culations detected by the post-embedding method. The two-

dimensional view and high sensitivity of SDS-FRL

revealed small irregularly shaped AMPA receptor clusters

within individual PF–PC synapses (Figure 3c). By using

conventional shadowing, IMPs of variable sizes were dis-
cernible, and small clusters of relatively large IMPs were

preferentially labeled for GluR1–GluR4 and for GluR2

AMPA receptor subunits (Figure 3a). These clusters were

located not only laterally but also centrally in some

synapses. A similar observation was made in synapses

between Schaffer collateral fibers and hippocampal pyra-

midal cells. By contrast, AMPA receptor density at CF–

PC and PF–interneuron synapses was homogeneous over

postsynaptic areas (Figures 3d) [11**]. In mossy fiber–

granule cell synapses in the cerebellum, double labeling

for the NMDA receptor subunit NR1 and GluR4 revealed

that they were segregated into distinct clusters (Figure 4a).

Such microdomains within the synapses might reflect

selective anchoring of these receptors to the subsynaptic

actin cytoskeleton by different scaffold proteins

[31*,32,33*].

Presynaptic localization of receptors

The pre-embedding immunogold method can reveal

presynaptic localization of receptors [34–39]. Group II

metabotropic glutamate (mGlu) receptors (mGlu2/3) are

often observed in the extrasynaptic sites around glutam-

materic presynaptic elements, most of which are located

relatively far from the presynaptic active zone [34]. By

contrast, group III mGlu receptors (mGlu4, mGlu6,
mGlu7 and mGlu8) are localized mainly to the presynaptic

active zone [34]. Interestingly, the GABA_B receptor sub-

units GABA_B1 and GABA_B2 are also frequently present in

the presynaptic active zone of glutamatergic synapses

[35–39], indicating heterosynaptic interaction by

spilled-over GABA [36]. Although the post-embedding

method can also detect presynaptic and postsynaptic

ionotropic receptors, it is often difficult to decide whether

the presynaptic active zone is labeled because the immu-

nogold particles for the postsynaptic sites can be up to

30 nm away from the epitopes [28]. Furthermore, the

resolution of this method can be limited by projection

artifact caused by projecting the entire thickness (e.g. 60–

90 nm) of the section onto a single plane using the

immunogold particles derived only from the surface

(Figure 1b). Thus, statistical analyses of the distribution

of particle distance from the synaptic membrane and
detection of a Gaussian peak that indicates presynaptic

localization are necessary [28,40]. This is one of the

reasons for the relative lack of morphological evidence

with regard to presynaptic ionotropic receptors as com-

pared with the substantial data collected by electro-

physiological studies [41]. The SDS-FRL method

clearly separates the presynaptic and postsynaptic mem-

branes, making it easy to demonstrate presynaptic mol-

ecules on the P-face [42]. Immunolabeling for GABA_A

receptors is observed on the P-face of the presynaptic

active zone (Figure 4d), which opposes the postsynaptic

E-face that exhibits characteristic IMP aggregation (red

in Figure 4d). The presynaptic active zone is also readily

identified by the labeling for the cytomatrix at the active

zone-associated structural protein CAST [42].

Extrasynaptic receptors

Accumulating evidence supports the functional signifi-

cance of extrasynaptic ionotropic receptors [43,44], whose

channel properties can differ from those of the synaptic

receptors. Although most of these reports are based on

electrophysiological data, certain post-embedding immu-
nogold studies revealed the contribution of different

GABA_A subunits to the extrasynaptic receptors [45,46].

Furthermore, recent pre-embedding immunogold studies

have revealed a high level of extrasynaptic AMPA rece-

ceptor immunoreactivity in hippocampal neurons [47*].

Using SDS-FRL, we detected extrasynaptic receptors

with varying densities depending on cell types. For

example, in the dentate gyrus of the hippocampus, we

found high levels of immunolabeling for AMPA receptors

in extrasynaptic sites (arrows in Figure 4b; ~100 particles

μm⁻²), whereas in the molecular layer of the cerebellum,

the density of the extrasynaptic AMPA receptors was very

low (~10 particles μm⁻²) [11**]. High sensitivity and

exceptionally low background (usually <2 particles

μm⁻² for AMPA receptors) facilitated measurement of

the density of the sparse extrasynaptic receptors. SDS-

FRL is also useful for demonstrating the two-dimensional

relationship between distributions of different molecules
in extrasynaptic sites, as shown for GABA_B receptors and Kir3.2-containing K⁺ channels, which are selectively colocalized in the spines of hippocampal pyramidal cells [48*].

**Relationship with distinct structures other than synapses**

Recently, considerable interest has been shown in understanding the functional roles of gap junctions in the brain [4]. Gap junctions have several distinctive morphologies in freeze-fracture replica materials [9*,49,50]. GABA_A receptor labeling immediately adjacent to gap junctions (Figure 4c) suggests a possible functional link between these junctions and inhibitory synapses. Hence, replica immunolabeling is especially useful for examining such spatial relationships between various gap junctions and receptors.

**Conclusions and outlook**

The three immunogold electron microscopy techniques — pre-embedding, post-embedding and SDS-FRL — should be used in combination, depending on the purpose and based on knowledge of the advantages and disadvantages of each method. In our SDS-FRL method, we could detect ~1 gold particle for each AMPA receptor channel, enabling us to estimate the exact receptor number and density at high resolution among various types of excitatory synapse. Calibration of the replica labeling sensitivity for the other receptors should be established in the future. The high reproducibility of SDS-FRL has also facilitated detection of changes in AMPA receptor numbers in different conditions. With careful evaluation of the potential sampling problems, SDS-FRL might prove to be a powerful method that can complement the conventional immunogold methods.

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References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


Using freeze-fracture replica immunogold labeling (FRIL), the authors demonstrated connexin-Cx36-containing gap junctions at the mixed glutamatergic and electrical synapses between presumptive mitral or tufted cell dendrites in the olfactory bulb.


This is the first measurement of the synaptic AMPA receptor density in the brain. Using a combination of two-photon uncaging, electrophysiological noise analysis and electron microscopy, the authors analyzed the number and density of functional AMPA receptor channels in single synapses; the results were similar to those revealed using immunogold particles in SDS-FRL.


Using SDS-FRL that has almost one-to-one detection sensitivity for AMPA receptors, the authors gave good estimates for the number and density of AMPA receptors in three different types of synapse in the cerebellar molecular layer. The results indicated input-dependent and target-dependent regulation of AMPA-receptor-mediated synaptic strength.


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