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REVIEW

Microfabrication Technologies for Integrated Nucleic Acid Analysis

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In any field, the prediction of future technology is a risky venture. One need only skim through a pile of popular scientific magazines from 20 years ago to resurrect a long list of beautiful, but unimplemented, technologies, ranging from helium-filled sky cranes to weeds that exude high-grade motor oil. In the face of uncertainty, technology prediction relies on three basic ideas. New technologies are (1) driven by demand, (2) in competition with the economics of established methods, and (3) limited by the available materials and systems for designing and fabricating tools.

In basic science, few areas have witnessed technical changes of the magnitude observed recently in DNA and RNA analysis. To continue this advance, future analytical devices must balance cost and accuracy with the rapidly increasing demand for genetic information. The long-term potential of any proposed nucleic acid analysis system will be linked to the efficiency of its construction methods. Photolithographic microfabrication is a mature technology developed and optimized by the computer microprocessor industry. The modern silicon-based microprocessor is an example of a monolithic integrated system, with each device containing large numbers of compatible components formed on a single substrate. Uniform replicate devices are produced economically in large batches using photographic templates. In a similar manner, photolithographic microfabrication may provide a candidate technology to build integrated nucleic acid analysis systems having improved sample throughput, accuracy, and cost efficiency.

This paper presents a perspective of nucleic acid analysis, its compatibility with silicon microfabrication strategies, and future prospects for merging the two. Microfabricated devices for biochemical and fluidic manipulation are undergoing rapid development in many laboratories around the world (Ramsey et al. 1995; McIntyre 1996). Although an attempt to cover the range of published research efforts has been made, this is not a comprehensive review.

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The Demand for Nucleic Acid Analysis Is Essentially Unlimited

The biological and biomedical sciences, from agriculture to epidemiology to taxonomy, have enthusiastically incorporated DNA and RNA analysis into their experimental domains. Extraction of nucleic acid-based information from biological samples has become accessible worldwide, even for laboratories with limited resources and basic technical knowledge. Rather than satisfying demand, this initial burst of information has stimulated scientific appetites for an even greater breadth and depth of data (Olson 1993). For example, in genetics, variations in DNA sequence provide an enormous resource for the analysis of inheritance, mutation, and disease (Botstein et al. 1980; Cooper and Clayton 1988; Bowcock et al. 1991; Tanksley 1993). Similarly, in developmental biology, the dynamic expression of RNAs across tissue types and over time can address fundamental questions of pattern formation and organogenesis (Ringwald et al. 1994; Schena et al. 1995). Within any experimental program, analytical demand is constantly extended by the need for contiguous information, broader surveys of individuals, multiple-tissue analyses, or replicate samples.

In the field of medicine, analytical demand continues to increase as new knowledge is gained by research scientists. Because clinical science is concerned with what is wrong with a "particular" human, knowledge of individual variation is essential to clinic-level diagnosis. As a consequence, human genetic analysis is rapidly developing into the study of hundreds to thousands of individuals in populations (Davies et al. 1994; Lander and Schork 1994). Pathogen isolate analysis also will be increasingly

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important for accurate clinical diagnosis and treatment (Seillier-Moiseiwitsch et al. 1994). At some point in the future, clinical applications of genetic knowledge may impact the majority of individuals worldwide.

If made sufficiently inexpensive, clinical, environmental, and agricultural nucleic acid analyses may easily increase to hundreds of millions of individual reactions per year.

Nucleic Acid Analysis Is Consistent and Amenable to Automation

The biochemistries for extracting nucleic acid information are relatively uniform. Samples are aqueous and within a defined range of volume (1 ml–0.1 μl), temperature (0°C–100°C), and pH (6–10). Processing mechanics involve volume measurement, liquid mixing, temperature control, molecular weight separation, and molecular detection. Throughout the processing chain, samples must remain distinct and free of external contaminants. Most reactions are defined by the mixing of nucleic acid samples with substrate-specific enzymes under controlled temperature. Often, enzymatic and thermal processing reactions are linked in series. Alternatively, a sample may be divided into subsamples, with each division undergoing a unique set of process steps. The end products of the reactions are characterized either by hybridization to known nucleic acid sequences or electrophoretic determination of polymer length. Although generally uniform, some variation occurs among the specific analysis methods—for example, in complementary-strand binding parameters, polymer size range, electrophoretic resolution, and product detection sensitivity.

The consistent processing characteristics of nucleic acids allow for "industrial-style" sample handling. Such effort is often economically favorable, as the reduced cost per unit of data can outweigh the capital investment in complex, high-throughput machines. The most significant recent improvements in sample handling result from bundling groups of reaction tubes into standardized rectangular arrays (8 × 12 or 16 × 24 places). The tube arrays are matched with robotic handling and pipetting equipment having the same format (Uber et al. 1991; Watson et al. 1993). A second major advance has occurred in electrophoretic analysis. The introduction of automated gel readers and loaders has reduced labor costs for electrophoresis as well as improving data detection, handling, and error rates (Hunkapillar et al. 1991; Ansorge et al. 1992; Wilson et al. 1994).

Not surprisingly, high-throughput sample analysis based on linking robotic liquid handling equipment and automated electrophoresis has been successful in reducing costs (Fraser et al. 1995). The incremental advances based on these technologies, however, may be approaching their limit. As an example, although clearly an improvement over individual reaction tubes, molded plastic tube arrays have a lower size limit and packing density. The factors limiting conventional microtube sample handling include (1) surface evaporation in open vessels, (2) fabrication precision of plasticware, and (3) three-dimensional location accuracy of robotic pipetting equipment. Additionally, the sample processing and sample analysis instruments have been designed to stand alone, resulting in distinct steps in a processing stream rather than a fully integrated system.

The capital investment in specialized automated equipment, especially dedicated robotic devices, can be significant (Adams et al. 1994; Hall et al. 1996). As a result, the implementation of these technologies, although effective, may remain restricted to well-funded research laboratories or centers.

Monolithic Integrated Devices

Ideally, new systems for nucleic acid analysis will address several of the limitations in current technology, including cost inefficiencies. Large-scale nucleic acid testing will demand instruments with technical simplicity, assay reproducibility, and inherent quality control, as well as high sample throughput. Many of these desired functions are consistent with fabrication technologies for "monolithic integrated" devices.

Systems that use interchangeable units to perform diverse processing steps on a single, unified platform can be described as monolithic and integrated. Integrated systems use modular components having standardized connections and a consistent format. Each component performs a unique, fundamental function and meets preset criteria for construction. Consequently, during the design stage individual components are assumed to be compatible in the final device.

From the design and testing standpoint, integrated systems are extremely powerful. Once each component is established, the units can be linked in a logical pattern to accomplish a specific task. Integrated systems are remarkably adaptable to changing demand, as complex devices can be modified rapidly by rearranging existing components. Design

engineers can operate using a common "language" that includes agreement on interconnection methods, component response characteristics, and device package formats (Petersen 1982; Kovacs et al. 1996). In this environment, improvements in individual components can be pursued by independent research teams, with each team assured of component reintegration into the complete system.

Monolithic refers to the fabrication of all components into a single structure, either as sequential application of layers to a substrate or by surface bonding of matched substrates. In monolithic construction, sequential fabrication steps must remain compatible with each other, as each new layer is built on a completed underlying framework. A successful example of a monolithic device is Polaroid instant film. In Polaroid film, three separate steps of traditional photography—light capture, chemical conversion of the captured light into an image, and permanent presentation of the image—are all contained within a single, multilayered, plastic package. Monolithic fabrication can support powerful technologies, as it has the potential for device uniformity, sealed systems, and reduced operator interaction. An ideal technology for nucleic acid analysis would incorporate the simplicity and adaptability of integrated devices into an inexpensive, standalone monolithic package.

Silicon-Based Fabrication of Monolithic Integrated Devices

Silicon fabrication is a mature technology for making complex electronic circuits, such as computer microprocessors, and is a superb example of a monolithic integrated construction system (Jaeger 1993). Within a silicon microprocessor individual electronic circuitry components—transistors, diodes, capacitors, resistors—are fabricated directly on a crystalline silicon substrate (or wafer). Hundreds or thousands of copies of a particular component are made simultaneously across the entire wafer surface. The components are made by sequential deposition, ion implantation, or etching of thin layer materials in defined patterns. Materials that are commonly used include silicon oxide, silicon nitride, and various metals and alloys. The mechanical and electrical properties of the thin layers are known and reproducible. During the fabrication process each layering step is chosen to be compatible with those occurring before and after. Ultimately, extremely complex patterns of components can be built using object line widths of less than 1 μm (Wolf and Tauber 1986).

The technology of silicon fabrication is essentially a photolithographic method for making machines. Once a "template" or "stencil" pattern has been prepared, additional copies of the machines are replicated at minimal cost and effort. The density of components is limited by line-width considerations and the designing abilities of the engineers. Complete devices are made in batches and can often exceed thousands of replicates per fabrication run. Additionally, silicon fabrication has benefited from massive industrial commitment over the past 20 years. The characteristics of the fabrication steps are known and have been incorporated into intelligent design software or computer-aided design and manufacturing packages (CAD/CAM).

Photolithographic fabrication of silicon has several characteristics of a process compatible with molecular biology instrumentation. First, high-throughput nucleic acid analysis is a repetitive task, requiring numerous identical devices with uniform characteristics. Second, the "mix and match" flexibility of integrated designs can accommodate rapid changes in sample handling procedures. Also, photolithographic methods give consistent reproduction with low numbers of failed devices. Table 1 lists several of the positive and negative aspects of developing silicon devices for nucleic acid analysis.

In addition to conventional circuitry components, a wide range of devices can be made by photolithographic patterning on silicon or glass substrates. Recently developed silicon devices can sense temperature, stress, magnetic fields, and radiation, as well as optic and acoustic signals. Because these devices interact with mechanical variables, specialized machining techniques have been developed to build silicon microstructures accurately (Petersen 1982; Nakagawa et al. 1990). Individual components such as pumps, valves, fluid channels, and chromatographic and liquid electrophoresis separation systems have been built as research items. Table 2 gives a summary of published devices having potential application to nucleic acid analysis. Salient among these are sophisticated systems for capillary electrophoresis of DNA, sample loading to electrophoresis columns, and controlled enzymatic reaction chambers. In each case, the individual components have solved challenging design problems and have provided valuable test platforms for understanding miniaturized biochemistry, sample handling, and product detection.

Two items must be mentioned concerning these existing devices. First, while sharing many aspects in their production, silicon-based and glass-based

Table 1. Silicon Device Characteristics

Positive aspects	Negative aspects
Known physical and electrical properties	Biocompatibility unknown for many materials
Large variety of machining technologies	Construction primarily limited to planar devices
Photolithographic reproduction	Reduced size requires increased sensitivity of detectors
Computer-aided design software	Few demonstrated fluidic devices
Chemical synthesis on Si or SiO surfaces	Fragile structures
Batch fabrication	Size reduction of many biochemistries unknown
Reduced reagent costs by reduced volumes	Cost savings only with large numbers of identical units
Integration of sensors and control circuitry	Devices not at human operator size scale

microfabricated structures have significant differences and have not been shown to be fully integratable. In particular, the high voltages used during capillary electrophoresis on glass structures pose serious problems for closely associated silicon semiconductor structures. Second, silicon or glass photolithographic construction alone does not equate with full-fledged system integration. The development of a single microfabricated component, although useful, still requires additional matched components to provide complete processing devices.

First-Generation Devices for Nucleic Acid Analysis

Although a common vision of several research groups, complete silicon-fabricated nucleic acids analysis systems are still at the earliest stages of development. One promising device is an integrated glass system combining DNA restriction enzyme digestion and capillary electrophoresis (Jacobson and Ramsey 1996). An alternative format using high-density arrays of synthesized oligodeoxynucleotides has been demonstrated as a DNA sequence detector (Fodor et al. 1993; Hacia et al. 1996) and is available commercially.

A multicomponent, integrated device is being prepared by a collaborative group at the University of Michigan and includes the elements in Figure 1. The sections in the diagram represent fundamental process components fabricated on silicon. Sample and reagent are injected into the device through entry ports or reservoirs (A), and individual liquid drops are pumped through channels (B) to a thermally controlled reactor, where mixing and restriction enzyme digestion or DNA amplification occurs (C). To date, a significant effort has focused on methods for movement of individual drops within

Table 2. Microfabricated Components with Application to Nucleic Acid Analysis

Component	References
Gel electrophoresis	Zeineh and Zeineh (1990); Heller and Tullis (1992); Effenhauser et al. (1994); Woolley and Mathies (1994, 1995); Webster et al. (1996)
Capillary electrophoresis	Manz et al. (1992, 1995); Effenhauser et al. (1993); Fan and Harrison (1994); Jacobsen et al. (1994a,b); Jacobson and Ramsey (1995); Ocvirk et al. (1995); von Heeren et al. (1996)
Synthetic oligonucleotide arrays	Fodor et al. (1993); Schena et al. (1995); Hacia et al. (1996)
Continuous flow pumps	Lintel (1988); Esashi et al. (1989); Matsumoto and Colgate (1990; Nakagawa et al. (1990); Pfahler et al. (1990); Smits (1990); Wilding et al. (1994); Olsson et al. (1995)
Discrete drop pumps	Burns et al. (1996)
Enzymatic reaction chambers	Northrup et al. (1994); Wilding et al. (1994b); Cheng et al. (1996)
Optical/radiation detectors	Belau et al. (1983); Wouters and van Sprakelaar (1993); Webster et al. (1996)
Multicomponent systems	Harrison et al. (1992, 1995); Northrup et al. (1994); Jacobson and Ramsey (1996)

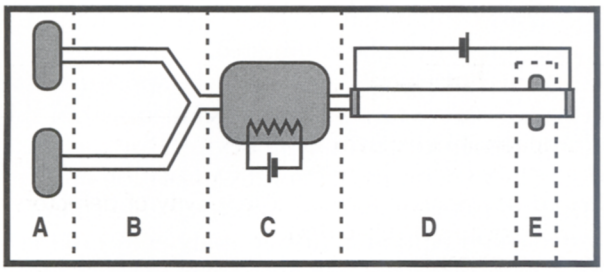

Figure 1 Schematic of a simple integrated system for DNA amplification and product detection.

the silicon channel format. The drop movement is controlled by simple heating, as differential heating of the two ends of a drop in a capillary tube produces motion (i.e., a thermocapillary pump; Burns et al. 1996). After reaction, the biochemical products are moved by the same pumping method to an electrophoresis channel (D), where DNA migration data are collected by an integral photodiode (E). The output data are sent off the integrated device for signal processing and DNA band identification.

Additional components can be added to the system, provided the channel connection format remains consistent. Under development are low temperature polymer-based channels (Fig. 2A,B; F. Man, D. Jones, and C. Mastrangelo, unpubl.), Peltier cooling surfaces, optical sensors, and ultraviolet filters for continuous spectrophotometric analysis (B.

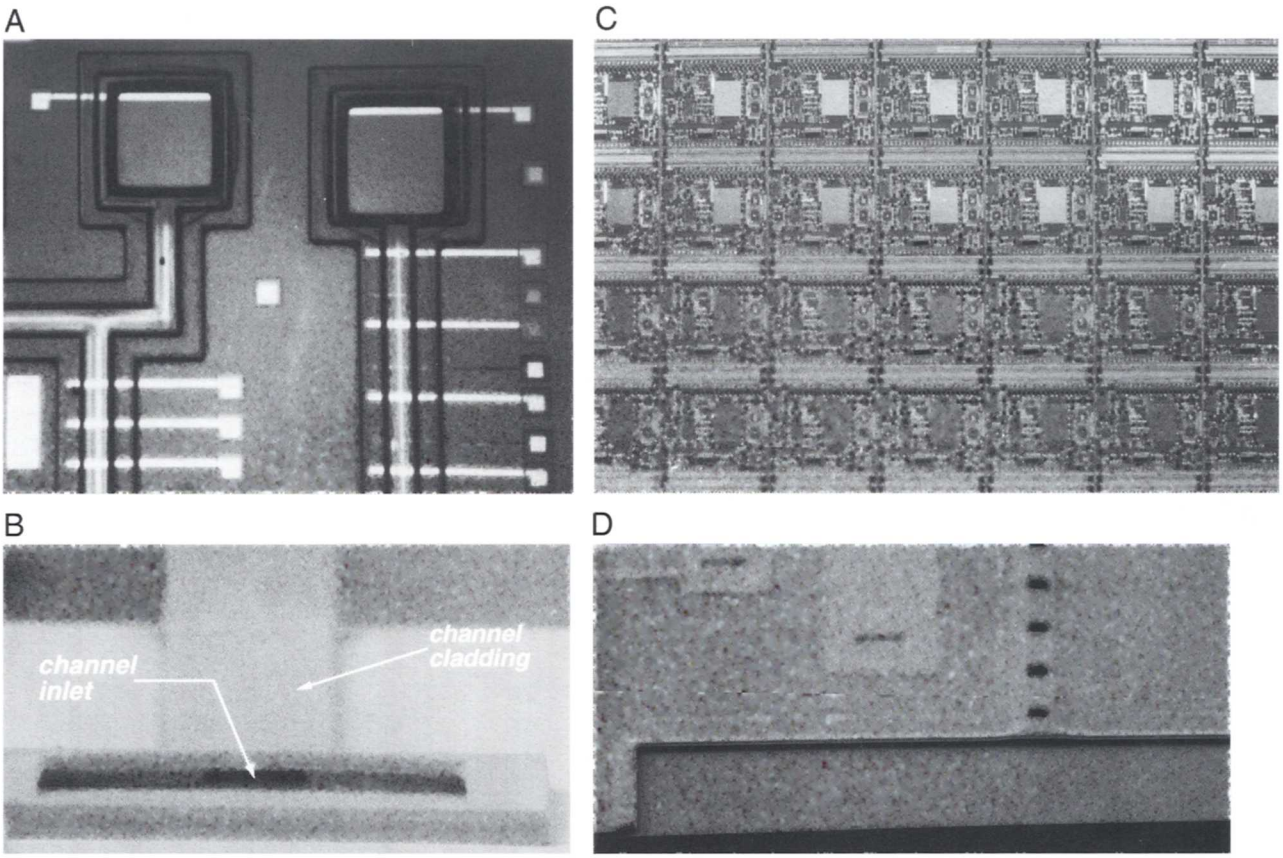

Figure 2 (*A*) Optical photograph of a silicon wafer with two liquid reservoirs (1000 × 1000 × 25 μm), each connected to a 200 × 25-μm channel. The channel and reservoir structures are made of a low-temperature polymer (p-xylylene) using a sacrificial etch procedure. Platinum electrophoresis electrodes are visible within each reservoir. Additional platinum surface electrodes and photodiode detectors have been placed beneath the channels. (*B*) Scanning electron micrograph of a p-xylylene reservoir and channel similar to those shown in *A*. The interior channel opening is ~100 × 25 μm. (*C*) Optical photograph overview of a silicon wafer with several 1.25 × 1.25-cm DNA processing units. Within each unit are ~30 different electrophoresis channel and photodiode configurations. (*D*) Scanning electron micrograph of an example electrophoresis channel device from the wafer in *C*. The wafer has been cleaved within one electrode chamber reservoir to provide a view of the electrophoresis channel. Other components visible to the *left* are connections for the integral electrodes and photodiodes. The channels are made using a silicon nitride sacrificial etch process and have an interior cross section of 40 × 5 μm.

Johnson and M. Burns, unpubl.). Additional effort will be essential to establish precise temperature and drop-motion control and for the design of thermally isolated reaction chambers.

Using photolithographic fabrication, a silicon wafer having >30 different electrophoresis channels and integral detectors within a 1.25 × 1.25-cm unit area has been developed. The devices provide a reproducible test platform for understanding gel electrophoresis at a micron-size scale (Webster et al. 1996). The silicon components have provided considerable preliminary information on channel and detector formats. Figure 2C shows the overall arrangement of components across several unit repetitions on a single wafer. Figure 2D is a scanning electron micrograph of a portion of one of the components, including the opening to one electrophoresis channel, an associated photodiode, buffer reservoir, and external contact points for electronic control.

Future Prospects

Although success for these devices is not assured, silicon microfabrication as a tool-making technology is compatible with many aspects of nucleic acid analysis. First, the economics of silicon production rewards large-scale batch construction of devices. Photolithographic fabrication becomes less expensive per item as the number of duplicate items increases. Second, many of the diverse components that must interact with nucleic acid samples during processing have the potential to be made on silicon. The individual components can be designed to replicate fundamental, robust biochemistry processes that occur in a conventional laboratory—but on a nanoliter scale. Such processes include liquid drop pumping, volume measurements, sample mixing, optical detection of fluorescent molecules, heating and cooling, and electrophoresis. Third, silicon-based integrated systems are compatible with parallel processing of multiple samples. Because each individual sample is restricted to a dedicated set of components, the labor costs, bottlenecks, and cross-contamination that occur in nonintegrated systems can be eliminated.

A true "black box" system can be approached as more laboratory functions are included within the monolithic integrated silicon device. One such task would be to perform DNA or RNA isolation and purification prior to analysis. Another, more complex design could support multistep analyses by linking a set of tasks into a functional group or module. Once developed the modules can be replicated at multiple locations across the silicon wafer and perform consistent processing as required. Figure 3 is a schematic diagram of a multistep complex system. In this example a set of components for DNA amplification and product detection are linked as a "sequence-tagged site module." Four replicates of the module assay subsamples from a single long-range amplified product prepared in a previous reaction (Barnes 1994).

The linking of individual process steps can be expanded to increasingly complex experiments. In the ideal case, data detection, analysis, and decision making will reside within the integrated system. To achieve this, the biochemical steps may be controlled by electronic circuitry fabricated in the silicon substrate (Wouters and van Sprakelaar 1993). The integration of information processing and sample handling on a single platform has the potential to significantly reduce human interaction. At this stage in development the devices will have

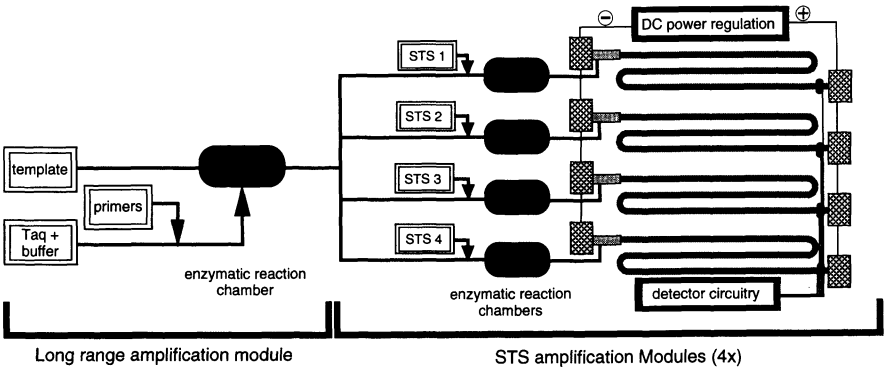

Figure 3 Schematic diagram of an integrated device for multiple amplification analysis. The original template DNA sample is introduced at the *left* and is amplified in a reaction chamber. The product of the reaction is divided into four subsamples. Separate sequence-tagged site (STS) amplification reactions are performed on each subsample and are analyzed by gel electrophoresis. The amplified products are observed using on-wafer detectors, and the product information is exported from the device for analysis. Sample reservoirs are shown as rectangles, reaction chambers as shaded ovals, interconnecting channels as solid lines, electrophoresis gels as shaded boxes, and detectors as small, solid ovals.

become "intelligent," not only producing data from raw materials but making decisions about conducting the tests.

A simple, portable format may bring nucleic acid analysis out of the laboratory and into the clinic or field. Such self-contained, stand-alone devices would be equivalent to biological "sensors" that give information at the site of use about living objects. Because silicon devices can be produced at central facilities, the quality of the analysis would be consistent, regardless of the nature of the individual sampling environment.

As with other well-meaning technology predictions, the adoption of integrated silicon devices for nucleic acid analysis may be premature. It is still not certain that current machining technology is sufficiently advanced for making large numbers of biologically compatible components. At a more fundamental level, silicon fabrication of integrated devices may not be cost effective relative to other nucleic acid analysis technologies. Because demand for the instruments is assured, the success or failure of silicon-based microfluidic devices may rest primarily on the relative economics of their production. Regardless of the specific devices implemented, however, only by making nucleic acid analysis inexpensive and simple will research and clinical applications receive full benefit of recent basic advances in science.

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