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REVIEW

Advances in genomic study of cortical projection neurons

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Abstract The mammalian neocortex gives rise to perception and initiates voluntary motor responses. The cortical laminae are comprised of six distinct cellular layers of local circuit neurons and projection neurons. To explore molecular identities of the distinct cortical projection neurons, discovery-orientated genomic approaches have been adopted. Microarray analysis of dissected cortical tissues has been applied to identify cortical layer markers. Early neuronal cells were sorted by FACS from GFP-labeled embryonic brains for gene expression profiling. Laser capture microdissection of retrograde-labeled projection neurons, when coupled with optimal RNA amplification technology, has become a valuable strategy for neuronal isolation and gene expression analysis in differentiated neurons. RNA sequencing technology is promising not only for the determination of gene expression, but also for discovery of posttranscriptional modifications of the complex neural system. There is no doubt that advances in genomic studies are opening up novel research avenues for our understanding of the cortical neuronal functions.

Keywords expression profiling, pyramidal neurons, RNA amplification, cortex, retrograde labeling

1 Introduction: cortical projection neurons

Mammalian neocortex gives rise to perception and initiates voluntary motor responses. The cortical functions are processed through precise synaptic connections between different regions of the cortex, and between the cortex and other parts of the brain. The disruption in the development and plasticity of cortical connections underlies many neurological and psychiatric disorders in humans (Harel and Strittmatter, 2006; Geschwind and Levitt, 2007; Polleux et al., 2007). The cortical neurons, classified into local circuit neurons and projection neurons, are arranged in six layers parallel to the surface of cortex. Projection neurons, also known as pyramidal neurons by their morphology, arise from asymmetric division of the neural stem cells, and acquire their laminar positions and layer-specific identities based on the timing of the terminal mitosis. Projection neurons have distinct connectivity in different layers. Layer IV is well developed in primary sensory cortical areas that are the major recipient of thalamocortical fibers. The upper layers (II and III) consist of pyramidal neurons making connections solely with other cortical neurons, either within the ipsilateral hemisphere or through the corpus callosum to the contralateral side of cortex. Majorities of layer V pyramidal neurons project to subcortical sites except for the thalamus, which receives fibers from the layer VI (O'Leary and Koester, 1993). Moreover, local differences in the laminar pattern of connectivity separate the cortex into functionally distinct areas. For example, layer V neurons of the frontal cortex project to motor nuclei in the brain stem and spinal cord, while those in the visual cortex project only to the tectum. This difference in subcortical projections was acquired by axonal pruning, an early postnatal process of developmental degeneration (O'Leary and Koester, 1993).

To have a better understanding of the development of neuronal identities and synaptic circuits, an in-depth knowledge of the principles and mechanisms governing gene expression programs is essential. Recently, a large number of genes with different laminar expression patterns in the cerebral cortex have been identified (Molyneaux et al., 2007, and Fig. 1). Area dependent gene expressions in neocortex have also been determined in both rodents (O'Leary et al., 2007) and humans (Johnson et al., 2009, Zhu et al., 2010). By determining gene expression at a high throughput (Schulze and Downwad, 2000; Greenberg, 2001), microarray screening has contributed greatly to the exploration of the genetic programs that regulate the molecular identities, dendritic arborizations and axonal projections of cortical pyramidal neurons. This article is

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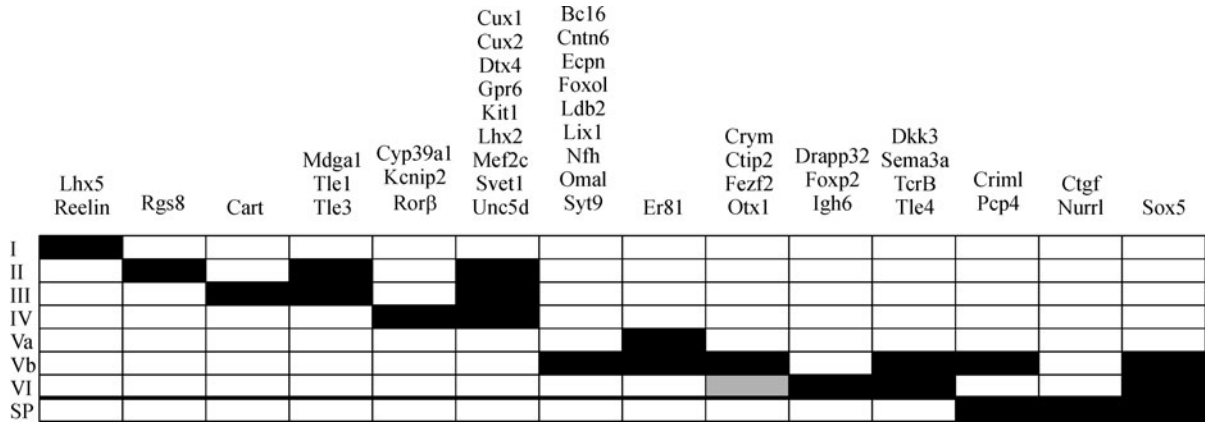

Fig. 1 Selected genes with layer specific expression in neocortex of rodents. Dark and light shadows represent the strong and weak expression, respectively. Genes are selected from the article (Molyneaux et al., 2007) and visually verified by using Allen Brain Atlas (http://mouse.brain-map.org/).

aimed to provide a short review on the recent development of genomic studies of the cortical projection neurons with a focus on the methodologies.

2 Gene expression profiles of cortical tissues

Since isolation of highly purified neural cell populations from complex, heterogeneous brain tissues has been a challenge, the most intuitive and productive approach has involved the selection of specific brain regions for microarray studies. The mammalian neocortex is organized into many function areas that can be dissected according to their cortical locations. Adequate RNA can be obtained from tissue samples and used for genechip hybridization directly after labeling. Region-specific genes in rodent (Sandberg et al., 2000; Zapala et al., 2005), primate and human brains (Evans et al., 2003; Khaitovich et al., 2004) have been successfully identified based on this approach. Although whole-tissue analysis inevitably includes heterogeneous cell types, which may limit detection of rare transcripts, it remains a reasonable approach when coupled with extensive verification by quantitative real-time PCR and in situ hybridization (ISH). Databases of large scale ISH are available now in the websites for gene expression in rodents (Gray et al., 2004; Visel et al., 2004; Magdaleno et al., 2006; Lein et al., 2007).

To validate genomic approach for studying differentially expressed genes in brain tissues, the influence of tissue complexity was evaluated with real-time PCR for the detection of regulated transcripts by comparing a rodent cell line, hypothalamus and cortex. As tissue complexity increased, distinguishing regulated genes became increasingly more difficult. However, cDNA microarray studies using regional brain dissections and appropriate numbers of replicates could identify genes that only have 2-fold of differences (Wurmbach et al., 2002). The power of transcriptional profiling was further illustrated in a study of a mouse model for Rett syndrome. Global gene expression was compared for forebrain, cortex and hippocampus between wild-type and Mecp2 mutant mice. Although no dramatic changes were observed in the transcription for mice displaying Rett-like symptoms, the combination of a small set of genes was able to distinguish between the mutant and wild-type mice (Tudor et al., 2002). This indicates that a subtle difference in gene expression may be associated with the phenotype and can be identified by microarray screening.

Microarray screening proved to be a successful approach for exploring layer and area specific genes in the cortex. To discover the genetic basis for the subcortical projections from the layer V pyramidal neurons, the mouse brain was divided into four regions (upper and lower layers of neocortex, hippocampus and striatum). The striatum was taken as a control for non-pyramidal neurons. Deep layer selective genes were identified by comparing the gene expression profiles among the four tissue samples, and were verified by ISH subsequently. Further study using axonal tracing and *in vivo* gene manipulation found that Fezf2 (also known as Zfp312) is expressed in the early progenitors of cortical projection neurons, and is a marker of subcortical projection neurons in the layer V (Chen et al., 2005).

In addition to the classic six layers of the cortical plate, a subplate of neurons consisting of transient cells is located under the layer VI and above the white matter/intermediate zone. The heterogeneous cell population has been an obstacle for molecular characterization of the subplate cells. By comparing gene expression profiles of the subplate and layer VI in visual and somatosensory cortices of postnatal mice, novel markers for subplate neurons,

including MoxD1 and complexin 3, were discovered (Hoerder-Suabedissen et al., 2009). The expressions of these markers were further examined in the reeler mutant and P35 knockout mice, where the subplate was displaced in relation to the cortical plate. The disparate locations of different marker-positive cells in the mutant mice suggest the presence of subpopulations in subplate cells.

The axon projection of a cortical neuron is not only dependent on the cortical layer, but also on the cortical area, where the neuron is located. The discovery of area-restricted genes is especially important for our understanding of axon pruning, a postnatal process of neuronal maturation that is specific to each cortical area (Low and Cheng, 2006). Microarry screening was performed on primary somatosensory and visual cortices dissected from newborn mice. More than 20 genes were differentially expressed that were later confirmed by real-time PCR. Among those genes, transcription factor Bcl6, is expressed by specific projection neurons in the somatosensory cortex, while transmembrane protein Ten_m3 is expressed preferentially in visual cortex (Leamey et al., 2007). However, the functions of these area-dependent genes remain obscure regarding to their possible contributions to the cytoarchitecture and connectivity of their respective areas.

Region and area specific genes were also studied in primate and human brains by high-throughput screening (Mirnics et al., 2000; Mirnics et al., 2005; Bunney et al., 2003). Microarray analyses on human postmortem brains suggest that large numbers of genes exhibit a significant difference in expression between cerebral cortex and subcortical regions. Thus, the regional profile of gene expressions may be accounted for by particular sets of genes. Cortex-enriched or cortex-restricted genes include transcription factors, immune system related proteins and axon guidance molecules. By contrast, among the neocortical areas, there are very few genes that show significant area-specific expression patterns (Evans et al., 2003). Genes that have area dependent expressions in primates include occ1 that is expressed specifically in the visual cortex (Yamamori and Rockland, 2006). The occ1 expression in excitatory neurons of visual cortex is activity-dependent and strictly regulated by thalamocortical projections during brain development. In contrast, gdf-7 is specifically expressed in the primate motor cortex (Watakabe et al., 2001).

A genome-wide, exon-level expression analysis of 13 regions from a mid-fetal human brain revealed that the regional differences in gene expression in prenatal brain are more robust and complex than that of the adult (Johnson et al., 2009). Interestingly, more than 200 genes were identified with putative expression differences for the frontal lobe in human fetal brain. Several of the genes identified have been previously implicated in complex social and emotional disorders originating from the frontal lobe. For example, CNTNAP2 is related to the language delay in autism, and is a target of the language-related transcriptional repressor FOXP2 (Vernes et al., 2008).

3 Purification and characterization of homogeneous populations of neurons

Since the cortical tissues used for extracting RNA contain many cell types, the ratios of gene expression only reflect the average of all cell type. Thus, the gene expression in cortical projection neurons is very much diluted by mRNA from other cells, and only highly abundant transcripts like Fezf2 may be identified (Chen et al., 2005). To detect low-abundant messages, projection neurons must be labeled and isolated prior to genomic studies.

A key advantage in the study of projection neurons is that they can be labeled alive by retrograde tracers in the absence of antigenic markers. Fluorescence neural tracers injected into axonal projection fields can be taken up at axonal terminals and transported back to soma along the axon (Vercelli et al., 2000). To label and isolate the subcortical projection neurons and the callosal projection neurons, Arlotta injected green fluorescent microspheres in one hemisphere of cortex and red microspheres in the pyramidal decussation, respectively. Different populations of projection neurons were labeled (Fig. 2). After dissociating the neuronal cells in culture, the callosal neurons and corticospinal projection neurons were separately isolated by fluorescence activated cell sorting (FACS) (Arlotta et al., 2005). Gene expression profiling with microarrays identified multiple transcripts involved in the differentiation of subcortical projection neurons, including Fezf2 and Ctip2 (Molyneaux et al., 2005). However, the callosal projection neurons, labeled as such, reside both upper layer (III) and the deep layer (V) (Fig. 2), thus representing a heterogeneous population of cells when isolated by FACS sorting.

Neuronal cells can also be labeled genetically by reporters driven under a cell type-specific promoter. Using the transgenic mice that express enhanced green fluorescent protein (EGFP) under the control of an S100beta promoter, astrocytes, neurons, and oligodendrocytes from developing and mature mouse brain were isolated separately based on FACS sorting and immunopanning purification. The gene expression profiles of these three neuronal cell types were obtained, and cell type specific markers were identified (Cahoy et al., 2008). By a similar approach, sensory (Colosimo et al., 2004) and motor (Fox et al., 2005) neurons from *C. elegan* were successfully labeled and FACS-sorted for the genomic studies. Labeling and FACS sorting have also been applied to *in vitro* cell culture to obtain homogeneous cell populations from human brains. The lateral ventricular wall from adult patients undergoing therapeutic lobectomy was dissociated in culture. The cells were transduced with

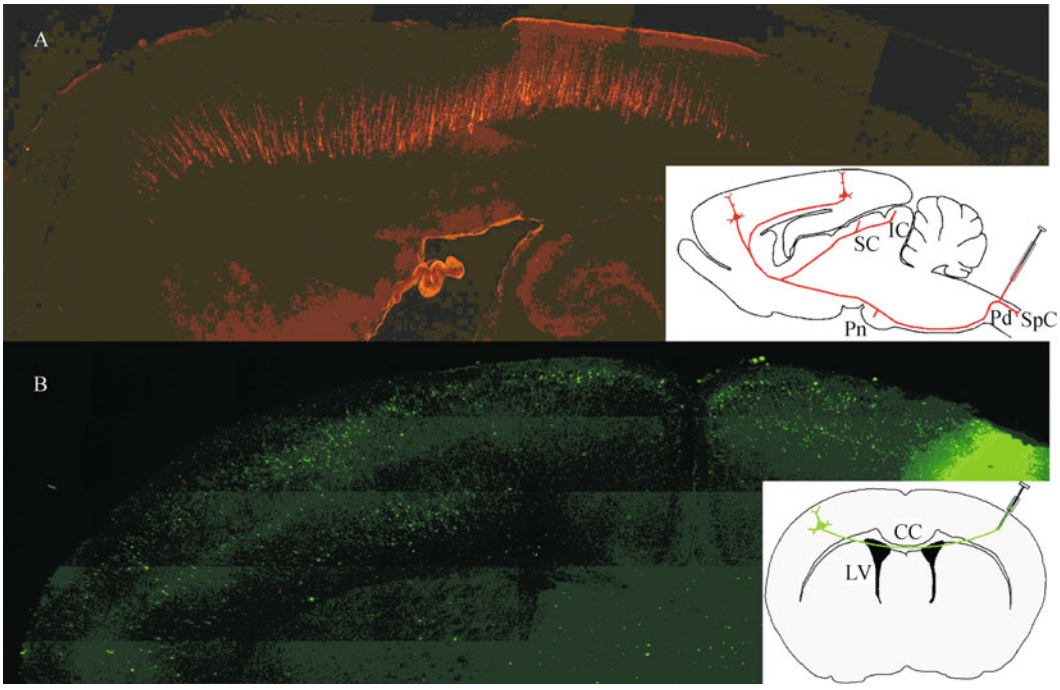

Fig. 2 Retrograde labeling of cortical projection neurons. Corticospinal projection neurons were labeled by Red Retrobeads injected at the pyramidal decussation. Corticocortical neurons in left cortex were marked by Green Retrobeads injected in the right hemisphere. The labeled neurons were isolated by FACS (Arlotta et al., 2005), or by LCM (JG Chen, unpublished results).

a GFP reporter driven under the early neuronal promoters of tubulin Tα1 and nestin. Neuronal progenitor cells from the adult human brain were successfully obtained by FACS sorting (Roy et al., 2000).

Bacteria artificial chromosome (BAC) is a large segment of DNA that contains most cis-regulatory elements required for a gene expression *in vivo*. BAC-mediated transgenesis permits expression of a cell-type specific reporter in mice (Heintz, 2000). Currently, the gene expression nervous system atlas (GENSAT: http://www.gensat.org/index.html) has a repository of BAC-transgenic mice with hundreds of genetically GFP-labeled neuronal types (Gong et al., 2003). Thus expression profiles of distinct neuronal populations may be acquired with the sorting of GFP-labeled cells, followed by RNA extraction and microarray hybridization. For example, based on the expression of GFP, Lobo et al. were able to dissociate striatonigral and striatopallidal neurons from juvenile and adult mice by FACS-sorting. The approach allowed for identification of numerous genes by microarray screening, which demonstrated a specific disruption of striatonigral pathways in the Ebf1 knockout mouse (Lobo et al., 2006). The study provided support for the use of FACS-array for the isolation and analysis of neurons from young and adult mice. It is not clear, however, whether dissociation of projection neurons by digestive enzymes from adult mice may induce injury to the long-range axons, and alter the gene expression profiles in the differentiated cells.

4 Isolation of neurons by laser capture microdissection: limitations of RNA amplification

Since differentiated neurons have axon-dendritic processes and rely on close contact with other cells in the brain, cells dissociated from adult tissue are not always optimal for preserving the RNA profiles. Neurons dissected directly from fresh-frozen brains would be better to represent the *in vivo* physiology. In addition, cell-type specificity may be lost in some populations of cells, either collected from whole tissues or selected by FACS sorting. For example, the hippocampus neurons considered to be homogeneous turned out to include distinct groups based on the gene expression profile (Kamme et al., 2003). Theoretically, maximum specificity for neuronal cell types may only be achieved from the analysis of single neuron.

In the past, single-neurons were obtained from micro-dissection using a needle aspiration and other manual methods. Laser capture microdissection (LCM) (Emmert-Buck et al., 1996, Böhm et al., 2005) allows selective isolation of cells under direct microscopic visualization (Espina et al., 2006). Thus, histologically-pure cell populations can be harvested directly from fresh-frozen tissue sections. As illustrated in a landmark paper by Luo et al., neighboring small and large dorsal root ganglia neurons were individually captured by LCM from Nissl-stained sections and used for the analysis of differential

gene expression (Luo et al., 1999). Retrograde labeling by tracer molecules makes projection neurons visible and guides the LCM. For example, dopaminergic (Yao et al., 2005) and motor (Cui et al., 2005) neurons have been marked by the retrograde tracers and isolated for microarray analysis. With a similar approach, intermingled long-range projection neurons were labeled and harvested separately from zebra finches by LCM (Lombardino et al., 2006). We found recently that cortical projection neurons in mice may be labeled by Retrobeads (Lumafluor Inc., USA) and isolated by LCM (Fig. 2, and JG Chen unpublished results). It is worth noting that expression of GFP cannot label neurons for the purpose of LCM, only nuclear localized GFP can guide LCM. Studies in transgenic mice with nuclear GFP that was selectively expressed in the layer V of cortex found that large pyramidal neurons in motor cortex have higher demands for ribosomal synthesis and ATP metabolism (Rossner et al., 2006).

Although LCM can isolate a neuron closely representing the *in vivo* state, only a very limited number of cells may be isolated from a frozen section by the microdissection and a typical RNA yield is less than 10 pg of RNA per cell. However, microgram RNAs is required for current platforms of microarray analysis. Thus LCM has been tightly linked to the available options to achieve reliable and accurate RNA amplification (Eberwine et al., 1992; 2001). There is a technical limitation inherent to RNA amplification, however, which is a loss of linearity for highly diluted transcripts (Sugino et al., 2006). Transcripts with lower levels of expression may be too rare to be detected with microarrays, leading to the possibility of obtaining different transcription profiles from the original cell sources. Therefore, a critical mass of cells is needed to detect rare transcripts with high-throughput technologies. Attempts to estimate this number have been reported, which indicated that several hundreds of cells were required to start the amplification with (Sugino et al., 2006; Lobo et al., 2006).

The first and frequently used method for RNA amplification is based on linear amplification of a cDNA template into a complementary RNA (cRNA) using T7 RNA polymerase. Several protocols based on the *in vitro* transcription (IVT) have been developed and are commercially available, which include: i) Arcturus RiboAmp™ system, ii) Ambion MessageAmp™, iii) Epicenter TargetAmp™. Protocols proposed by Arcturus, Ambion and Epicenter are adapted from the IVT method first described by Eberwine et al. (Eberwine et al., 1992, van Gelder et al., 1990). Although T7 based linear amplification maintains more than 90% similarity as compared to the original expression profiles (Rudnicki et al., 2004), a second round amplification with random primers is needed to obtain enough materials for microarray screening (Wilhelm et al., 2006); and this will cause the preferential amplification of 3′ end of the transcripts. The bias may not be critical to Affymetrix 3′IVT expression arrays like human 133 and mouse 430, since the probes designed in these arrays are close to the 3′ end of the transcripts. However, the IVT amplified products are obviously not suitable to the recently developed arrays that are designed for whole-transcription expression analysis, including exon 1.0 ST arrays and gene 1.0 ST arrays from Affymetrix.

To overcome the obstacle, a new RNA amplification method (called Ribo-SPIA), based on the linear isothermal amplification of double-stranded cDNA using the RNA-dependent DNA polymerase activity, has been developed (Kurn et al., 2005). The amplification is carried out by a chimeric primer with a randomized 3′ DNA portion to anneal the primer randomly across the full length of transcripts. Thus, the signal intensity ratio of the 3′ probe over the 5′ probe for the amplified transcripts can be as close to one for this method. A microarray study comparing four amplification strategies suggested that Ribo-SPIA is superior to other IVT based amplification methods (Clément-Ziza et al., 2009). The commercial product for this amplification is available from Nugen (WT-Amplification™ pico system), and has been used to perform gene expression profiling experiments coupled with LCM (Watson et al., 2008). However, vigorous testing of the amplification method is needed for its full acceptance into the research community.

The composite strategy combining LCM, RNA amplification and microarray analysis has been applied to the studies of expression abnormalities associated with neuronal disorders in humans. Pyramidal neurons in layer III were captured from schizophrenia patients by LCM and undertook two rounds of T7-based RNA amplification prior to measuring gene expression profiles by human genechips (Pietersen et al., 2009). Similarly, the neuropathological hallmarks of Alzheimer's disease (AD) were characterized by a single neuron biopsy and RNA amplification (Chow et al., 1998; Ginsberg et al., 2000, 2006; Mufson et al., 2002; Liang et al., 2008). Functionally discrete postmortem brain regions in AD-afflicted individuals were collected by LCM and analyzed by Affymetrix human microarrays. The study revealed the abnormal expressions in genes involved in tangle and plaque formation, and provided a reference data set useful for the identification of new targets of AD (Liang et al., 2008). A combination of LCM and T7-based linear amplification was also performed in the degenerating spinal motor neurons isolated from autopsied patients with sporadic amyotrophic lateral sclerosis. The gene expression profile from microarray screening, which was confirmed by real-time PCR and in situ hybridization, suggested that cell death-associated genes were upregulated in this neurodegeneration disease, while cytoskeleton/axonal transport and cell surface antigens/receptors were downregulated (Jiang et al., 2005).

5 Perspective

Since the invention of microarray technology in the last century, the high-throughput analysis of gene expression has come a long way. From the early cDNA spots on glass or nylon membranes, to genome-wide gene expression array of oligonucleotides (e.g. Affymetrix genechips), the expression of thousands of annotated genes can now be determined simultaneously. Later generations of microarrays (referred to as "tiling arrays"), which consist of probes designed to interrogate a genome systematically, irrespective of gene annotation, are helpful for the discovery of unknown transcripts (Bertone et al., 2005). Thus, microarrays has provided new understanding of complexity of gene regulation and changed the way of research from hypothesis-driven study to discovery-oriented data mining.

The previous profiling approaches are limited by the probe sets available for hybridization in genechips. The most advanced technology of high-throughput sequencing for analyzing RNA population, also known as RNA-seq, use tagged libraries of short cDNAs prepared from cellular RNA. The RNA-seq does not require prior knowledge of the sequences to be profiled, and is able to comprehensively characterize unknown transcriptomes. In addition, RNA-seq can discover splicing and single nucleotide polymorphism associated with each transcript (Marguerat and Bähler. 2010), which may be especially important to the exploration of brain development and functions (Yeo et al., 2007). Currently, researchers at Yale University are applying RNA-seq to investigate the transcriptome in human brain, across brain regions and developmental stages, in order to understand molecular mechanisms underlining the development and cognitive disorders (http://hbatlas.org/). Although selective labeling and isolation of projection neurons in humans, and the RNA amplification methods suitable to the RNA-seq remain challenges, the future advances in genomic studies are likely to transform our understanding of the complex neuronal networks built by the projection neurons and local circuit neurons.

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