A microelectrode device to investigate the effect of electrical stimulation on neural progenitor cell growth, alignment, and differentiation

by

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Signatures have been redacted for privacy

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Abstract

A polyimide-based, biocompatible microelectrode device has been designed, fabricated, and tested. The microelectrode device is multilayered, with pairs of microelectrodes positioned underneath microgrooves. The substrate has been designed to enable cells to align along the microgrooves and then be selectively stimulated using particular pairs of microelectrodes. This design might enable the substrates to be used in a conduit to replace damage optic nerves with selective stimulation of cells based on signals received from devices such as a retinal chip. The microelectrode devices also serve as model devices to investigate the effect of electrical stimulation in conjunction with various physical and chemical cues on the growth and differentiation of neural progenitor cells (NPC) *in vitro*. This device has been used to electrically and physically stimulate individual neural progenitor cells to investigate the effect of electrical stimulation on NPC growth, alignment, and differentiation. In the future this microelectrode device may be used to selectively record signals from cells.

1 Introduction

Regenerating damaged or severed nerves in the central nervous system (CNS) is difficult; some reasons include the death of many of the cells that are damaged, the fact that the cells seem to be programmed not to regenerate, and the microenvironment within the developed CNS that chemically and physically resists regeneration (Chierzi and Fawcett, 2001; Schmidt and Leach, 2003). The first generation microelectrode device that has been developed in this work is capable of incorporating many of the strategies that have been researched to overcome these factors opposing regeneration: chemical cues incorporated to counter the microenvironment that resists regeneration and/or help re-program the cells to regenerate (Takahashi et al., 1999; Chierzi and Fawcett, 2001; Miller et al., 2002; Recknor et al., 2005), grooves, physical cues, to aid in aligning the cells properly (Kawana, 1996), helping them overcome any gap between the two ends of a severed nerve, and as a physical cue to differentiation (Miller et al., 2002; Yang et al., 2004; Recknor et al., 2005), and progenitor cells seeded onto the devices to become the neurons that will aid in the regeneration process by making connections with the damaged cells and extending their axons to make connections in the proper areas of the CNS (Young et al., 2000; Song et al., 2002b; Murakami et al., 2003; Recknor et al., 2005). These progenitor cells may also aid in regeneration by releasing factors into the microenvironment that serve to protect damaged cells (Koyama et al., 1997; Longo et al., 1999; Lohmann et al., 2000; Chierzi and Fawcett, 2001; Cornish et al., 2002).

The microelectrode device was specifically developed to investigate whether the addition of electrical cues is more effective in driving neural progenitor cells to preferentially accept a neuronal fate *in vitro* than physical and chemical cues alone; a combination of cues,

when compared to a single cue, has been shown to be more effective at affecting the release of biochemical factors from cells which may affect differentiation (Sontag, 2001; Miller et al., 2002; Recknor et al., 2005). This microelectrode device will also allow for the recording of cells during differentiation which produces the ability to ascertain whether neurons that are formed are functional. Future microelectrode devices will incorporate all the features of this first generation device (substrate flexibility, grooves, microelectrodes, and biocompatibility) and will be capable of *in vivo* transplantation. One day this device may be used to interface with a retinal chip; the chip receives visual input and could directly relay this information to a microelectrode device, which could then stimulate individual cells in the optic nerve based on the visual input received. Figure 1 is a conceptual drawing showing how this might look.

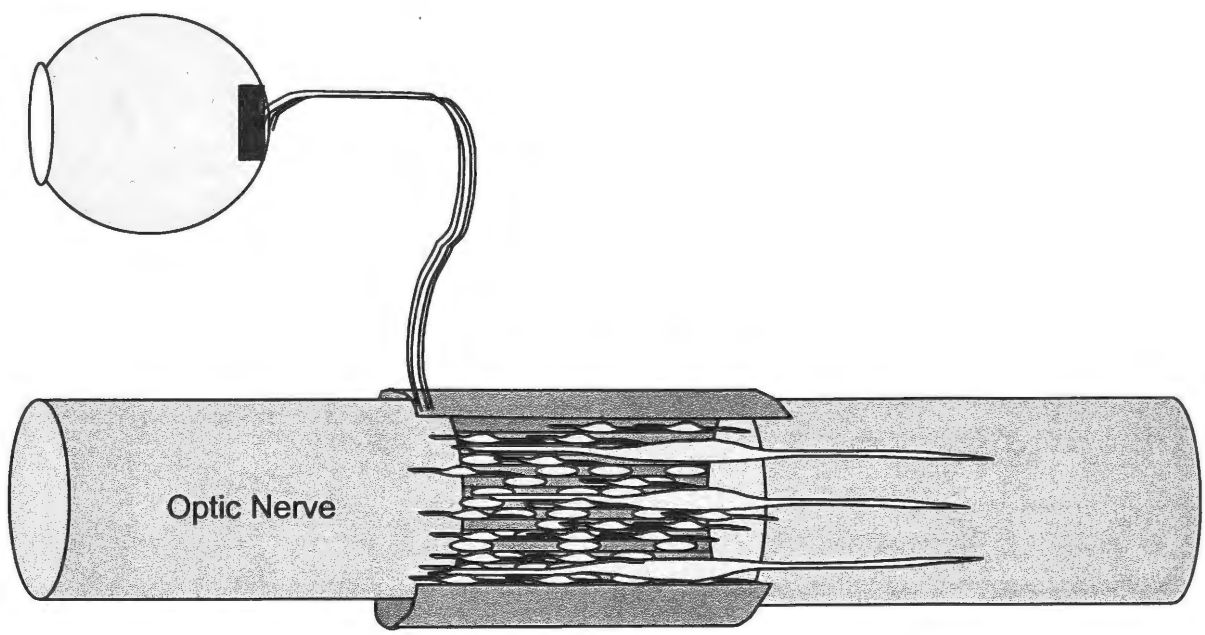


Figure 1: Conceptual drawing of how a future microelectrode device might look *in vivo*

This *in vivo* device can be tested in the optic nerve in the future because it is widely accepted as a good model for CNS regeneration studies; it is easily accessible for *in vivo* experiments and the functional recovery of the nerve can be tested (Chierzi and Fawcett, 2001).

3 Literature Review

Regeneration of the CNS is a multifaceted problem; there must be cells present in an environment permissible to regeneration that will extend axons and re-establish connections in the proper regions.

3.1 CNS Regeneration Strategies

The first of these challenges is to make sure that cells are present near the damaged area. There has been some success in rescuing the resident cells that are damaged during transection of the nerve. These cells have been rescued by the introduction of neurotrophins (chemicals that control and stimulate neurogenesis) such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), and the cytokine ciliary neurotrophic factor (CNTF), as well as chemicals that have a secondary effect on the cells, such as using chemicals that increase cellular levels of the second messenger cyclic adenosine monophosphate (cAMP) (Chierzi and Fawcett, 2001). Rescuing cells has also been accomplished by the addition of tissue or cells to the damaged area (Sauve et al., 1995; Wang et al., 2002; Murakami et al., 2003), it is thought that these donor cells secrete some of the previously mentioned factors that contributed to cell survival. A third method of rescuing damaged cells has been the application of electrical, magnetic, or electromagnetic stimulation to damaged nerves (Sisken, 1992; Sisken et al., 1993; Walker et al., 1994; Arias-Carrion et al., 2004); once again the stimulation is thought to induce the release of many factors including NGF (Koyama et al., 1997; Sontag, 2001).

Instead of relying on the resident cells as the sole means of regeneration, some researchers have introduced cells or tissue in a damaged area to supplement the possibly

small number of host cells present (So and Aguayo, 1985; Sauve et al., 1995; Young et al., 2000; Wang et al., 2002; Murakami et al., 2003). These donor cells are expected to regenerate and make connections in the host tissue. As long as these cells do not interfere with the host cell regeneration (i.e. there is no host rejection response), this method is a good combination of both approaches.

Once the cells are present at the site of damage, it is critical that the cells are capable of regeneration. Either the cells introduced must be capable of extending axons to the brain or the host cells must be re-programmed to regenerate. Stem cells under the right conditions have been shown to differentiate into neurons capable of extending axons and making functional connections (Ray et al., 1993; Gage et al., 1995; Takahashi et al., 1999; Young et al., 2000; Murakami et al., 2003; Silva et al., 2004; Recknor et al., 2005). Coercing the host cells into a regenerative state is the other option. Electrical stimulation has been used to elicit a regenerative response in both peripheral and optic nerves (Politis et al., 1988; Sisken, 1992; Sisken et al., 1993; Walker et al., 1994; Longo et al., 1999; Chierzi and Fawcett, 2001). The introduction of tissue or cells capable of regeneration has also proven somewhat effective in causing regeneration to occur, but whether these donor cells persuade the host cells to regenerate or merely create an environment suitable for regeneration to occur is unclear (So and Aguayo, 1985; Sauve et al., 1995; Murakami et al., 2003).

Different aspects of nerve regeneration such as stem cells as well as electrical and physical cues are described in greater detail below as they are relevant to our approach.

3.2 Adult Hippocampal Progenitor Cells

As previously mentioned, the major aim of this project was a proof of concept device for electrically stimulating neural progenitor cells to preferentially adopt a neuronal fate. Neural progenitor cells are more committed to adopting neural cell fates than true stem cells but are still self-renewing and multipotent; typical fates of neural progenitor cells include oligodendrocytes, astrocytes, and neurons. The neural progenitor cells chosen for this project were adult hippocampal progenitor cells (AHPCs) (Gage et al., 1995; Palmer et al., 1997; Takahashi et al., 1999; Young et al., 2000; Song et al., 2002b). These cells are self-renewing and can adopt neuronal and glial fates. AHPCs can be directed to increase their adoption of a neuronal fate with the introduction of agents such as retinoic acid (Palmer et al., 1997; Takahashi et al., 1999; Song et al., 2002a), and co-culturing of AHPCs with hippocampal astrocytes (Song et al., 2002a). These newly formed neurons are functional and are capable of forming functional synapses (Song et al., 2002b). This project is the start to the investigation of the effects of electrical stimulation on this population of neural stem cells. One study has shown that AHPCs stimulated with extracellular potassium developed into a greater proportion of neurons than controls without stimulation (Deisseroth et al., 2004). This is a very promising finding for this project because one action of the extracellular potassium is to depolarize the cell. The electric stimulation performed in this project will also depolarize the AHPCs and explore one possible mechanism of controlling differentiation.

3.3 Physical and Chemical Cues

The effect on the microenvironment is important because when the cells are capable of regeneration, the microenvironment must be engineered chemically and physically to allow the regeneration-capable cells to extend axons and reestablish the original connections in the brain. Neurotrophic factors and cell adhesion molecules are a method of chemically engineering the microenvironment, which has had successes in promoting the extension of axons (Takahashi et al., 1999; Chierzi and Fawcett, 2001; Cornish et al., 2002; Wang et al., 2002; Recknor et al., 2005). In order to create a physical environment in which cells can freely regenerate within, polymer materials have been employed in a variety of ways; three dimensional scaffolds in which cells grow (Novikov et al., 2002; Luo and Shoichet, 2004; Silva et al., 2004; Yang et al., 2004), polymer tubes (Kanemaru et al., 2003), and polymer cylinders with parallel running microgrooves on the inside surface (Miller et al., 2002; Recknor et al., 2004; Recknor et al., 2005) have been tested, each with some success. The combination of physical and chemical cues in the form of microgrooves with laminin attached selectively to the grooves has been shown to control the growth and alignment of dorsal root ganglia.(Miller et al., 2001a; Miller et al., 2001b; Rutkowski et al., 2004) Accelerated oriented growth is one of the key factors in successful regeneration, and the microelectrode device fabricated in this work incorporated microgrooves to align the cells as well as serve as a differentiation cue (Recknor et al., 2005). The positioning of cells within microgrooves also allows for spatial control and selective stimulation of individual cells in the microgrooves as opposed to the entire population. This is also important in the eventual application of using these devices in conjunction with retinal chips.

3.4 Devices for the Electrical Stimulation of Cells

Electrically stimulating cells can be performed with a variety of devices that are grouped into two main classes: intracellular and extracellular stimulation. Intracellular stimulation is most often performed by inserting a microelectrode into a cell through the cell membrane. Voltages and currents can be measured and induced with respect to a reference electrode placed outside of the cell. One advantage of this type of stimulation is the highly sensitive nature of neurons to produce an action potential when the threshold membrane voltage is exceeded. However, this technique is limited in that only one cell can be stimulated at a time with one electrode, the cell is damaged as the microelectrode punctures the cell membrane, and the connection between the microelectrode and the cell is degraded and cannot be used for long periods of time.

Extracellular electrical stimulation is performed using two microelectrodes, both placed outside of the cell. A voltage is applied at one of the microelectrodes with respect to the other. The strength of the electric field generated is approximately equal to the voltage applied between the microelectrodes, divided by the distance between the microelectrodes – as the distance between the microelectrodes increases the strength of the electric field decreases. The nature of the electric field is determined by the shape and position of the two microelectrodes.

There are a wide variety of voltages that can be used to stimulate cells; DC (constant voltage) (Bawin et al., 1986; Bikson et al., 2004), AC (sinusoidally varying voltage) (Bawin et al., 1984; Bawin et al., 1986), square pulses (Zeck and Fromherz, 2001; Fromherz, 2003), triangular pulses, monophasic pulses (pulses with only either positive or negative

components) (Zeck and Fromherz, 2001; Fromherz, 2003), biphasic pulses (pulses with both positive and negative components, etc. (Grumet, 1994; Grumet et al., 2000). The microelectrodes can take a variety of forms including: microwires placed on or near the cells slated for stimulation (Salimi and Martin, 2004), semiconductor-based multi-microelectrode arrays (Jimbo, 1992; Sisken et al., 1993; Kawana, 1996; Borkholder, 1998; Grumet et al., 2000; van Bergen et al., 2003; Bieberich and Anthony, 2004; Nam et al., 2004), and electrically conducting polymers (Schmidt et al., 1997; Kotwal and Schmidt, 2001). The effects of electrical stimulation range from morphological changes (Schmidt et al., 1997; Kimura et al., 1998; McCaig et al., 2002; Sauer et al., 2005) and changes in the electrical activity of the cells/tissue (Bawin et al., 1984; Bawin et al., 1986; Bikson et al., 2004) to the production and release of different factors (Koyama et al., 1997; Lohmann et al., 2000; Sontag, 2001; Sauer et al., 2005).

There has also been use of magnetic fields (Blackman et al., 1993; Trabulsi et al., 1996; Arias-Carrion et al., 2004) and electromagnetic fields (Walker et al., 1994; Longo et al., 1999; Lohmann et al., 2000; McFarlane et al., 2000; Tattersall et al., 2001) to stimulate tissues and cells. These studies have found varying degrees of effects on the electrical activity of cells/tissues (Trabulsi et al., 1996; Tattersall et al., 2001).

Unlike intracellular stimulation, currently there is no standard method for extracellular electrical stimulation of cells; almost every group has their own microelectrode arrangement, parameters for stimulation, aims, and outcomes. However, while the effect of electrical stimulation on neurons has been extensively investigated, its effect on neural progenitor cell differentiation has not been investigated. Our studies have also shown that patterned substrates can play a role in AHPC differentiation (Recknor et al., 2005). The

micropatterned substrates, coupled with microelectrode stimulation, will be investigated in this work to control neural progenitor cell differentiation selectively.

The overall objective of this project is to design, fabricate, and test a first generation microelectrode device that would help to investigate the efficacy of using a combination of physical and electrical cues to stimulate AHPCs to differentiate into neurons. The first generation microelectrode device was designed to have many of the same properties that future devices will have. This would allow the confrontation with many of the fabrication problems during this first phase, and the subsequent solutions to these problems.

4 A Microelectrode Device to Investigate the Effect of Electrical Stimulation on Neural Progenitor Cell Growth, Alignment, and Differentiation

4.1 Introduction

Regenerating damaged or severed nerves in the central nervous system (CNS) is very challenging (Chierzi and Fawcett, 2001; Schmidt and Leach, 2003). Many strategies have been researched to overcome factors opposing regeneration: chemical cues can be incorporated to counter the microenvironment that resists regeneration and/or help re-program the cells to regenerate (Takahashi et al., 1999; Chierzi and Fawcett, 2001; Miller et al., 2002; Recknor et al., 2005), grooves, physical cues, can aid in aligning the cells properly (Kawana, 1996), helping them overcome any gap between the two ends of a severed nerve (Miller et al., 2002; Yang et al., 2004; Recknor et al., 2005), and progenitor cells can be implanted that will become the neurons and aid in the regeneration process by making connections with the damaged cells and extending their axons to make connections in the proper areas of the CNS (Young et al., 2000; Song et al., 2002b; Murakami et al., 2003; Recknor et al., 2005). These progenitor cells may also aid in regeneration by releasing factors into the microenvironment that serve to protect damaged cells (Koyama et al., 1997; Longo et al., 1999; Lohmann et al., 2000; Chierzi and Fawcett, 2001; Cornish et al., 2002). Combinations of various cues have been shown to have more success than individual cues (Miller et al., 2001a; Miller et al., 2001b; Sontag, 2001; Miller et al., 2002; Recknor et al., 2004; Rutkowski et al., 2004; Recknor et al., 2005)

The first generation flexible microelectrode devices described here incorporate many of the elements that have been shown to promote CNS regeneration- electrical cues, physical

and chemical cues, and adult neural progenitor cells. There have been no studies conducted to investigate the influence of electrical cues on neural stem cell differentiation and the device was designed to selectively stimulate neural progenitor cells extracellularly and to investigate whether the addition of electrical cues is more effective in driving neural progenitor cells to preferentially accept a neuronal fate *in vitro* than physical and chemical cues alone. .

4.2 Materials and Methods

Substrates were designed and fabricated to selectively stimulate cells in microgrooves using microelectrodes. The microgrooves provide physical cues and allow for spatial control and alignment of cells.

4.2.1 Microelectrode Design

Figure 2 shows the design of the first generation device along with the dimensions. Figure 3 is a map showing which contact pads are continuous with each microelectrode pair. The four grooves connected to the contact pad pairs numbered 1-18 and 43-48 have an intra-microelectrode spacing of 5 μm, and the other four grooves have an intra-microelectrode spacing of 10 μm. The limitations of the photolithography processing equipment used precluded the resolution needed to accurately pattern the four grooves with and intra-microelectrode spacing of 5 μm; these grooves were unusable for stimulation and recording however they served as an internal control for electrical stimulation in the experiments. Polymeric substrates (polystyrene and polyimide) were used to make the substrates flexible for future *in vivo* use in conduits.

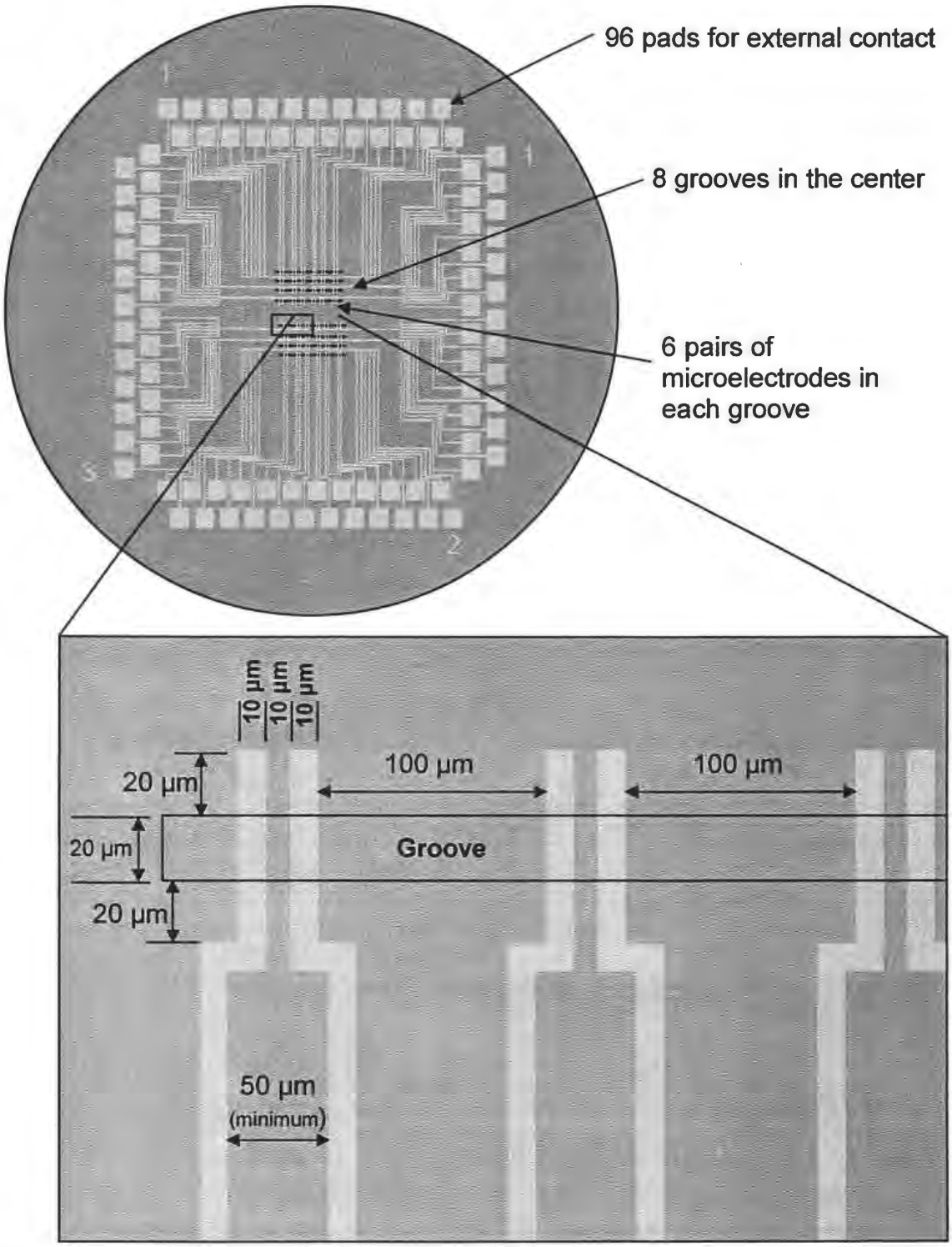


Figure 2: Microelectrode Device Layout with dimensions

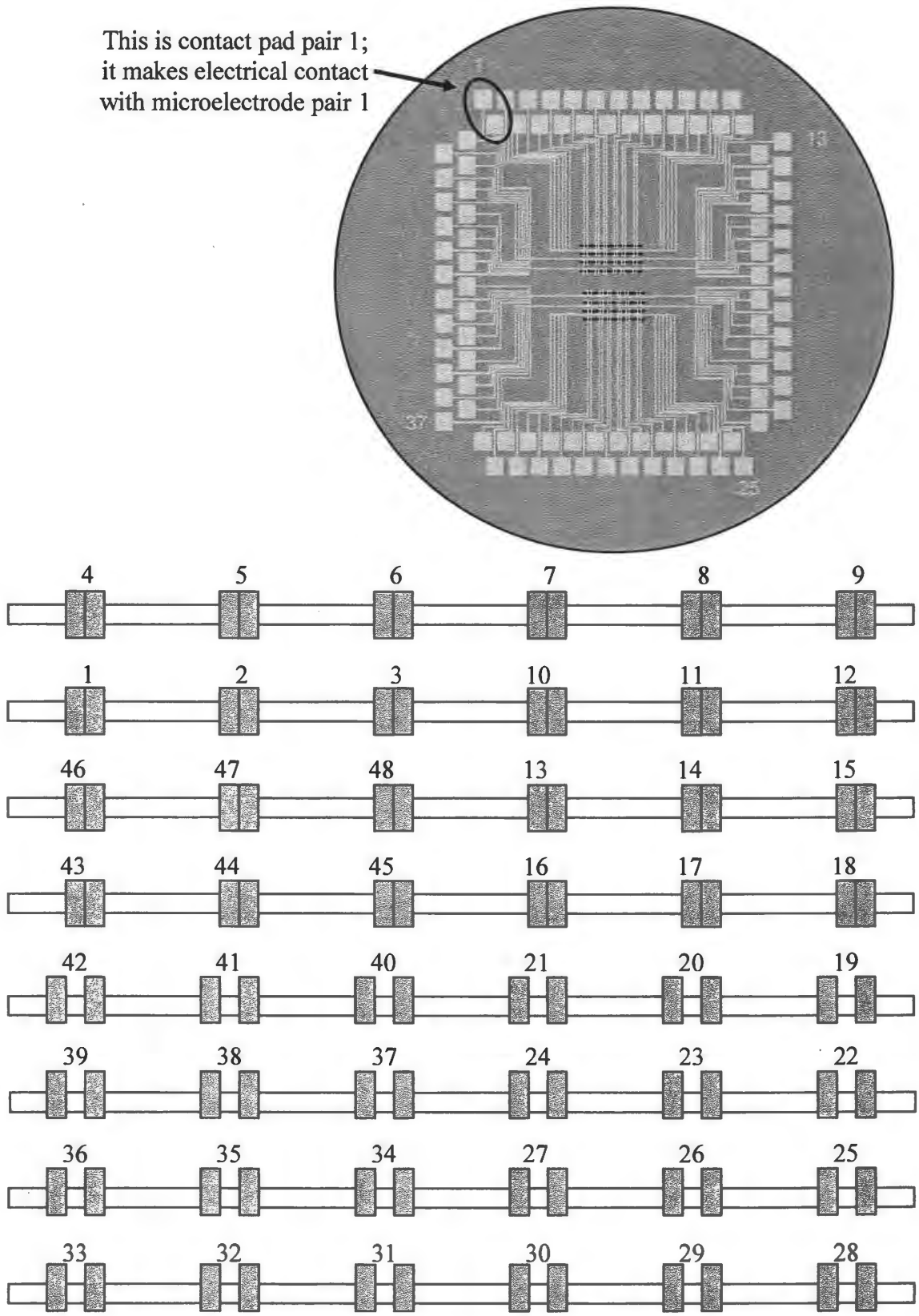


Figure 3: Microelectrode Device Layout: Contact Pad and microelectrode pair map

In order to study the effects of electrical stimulation on the differentiation of the AHPCs, a layer of gold was patterned into microelectrode pairs with a contact pad for each microelectrode of the pair. Gold was used because it is biocompatible, stable in culture media for long periods of time, malleable, readily available, and easy to process. Other microelectrode materials that were of interest were indium-tin-oxide (ITO) and black platinum. ITO has better effective microelectrode area than gold mainly because of its lower resistivity, but there are some questions about its toxicity and the brittle nature of the material therefore prevented its use in future devices that will be curled into a cylinder shape to be inserted into conduits (Borkholder, 1998). Black platinum can be added to the surface of gold microelectrodes by using an electroplating process; this improves the effective electrode area because of its lower resistivity and the increase in surface area due to the nature of its uneven surface. Black platinum is not very durable and can be removed easily (Borkholder, 1998). Future microelectrode devices may still incorporate a black platinum layer very easily if deemed necessary for recording purposes.

Because there is a need for a structural differentiation cue and also for electrical isolation between the gold wires connecting the microelectrode pairs to the contact pads, a top layer of polyimide was incorporated in which grooves were patterned. The microgroove widths of 20μm were determined based on earlier experiments that studied the effects of physical cues on AHPC alignment and differentiation (Recknor et al., 2004; Recknor et al., 2005). Originally this layer was created from polystyrene; the process to create this layer was lengthier, costlier, and less dependable than that for the photodefinable polyimide that was eventually chosen. The adhesion between this second polymer layer and the first polymer layer was also much better in the case of polyimide on polyimide, when compared

to both polystyrene on polystyrene and polystyrene on polyimide. One requirement of the photodefinable polyimide layer is that the processing must take place in a climate controlled environment; the process is both humidity and temperature dependent.

The last step in processing the microelectrode device was to remove it from the silicon wafer that it was created on, mount it for experiments, and sterilize it. The details of the fabrication process are provided below.

4.2.2 Base Polymer Layer

The bottom polymer layer allowed the creation of the entire device on a silicon wafer; this is important because most of the processing necessary to make this microelectrode device involves equipment used in integrated circuit fabrication. 3" silicon wafers were used, which were free of 3D structures that would interfere with the eventual removal of the microelectrode devices from the wafers. The base polymer layer allowed the removal of the device from the wafer once all processing was complete. Future microelectrode devices need to be flexible in order to be inserted into conduits around a damaged or severed nerve as was done in previous work; therefore the device needed to be made of a flexible polymeric material.

Two different base layers were investigated: polystyrene and polyimide. Polystyrene was initially used, as it is the polymer used most commonly in tissue culture dishes, and AHPCs grow well on polystyrene. However, after some testing with cells, polyimide was subsequently used. Polyimide is more resistant to the chemicals and temperatures used during the fabrication process than polystyrene, polyimide is biocompatible, and the polyimide did not have the adhesion problems between the two polymer layers of the

microelectrode device as did polystyrene. The polystyrene layer was created by dissolving polystyrene beads in toluene at room temperature to form a 10% polystyrene solution by weight. This solution was then filtered and directly applied to the surface of a clean silicon wafer; 4 ml of solution was cast on each 3" silicon wafer. The wafers were then covered with glass Petri dishes and dried for 24 hours under a hood.

To apply the polyimide layer, a clean 3" silicon wafer was centered on the chuck of a spinner (EC102 Headway Research Inc.). A 2 cm diameter pool of polyimide (PI2525, HD Microsystems) was then poured into the center of the wafer. The wafer was spun at 2000 rpm for 30s. The wafer was removed from the spinner chuck using flat-ended metal tweezers (36A-SA Tweezer, George Company, Fountain Valley, CA) and placed on a 130°C hotplate for 1 minute. The wafer was removed from the hotplate and placed into a wafer carrier (A82M, Entegris). When all the wafers were coated, they were cured in an oven that was set to ramp up to 240°C and held for 2.5 hours, after which the wafers were allowed to cool.

4.2.3 Microelectrode Layer

In order to stimulate cells electrically with the device, gold microelectrode pairs were patterned onto the base layer. A 0.25 μm thick gold layer was deposited directly onto the base polymer layer using a metal evaporator (TEMESCAL). Photoresist (AZ5214, AZ Electronic Materials USA Corp.) was then patterned on top of the gold using a glass/chrome mask with the desired pattern in chrome on a glass substrate and ultra-violet (UV) light from a mask aligner (Karl Suss MJB3 M4). This was followed by application of a developer (AZ312 MIF dil. 1:1.2; Clariant Corp.). The entire wafer was then submerged in a gold etch solution (4:1:40 by weight; potassium iodide (Fisher): Iodine (Fisher): de-ionized water) to

remove the gold outside of the photoresist pattern. The wafer was then rinsed in a cascade rinsing bath, dried with pressurized nitrogen, and wiped with a clean room wipe to remove any residue left from the gold etch. The photoresist layer was removed by soaking in acetone followed by a cascade rinse bath and dried with pressurized nitrogen. The microelectrode pairs were inspected using a light microscope to ensure that they were formed correctly. If the microelectrodes were not sufficiently separated in several places on one wafer, that wafer was submerged in the gold etch to remove the remaining gold and a new gold layer was deposited on this wafer to repeat the process.

4.2.4 Groove/Electrical Isolation Layer

Two different polymers were tested for the groove/electrical isolation layer: polystyrene and polyimide. The layer in either case was 4 μm in height, which resulted in 4 μm deep grooves; this allowed correlation with previous results (Recknor et al., 2005) involving only physical and chemical cues. The same 10% polystyrene solution was used as in section 4.1. A 3 cm diameter pool of this solution was placed directly on top of the patterned gold and polyimide covered wafers using a glass pipette. The wafer was then spun at 600 rpm for 30s. After spin coating, the wafer was allowed to dry for 5 minutes at room temperature. AZ 5214 photoresist was then patterned on the wafers using a mask that after developing, only left photoresist where the grooves and contact pads were located. The wafers were then coated with a 0.25 μm thick layer of aluminum in the metal evaporator. The wafers were individually placed in an acetone batch and agitated by hand for up to 10 minutes; when the areas of aluminum that were deposited on top of photoresist were

removed, the wafers were removed from the acetone bath. The wafers were placed in a cascade rinse bath and then dried with pressurized nitrogen.

Reactive ion etching (RIE) was used to etch through the polystyrene layer. Four wafers were placed in the chamber at one time and an oxygen plasma was used to etch away the polystyrene. Afterwards, the aluminum layer was removed using a PAN etch. The PAN etch is phosphoric acid, acetic acid, and nitric acid mixed with water at a ratio of 4:4:1:1 respectively. The wafers were individually placed in the PAN etch until all traces of aluminum were removed; this process usually took up to 10 minutes. This was followed by a final rinse in a cascade rinse bath and drying with pressurized nitrogen.

Creating this groove/electrical isolation layer using polyimide instead of polystyrene is much less expensive and less time consuming. A 2 cm pool of polyimide (PI2771, HD Microsystems) was poured into the center of the wafer at rest. The wafer was spun at 200 rpm and ramped up to 3500 rpm in 30s; the wafer was then held at 3500 rpm for 30s; this procedure resulted in a post-cure layer thickness of around 4 μm. The wafer was immediately placed on a 115°C hotplate to bake for 2 minutes, at which point it was removed and placed in a wafer carrier. It was very critical to control the humidity up to this point of the process in the range 40 +/- 10%. The wafers were then exposed in a mask aligner for 2 minutes and 12 seconds at a constant power intensity of 10 mW/cm^2; this was an effective dose of 1.32 J delivered to the polyimide. Each wafer was allowed to sit at room temperature for 30 minutes, allowing the crosslinking occurring within the polyimide to proceed. After crosslinking, it was placed into developer (AZ 312 MIF dil 1:1.2, Clariant) and agitated by hand for 75s. The wafer was removed from developer and placed in a cascade rinse bath for

3 minutes, then removed, dried with pressurized nitrogen, inspected under a microscope for proper groove formation and polymer removal, and placed in a wafer carrier. The substrates were examined by light microscopy and scanning electron microscopy (Hitachi S-2460N VP SEM). Any wafers with either malformed grooves, or with excess polymer in the grooves or in the contact pad areas were set aside to have the top polyimide layer removed in a 5 minute developer bath. All the acceptable wafers were placed into a curing oven (DX 300, Yamato) set to ramp up to 240°C and hold for 2 hours, followed by slow cooling of the wafers.

4.2.5 Seeding and Culturing Cells on the Device

Originally obtained from Fischer 344 rats by Dr. Fred Gage at the Salk Institute, the adult hippocampal progenitor cells (AHPCs) that have been grown on the microelectrode device were obtained from Dr. Don Sakaguchi's lab at Iowa State University. The AHPCs were maintained in media consisting of DMEM-F12 (Omega Scientific, Inc.), basic fibroblast growth factor (bFGF diluted 1:1250) (Promega Corporation), 1% N-2 supplement (Gibco), and 1.25% L-glutamine (Omega Scientific, Inc.) at 37°C, 5% CO_2, and 95% relative humidity. To prepare the devices for cell culture, they were adhered to a glass Petri dish and a small chamber was formed around the central groove area of the device using an o-ring. Sylgard 184 (Dow Corning), the adhesive, was used according to the manufacturer's directions; 1 part curing agent to 10 parts base. The microelectrode devices were placed upside down and a small drop of Sylgard 184 was placed in the center of the device. A 22mm glass coverslip, soaked in 70% ethanol and dried, was placed onto the Sylgard 184 and pressed down firmly using a ground-glass stopper. Four drops were then evenly spaced

around the perimeter of the microelectrode device, roughly 1 cm inside of the edge. The device was flipped over using tweezers and placed in the center of the inside of the lid of a 4" glass Petri dish. This was due to the height of the base of the 4" Petri dish; it was too high to fit into the device that makes contact with the microelectrode device during electrical stimulation. A 12mm o-ring, soaked in 70% ethanol and dried, was placed on a flat surface. Sylgard 184 was then placed on the top edge of the o-ring in small drops to ensure a good seal with the microelectrode device. The o-ring was picked up with tweezers and inverted before being placed around the groove area of the microelectrode device; once in place the o-ring was pressed firmly onto the microelectrode device. The device was then covered with the base of a 4" Petri dish and autoclaved for sterilization (Figure 4).

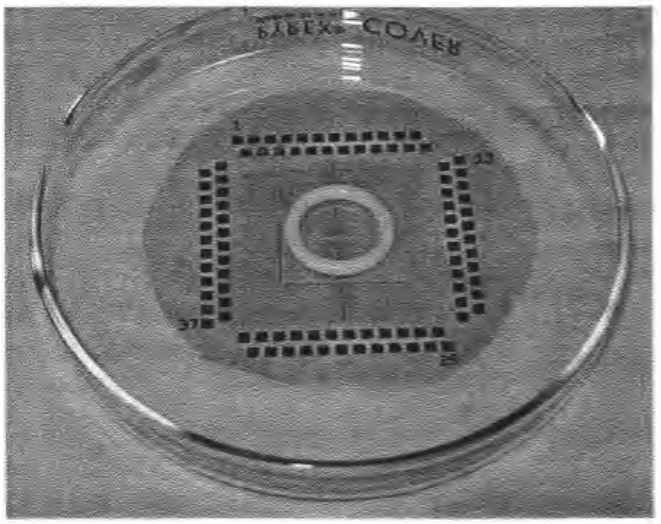


Figure 4: A completed microelectrode device

The devices were coated first with poly-L-lysine (a 0.01% solution was applied for 20-30 minutes, removed and dried at room temperature overnight) and then with laminin (a 0.01mg/mL solution was applied for 15-20 minutes, removed and dried at room temperature overnight). Cells were seeded in onto the substrates at densities of 2×10^5, 4×10^5, and 6 x

10^5 cells per microelectrode device. Cells were cultured under the conditions described above for two days on the microelectrode devices, stimulated, and then cultured for seven more days with a differentiation media (original media without bFGF); bFGF was removed from the original media after two days to promote differentiation.

4.3 Results and Discussion

The microelectrode devices were successfully fabricated using the procedures described above. Figure 4 shows a photograph of a polyimide device. Figure 5 shows SEM images of the gold microelectrodes in the microgrooves of the substrates. These images demonstrate that the device fabricated has the dimensions outlined in the device design in Figure 2.

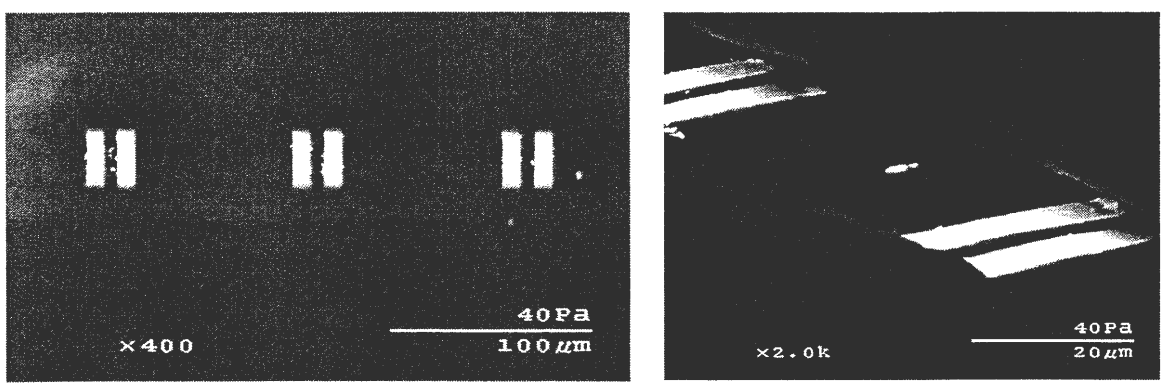


Figure 5: SEM images of the gold microelectrodes in the microgrooves of the substrates.

In order to electrically stimulate the neural progenitor cells, a connection was made between the stimulator (Grass SD9 Stimulator) and the microelectrode device through an interface device that was constructed and tested (Figure 6).

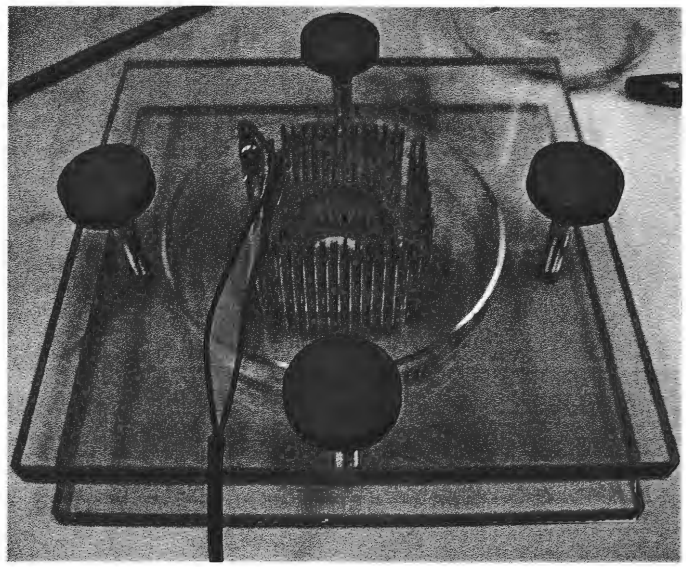
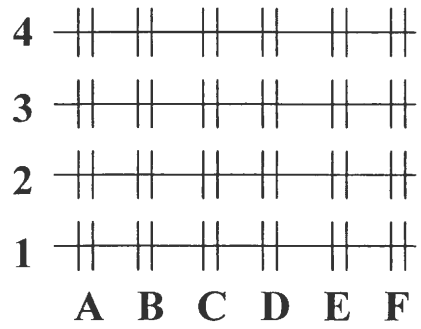


Figure 6: The interface device between the stimulator and the microelectrode device.

This interface device enabled a connection to each contact pad on the microelectrode device, and allowed simultaneous stimulation of each microelectrode pair. This interface device was used to stimulate cells during several tests of the microelectrode device. In the first experiment, which was aimed to test the effects of electrical stimulation parameters on the cells, 20,000 cells were seeded onto each microelectrode device in growth media on day 0 and were allowed to grow for two days; this was to ensure that some of the cells present would be near the microelectrodes. On day 2 the media was changed to differentiation media (original media without bFGF) and one microelectrode pair per groove was stimulated for 15 minutes with the following stimulator parameters: frequency = 100 Hz; duration = 1ms; voltage = 200 mV; biphasic pulses. The stimulation took place in a laminar flow hood in a cell culture room; during the stimulation the Petri dish covers were removed and a sterilized T25 flask cap was placed over the o-ring/groove area. The microelectrodes of the "top" four grooves of the device were not separated; therefore they received no stimulation, and served

as the internal controls for each microelectrode device. The microelectrode pair with the most cells in the vicinity was chosen for stimulation and recorded as a number followed by a letter corresponding to the microelectrode pair in Figure 7.



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25

Figure 7: The bold numbers and letters that designate the microelectrode pair that is stimulated in each groove (e.g. 2C would be the third pair from the left and the second groove from the bottom).

The microelectrode pair that was stimulated was noted so that any effects in the area could be monitored. On day 3, half of the microelectrode devices were chosen for another round of stimulation with the same parameters as day 2. When observing the electrodes that were stimulated on day 2, it was noticed that there were not as many cells present in the surrounding area; this prompted several changes to the parameters of the next experiment. The cells were allowed to grow for 7 days after the first stimulation and then were observed under a light microscope on day 9; there were not very many cells present near the groove areas. There was also no immunocytochemistry (ICC) performed for the first experiment. However, the experiment showed that the cells do grow on the devices. The next objective was to stimulate using revised parameters and to investigate the effects of this stimulation.

Experiment 2 proceeded just as experiment 1, but with the following adjustments: stimulation voltage was changed from 200 mV to 100 mV (the lower limit of the stimulator), since it was closer to a physiological voltage level; the stimulation time was changed from 15 minutes to 5 minutes in order to enable more cells to survive/remain in the microelectrode area; the seeding density was doubled to 40,000 AHPCs on each microelectrode device, also to increase the number of cells in the area of the microelectrodes; fluorescent ICC was performed to investigate the relative amounts of $TuJ1^+$ cells (early neurons).

More cells were present in the microgrooves on day 9 in the second experiment than in the first experiment. However, while observing the results of ICC of the second experiment it was observed that polyimide auto-fluoresces. The only change slated for the third experiment was the ICC method from fluorescent to DAB/nickel sulfate/HRP labeling. This will result in a permanent stain that is visible under a light microscope. To label cells using this method, AHPCs will be fixed with paraformaldehyde (Fisher) and labeled by the oxidation of diaminobenzidine (DAB; D5637-10G Sigma) and nickel sulfate (Fisher) by horseradish peroxidase (HRP); a primary antibody to the cell specific marker TuJ1 (1:750, Research Diagnostics INC, Concord, MA), which recognizes an epitope of type III β-tubulin characteristically found in early neurons, is bound with a donkey anti-mouse IgG HRP conjugated secondary antibody (Jackson Immunoresearch Labs), which will then be reacted with a DAB/nickel sulfate solution; Figure 8 depicts AHPCs labeled using this method. See Appendix B for the detailed ICC procedures used. The AHPCs of the third experiment formed large aggregates and there were too many cells on the devices. During ICC many of the cells detached; it is believed this happened due to the large number of cells present. Modifications for future experiments will include: seeding in differentiation media and

growing the cells for two days prior to stimulation and being gentler during the washes of the ICC procedure.

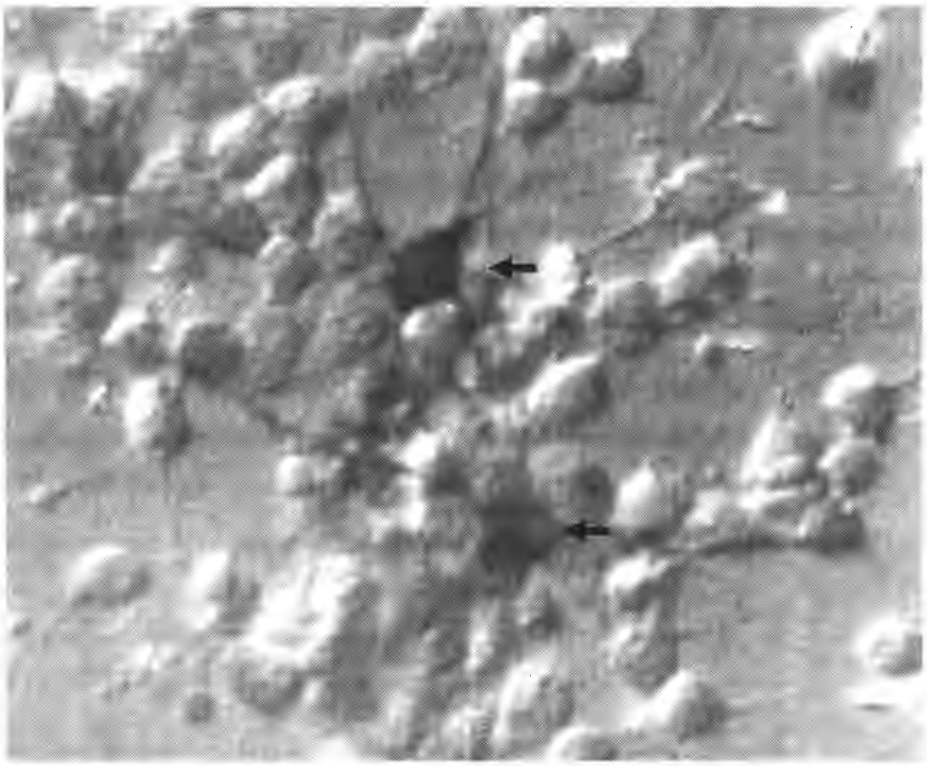

Figure 8: AHPCs labeled with permanent DAB staining of TuJ1

5 Conclusions and Future Work

A flexible polymeric microelectrode device that can provide physical, chemical, and electrical differentiation cues to neural progenitor cells was designed. A fabrication process was developed and optimized, and the new polyimide-based microelectrode devices were fabricated. These microelectrode devices were found to be capable of supporting the growth of adult hippocampal progenitor cells. The feasibility of using these devices to selectively stimulate neural progenitor cells was demonstrated. Preliminary investigations were conducted into the effect of the combination of electrical, chemical and physical stimulation on neural progenitor cell differentiation

Future work with these devices will include comprehensive testing of the electrical stimulation of cells and their effect on AHPC growth and differentiation. These devices can be used not just for stimulation but also for recording signals from cells. Further experiments are planned to stimulate cells using different cues and record responses of individual cells in the microgrooves using the microelectrodes. The additional advantage of the microgrooves is their ability to restrict mobility of cells to the grooves, thereby enabling accurate measurement of responses from specific individual cells. The eventual plans involve development of a second generation device that can be inserted into conduits and used for *in vivo* experiments.

6 Acknowledgements

I would like to thank my committee members Gary Tuttle and Tim Day, and group members Jen Recknor and Carlos Ariza, and my major professor Surya Mallapragada. I would like to thank Don Sakaguchi and Fred Gage for the AHPCs; Gary Tuttle for his guidance and assistance; and Charlie Drewes for the lending of stimulation equipment. I would also like to acknowledge the funding sources for this project: NIH and DOD.

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Appendix A: Detailed Microelectrode Device Processing Procedures

Abbreviations: Al = Aluminum, Si = Silicon, PS = Polystyrene, PR = Photoresist, PI = Polyimide, Au = Gold

Initial Preparation

Location: Anywhere

Top View **Side View**

Materials:

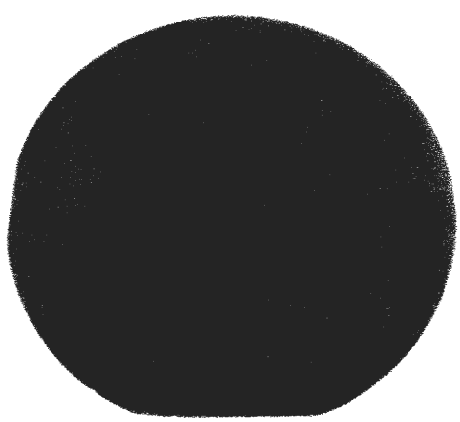
- 3" silicon wafers
- Razor blades
- Acetone squirt bottle
- Wafer carrier
- Wafer transport box
- Kim wipes or clean room wipes
- Metal tweezers

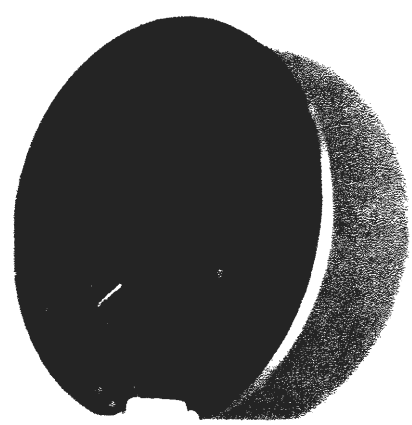
Notes:

- Starting multiples of 14 wafers works best because only 14 wafers fit into the evaporator at one time.
- Dr. Gary Tuttle can provide us scrap wafers to use. For our purposes we do not care about any processing that has been done to the wafers as long as the surfaces of the wafers are flat.
- Be gentle with the wafers they are very brittle

Procedure:

1	Scrape as much polymer, debris, etc from the surface of the wafer as possible.
2	Clean the surface of the wafers with acetone and Kim wipes or clean room wipes.
3	Place 14 wafers in each wafer carrier and place wafer carriers in transport boxes.

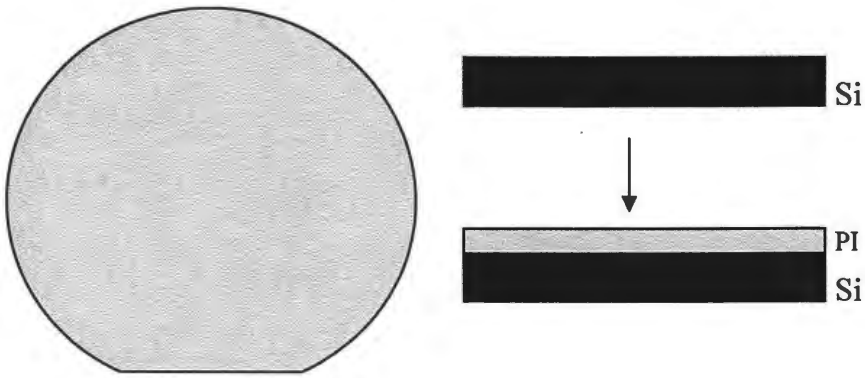




Polyimide Layer

Locations: NSF lithography lab (180C) and room 111 ASC I

Top View **Side View**



Materials:

- PI2525 Polyimide bottle
- Wafer carrier
- Wafer transport box
- Metal tweezers (2)
- Timer
- Clean Room Wipes

Equipment:

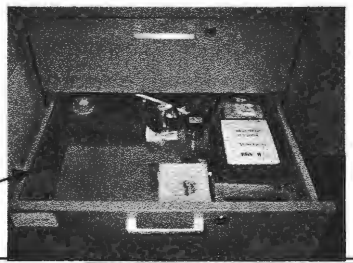
- Spinner (NSF lithography lab)
- Hot Plate (NSF lithography lab)
- Curing oven (rm 111)

Notes:

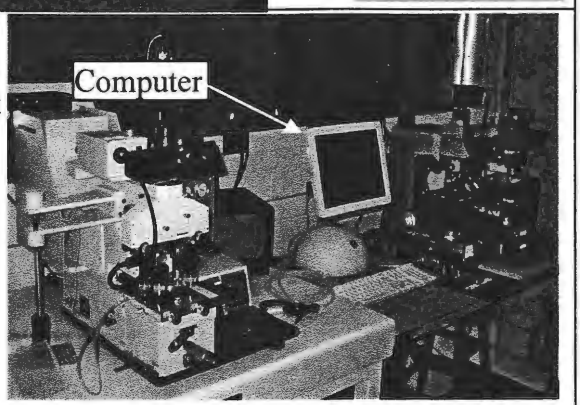
- You must let the polyimide warm up to room temperature overnight so that when you open it moisture does not condense inside the bottle. I lock it in the SKM group drawer in the NSF lithography lab overnight.
- The polyimide is VERY thick and expensive. Make sure you use the minimum necessary to spin on each wafer – make sure you get enough to cover the wafer though because the wafer is ruined if you don't cover the entire wafer.
- The spinner puts a vacuum on the wafer while it is spinning through the hole in the middle of the circular chuck – you must have a wafer on the chuck in order for the spinner to spin. The spinner is started and stopped with the foot pedal. It will stop automatically after the set spin time.

Procedure:

4 Get the PI2525 polyimide from the freezer in room 215 ASC I and put it into the locked drawer overnight to allow it to warm up to room temperature.

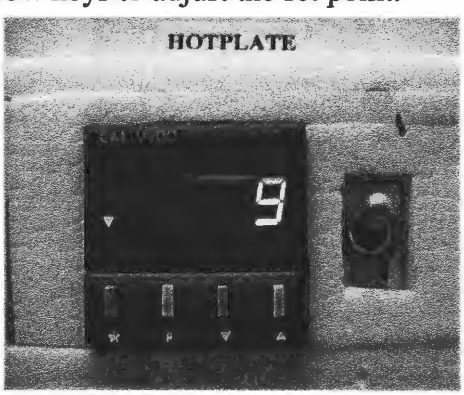


5 Log onto the spinner on the computer in the NSF lithography lab using Lab Logger. Put on a lab coat and safety goggles.

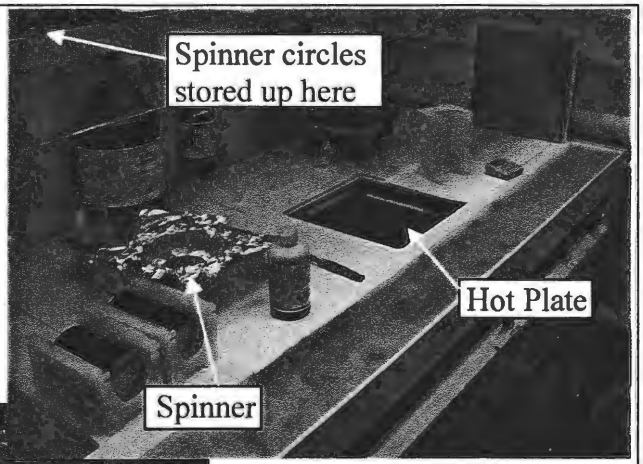


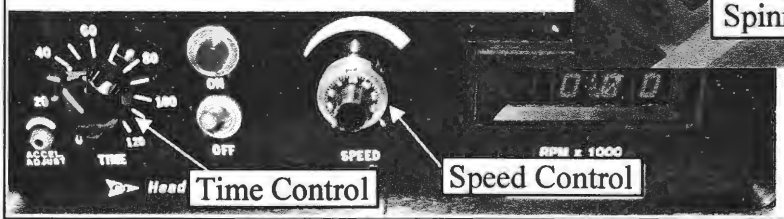
6 Turn on "Lights" and "Spinner Outlet" switches located on the front of the wet bench in the NSF Lithography lab.

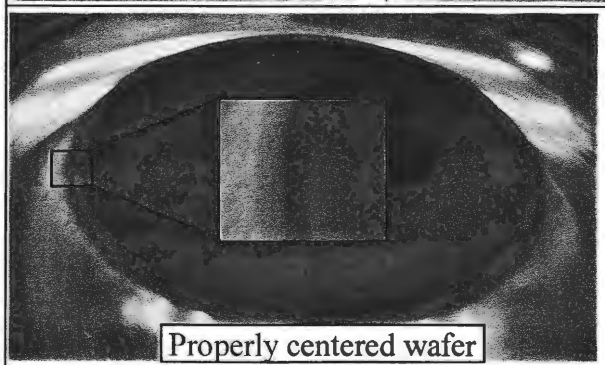
7 Turn on the hot plate switch, open the hot plate lid, and set the temperature set point to 130°C by pressing the "*" button and using the arrow keys to adjust the set point.

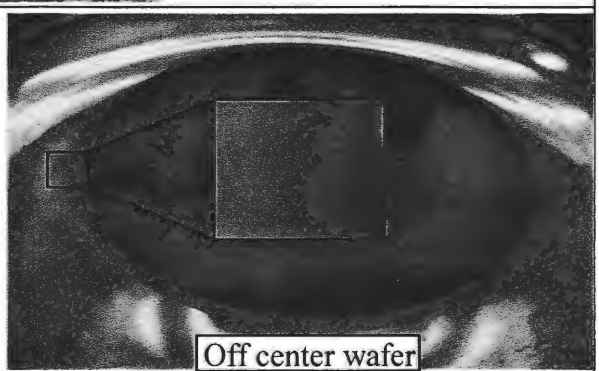


8 Cover the spinner with heavy duty aluminum foil
9 Place the large spinner circle on the spinner chuck.
10 Place a wafer in the middle of the spinner chuck then start the spinner using the foot pedal. Open the lower cabinet of the wet bench and adjust the spin speed to 2000 rpm and the spin time to 30s.



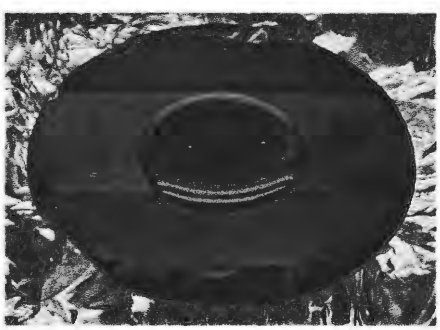




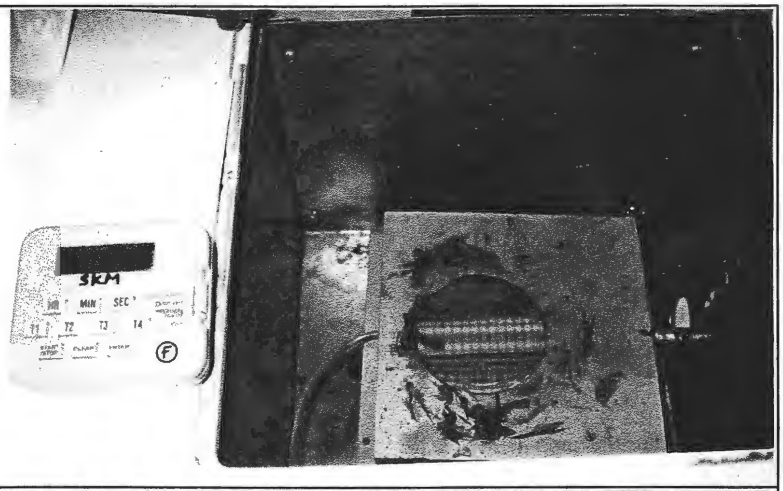


11 Wafers must be centered on the spinner circle to ensure full coverage. With a wafer in the middle of the spinner, start the spinner with the foot pedal. To ensure the wafer is in the center examine the edges of the wafer while it is spinning. The wafer will look solid around the edges while it is spinning if it is centered – if it is not centered it will appear fuzzy.

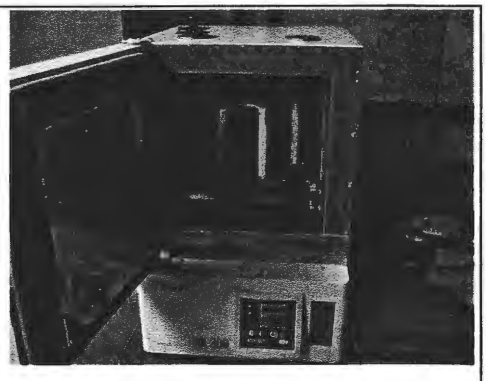
12 Pour a 2cm diameter pool of polyimide into the center of the wafer and wait a few seconds for it to spread out. Start the spinner with the foot pedal. Do not use more polyimide than is necessary to coat the wafer.



13 After the spinner has come to a stop, pick the wafer up with metal tweezers using a second pair of tweezers to hold the wafer steady. Touch as little of the wafer as possible. Place the wafer on the hot plate for 60 seconds.



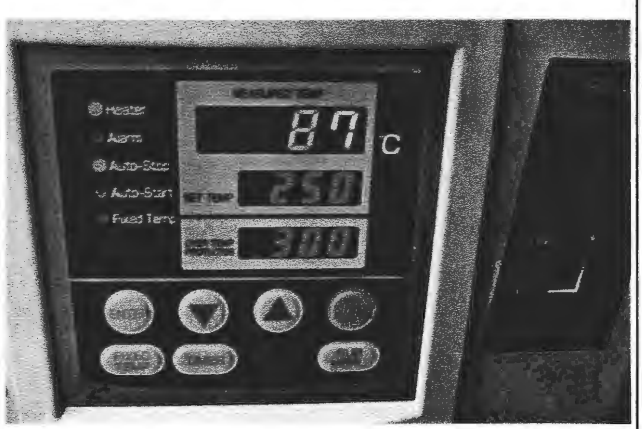
14 After 60 seconds, place the wafer into a slot of a wafer carrier standing on it's side (like a tall building) with the polyimide side of the wafer facing up. This is to ensure that the wafers do not stick to the carrier and cause burrs to form on the polyimide layer that will later cause problems while aligning the wafer with a mask.
15 Repeat steps 7-10 with all wafers.
16 Log out of the spinner using Lab Logger.
17 Place the wafer carrier into a transport box and take to room 111 ASC I.



18 Place the wafer carriers into the curing oven standing up.

19 Set the oven to go to 240°C and to turn off after 2 hours with the following procedure

- Turn Power on with black switch
- Use the arrow keys to turn the red light to the left of "Auto-Stop" on.
- Press "timer" then press "enter"
- The "set temp" display will blink, here you can set the temperature using the arrow keys. When you have set the desired temperature press the "enter" button
- Now the "set temp" display will blink again, here you can set the desired time, in hours, until the oven will shut off. When the time is set press "enter".



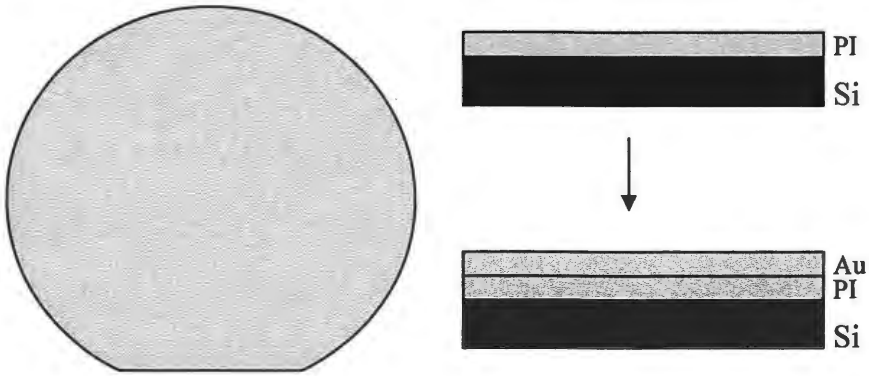
- Press "Start/Stop" twice to start the program, and the oven will automatically shut off.
- automatically shut off.

20 After the oven has cooled, remove the wafer carriers and place them into wafer transport boxes.

Gold Layer

Locations: NSF lab

Top View **Side View**

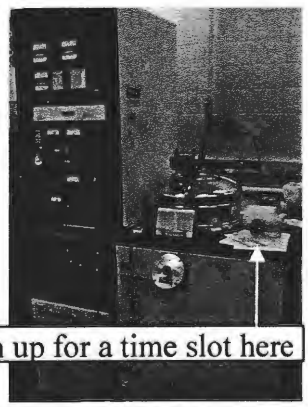


Materials:

- Gold in crucible
- Wafer transport box
- Metal tweezers (1)

Equipment:

- TEMESCAL metal evaporator (NSF lithography lab)



Notes:

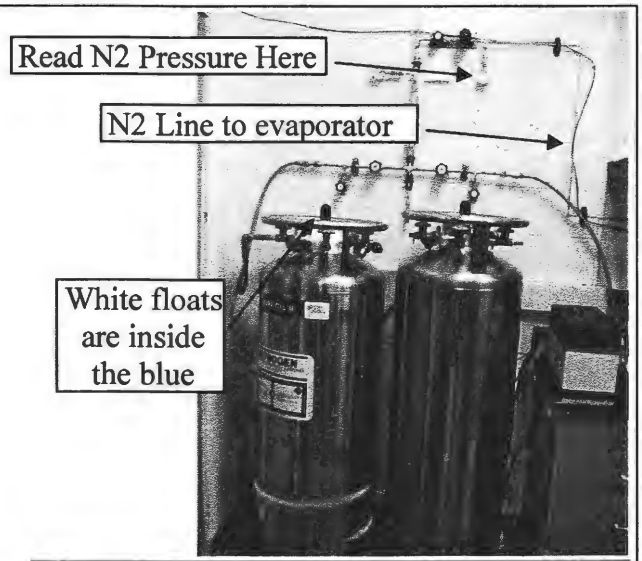
- Make sure to sign up for a time slot on the evaporator at least a day or two prior to use. Sometimes the schedule gets very busy and it may not be available for several days. If the EE 431 Fabrication Class is in session it will be impossible to use the machine during the week the class does Aluminum evaporations.
- Check the crystal health prior to pumping evaporator chamber down.
- Make sure to wear gloves when loading and unloading things from the evaporator chamber. Oils from your hands can increase the pump down time of the chamber.

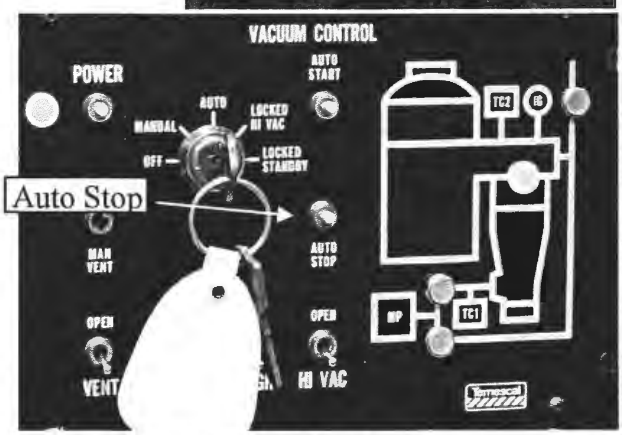
Procedure:

21 Log onto the evaporator on the computer in the NSF lab using Lab Logger. Put on a lab coat and safety goggles

22 Open the "Evaporator Log" in the "NSF Lab Stuff" folder on the computer in the NSF Lab (if there is more than one evaporator log file, open the most recently modified version). Fill in the next line available in the file with your evaporation parameters.

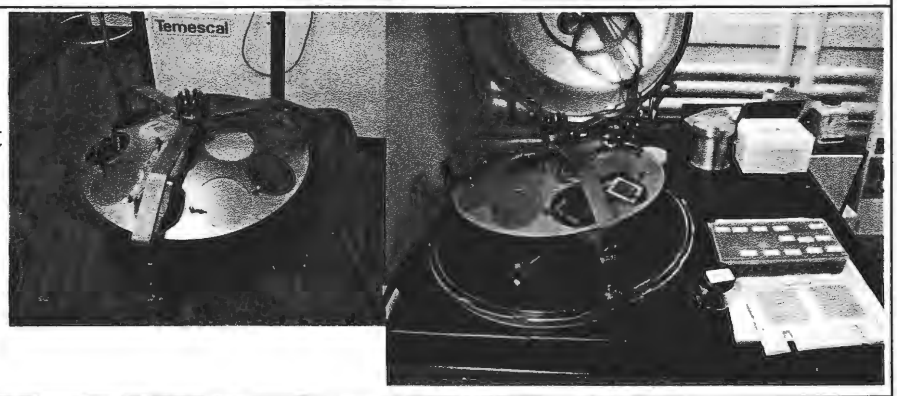
23 Make sure there is plenty of nitrogen in the dewers in the NSF Lab by checking to see that the pressure in the line is above zero and the white floats in the dewers are somewhere above the empty mark.



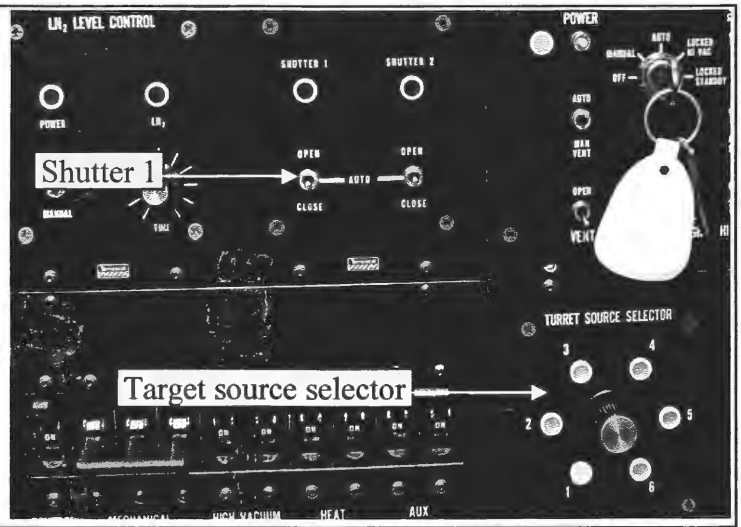


Press the "Auto Stop" button to pressurize the chamber.

24 Open the lid and remove the wafer holder unit, place it on the table.



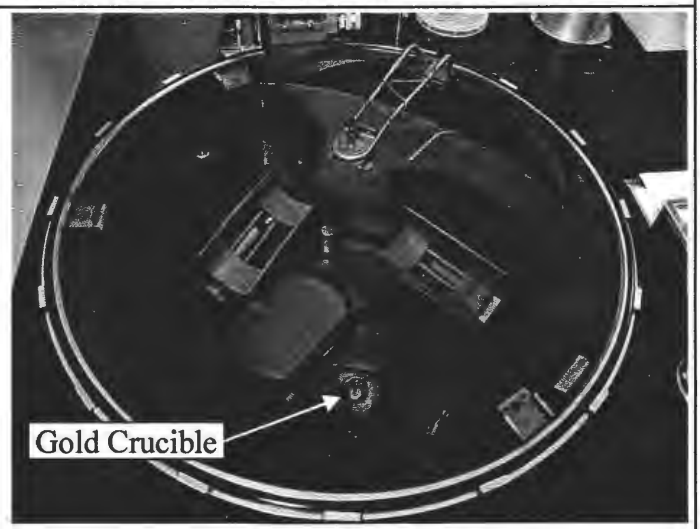
25 Open "Shutter 1" by flipping the switch up. Select crucible 1 with the lighted target source selector.



26 Remove the gold along with its crucible from the evaporator chamber and place it in the crucible box on top of the evaporator in a slot labeled Au.

27 Place the SKM group gold and crucible in the crucible 1 spot. Close shutter 1 by placing the switch in the middle "Auto" position.

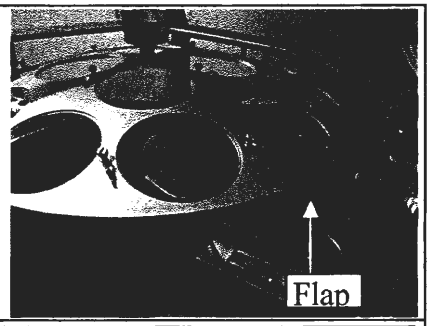
28 Replace evaporator crystal at this time if the crystal health parameter is low (See Dr. Tuttle).



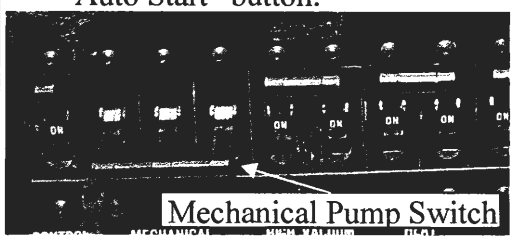
29 Remove scrap wafers from wafer holders that you need to use in the evaporator wafer holder unit.

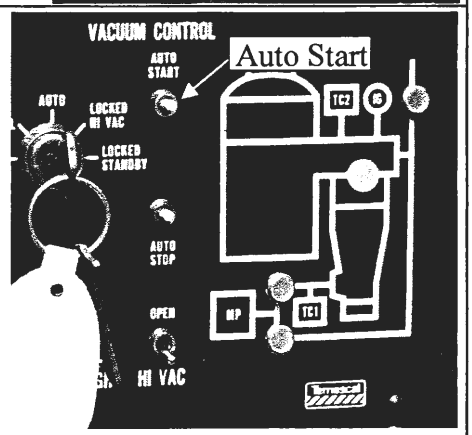
30 Set your wafers in the holders face down and place a spring loaded clip on each wafer to hold it in place.

31 Place the wafer holder unit back into the evaporator making sure to place the metal flap is over the crystal.

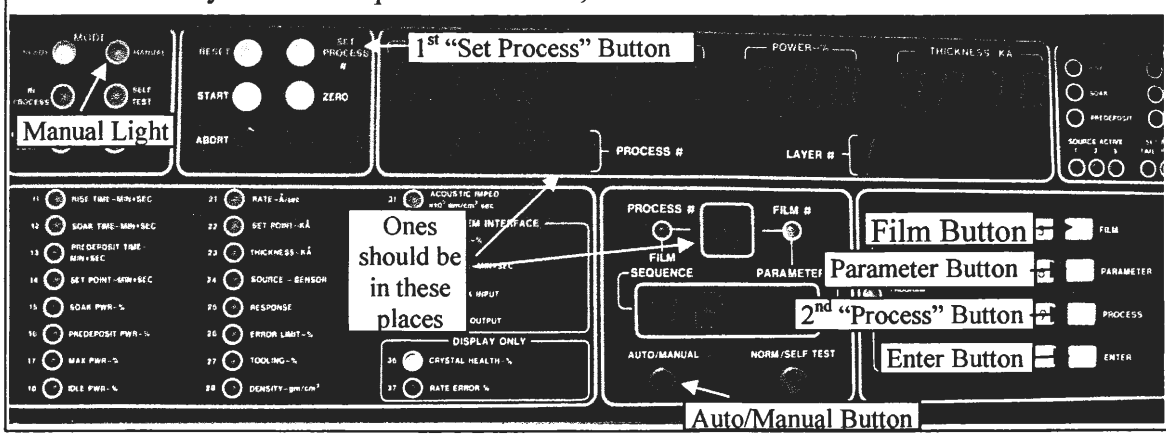


32 Wipe with a clean room wipe around the gasket of the evaporator lid. Close the evaporator lid, turn the mechanical pump on by flipping the switch up, and press the "Auto Start" button.

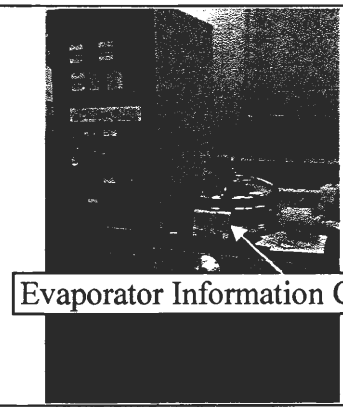




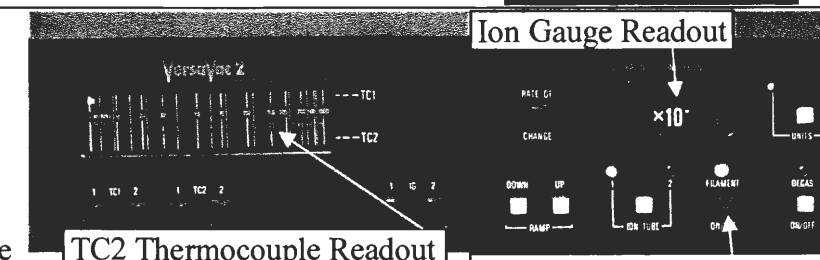
33 On the Evaporator Control Unit, push the "reset" button followed by the "auto/manual" button to put the evaporator in manual mode (See manual light). Set the process number to "1" in both positions on the controller, by pressing "Set Process" followed by "1" in the first position, and "Process" followed by "1" in the second position. Set the film number to 1 by pressing "Film" followed by "1". Check to make sure "density" and "acoustic impedance" are set at the correct values for gold as shown on the sheet of paper taped to the left side of the evaporator. If they need to be reset, press "parameter", followed by the corresponding parameter number, followed by the correct parameter value, and then "enter".



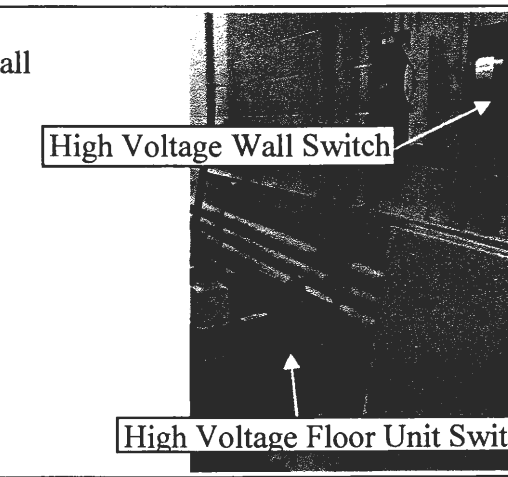
34 Fill out the Evaporator Information Card with your name, phone number, and estimated time of completion and place it on the evaporator lid.



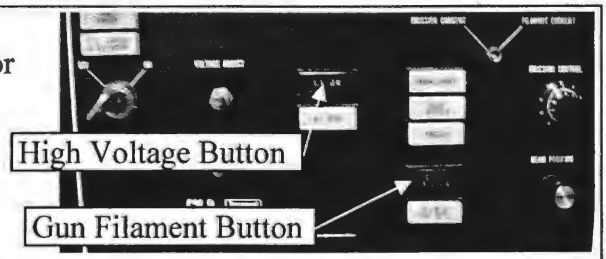
35 When the mechanical pump vavle closes and the cryo pump valve opens, the TC2 readout will decrease until there are no orange bars on the readout. Allow the evaporator to pump down for at least 6 hours or until the pressure reads at least $1x10^{-6}$ torr on the ion gauge (Turn the filament on to read the pressure).



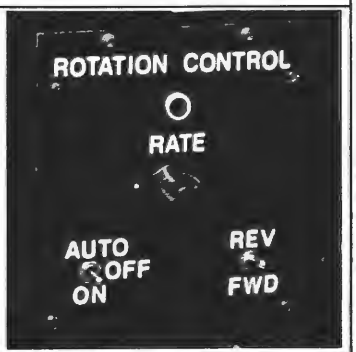
36 Turn on the High voltage switch on the wall (if it is off) then turn on the high voltage unit sitting on the floor.



37 Turn on the high voltage on the evaporator unit followed by the gun filament on the evaporator unit.

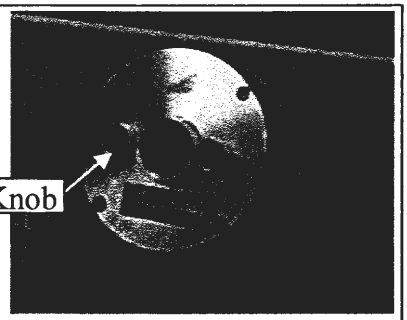


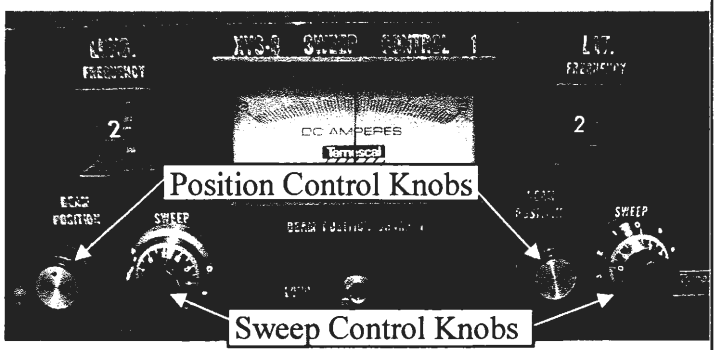
38 Turn on the wafer holder rotation motor by pressing the switch down.



39 Slowly ramp up the power with the hand-held controller to 12% - this should take about 2 minutes. Beware this controller is "sticky", you must quickly move it in the positive direction and pull it back to the middle to avoid ramping the power up too quickly.

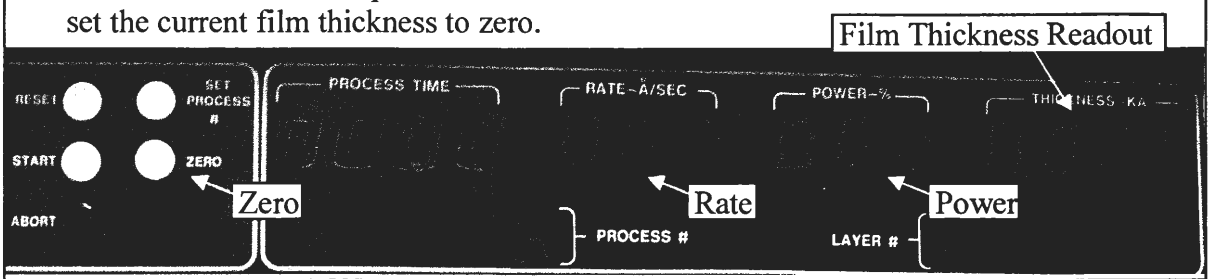
40 Check the beam position by twisting the viewing shutter knob and looking into the chamber. The beam should be moving around the gold in the crucible. If the beam moves outside the crucible, white sparks may appear – immediately move the beam into the crucible with the longitude and latitude position controls. The sweep length in both directions may also be controlled. You want a beam that is circling inside the crucible – do not leave the beam immobilized at a single point, the electron beam may burn through the metal and the crucible and damage the evaporator.



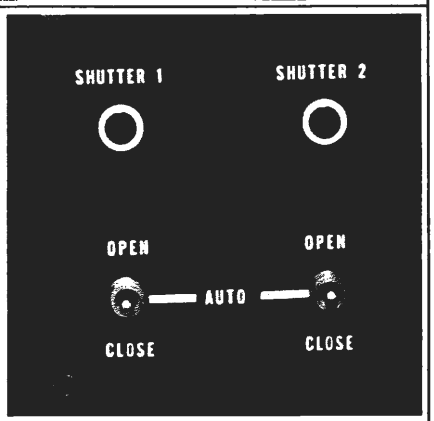


Proceed only after the beam is positioned properly (if you have problems, ramp the power all the way down and contact Dr. Tuttle).

41 Press "Zero" on the evaporator controller to set the current film thickness to zero.



42 Ramp the power up to 16% and wait for 1 minute in order for the gold to heat up. Open shutter 1 by flipping the switch up. Observe the "rate" displayed on the evaporator controller. An optimum evaporation rate is between 3 and 15 Å per second – Adjust the power as necessary to keep the rate fairly high. DO NOT EXCEED 30% POWER. Remember there will be some delay between the time you adjust the power and the time the metal heats up – don't increase the power too fast (i.e. 1% at a time is too fast) or you may get an enormous rate increase.



43 Monitor the "thickness" displayed on the evaporator controller and when it nears 2500 Å be prepared to close shutter 1 by flipping the switch to the middle position. Depending on the rate at which the metal is being deposited you will want to close the shutter before the "thickness" displays the 2.500 KÅ, because the shutter takes a few seconds to close. A good rule of thumb is to close the shutter X Å less than the desired thickness (X = rate * 3 seconds). Example: the rate is 15 Å/s. You want to close Shutter 1 when the readout is 2.455 KÅ (2500 – 15*3).

44 Immediately after closing Shutter 1, ramp the power down to zero by holding the hand held controller to the "decrease" side of the control. Turn off the gun filament and the high voltage on the evaporator controller. Turn off the high voltage floor unit.

45 Flip up the mechanical pump switch, turn off the ion gauge filament, and press the "Auto Stop" button. While the chamber is returning to atmospheric pressure remove your information sheets from the information card, and finish filling in the evaporator log on the computer – make sure to save the file and log out of the evaporator using lab logger.

46 Open the evaporator lid and remove the wafer holder unit. Replace the MRC's gold and crucible and take SKM Group's gold and crucible out of the evaporator.

47 Remove wafers from the wafer holder unit and replace the unit in the evaporator.

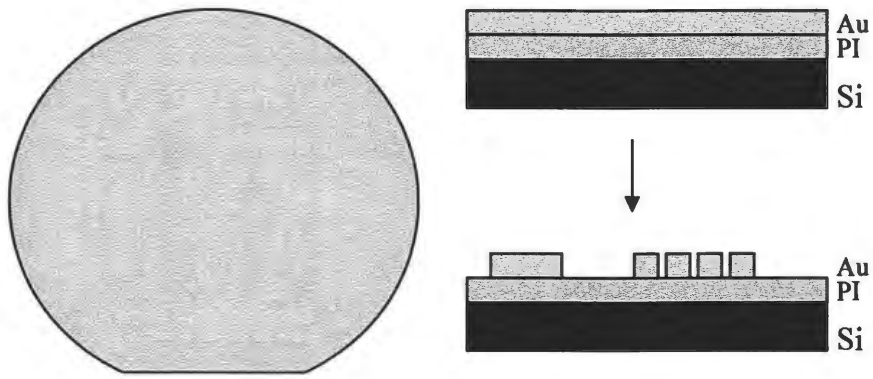
48 Press "Auto Start" and wait till there aren't any orange bars on the TC2 readout (~15-30 minutes).

49 Lock the gold in the SKM lockable drawer in the NSF lithography lab.

Patterning the Gold Layer

Locations: NSF lithography lab, and NSF lab

Top View **Side View**



Materials:

- AZ5214 Photoresist
- AZ312 MIF dil 1:1.2 developer
- 4" glass dish
- Small glass beaker
- Wafer transport box
- Metal tweezers (2)
- Mask #1
- Acetone and Methanol spray bottles

Equipment:

- Spinner (NSF lithography lab)
- Hot Plate (NSF lithography lab)
- Karl Suss i-line Mask Aligner (NSF lithography lab)
- Acetone bath (NSF Lab)
- Cascade Rinse Tub
- Microscope (NSF lithography lab)

Notes:

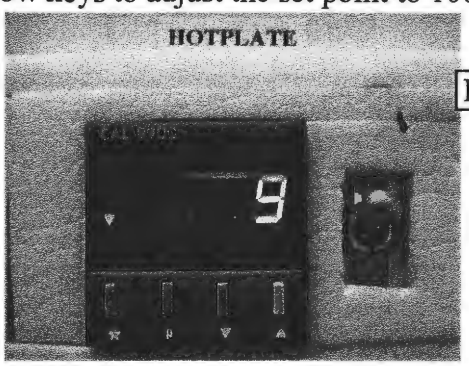
- If there isn't any gold etch left in the bottle you will have to make more by combining 40ml de-ionized water, 4 g potassium iodide, 1 g iodine. Let this mixture sit overnight to dissolve.

Procedure:

50 Log onto the spinner and the mask aligner on the computer in the NSF lab using Lab Logger. Put on a lab coat and safety goggles.

51 Turn on "Lights" and "Spinner Outlet" switches located on the front of the wet bench in the NSF Lithography lab.

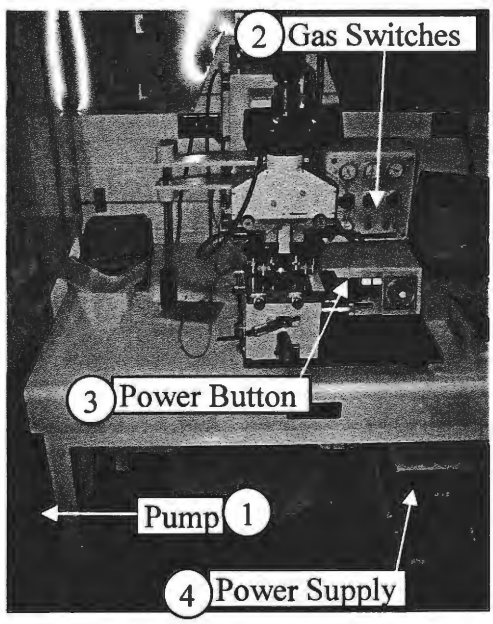
52 Turn on the hot plate switch, open the hot plate lid, and set the temperature set point to 100°C by pressing the "*" button and using the arrow keys to adjust the set point to 100.



53 Pour a small amount of AZ5214 photoresist from the large photoresist bottle into a clean small beaker.

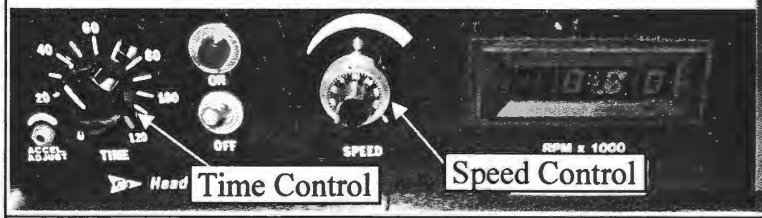
54 Turn on the mask aligner by first turning on the mechanical pump sitting on the ground to the left of the table, then flipping the two silver gas switches to the "on" position, then pushing in the red "power" button, and finally by turning on the mercury bulb power supply located underneath the table. This power supply will go through a self test and display "rdy" on its LED screen when it is ready. Press the small square button just below the power switch to try and ignite the mercury bulb. The power supply tries to light the bulb several times each time the button is pushed, it will display "Fire" each time. When the bulb has been lit, the readout will display "Cold". If it displays "rdy" again then you must press the small square button again to try and light the bulb. It will display the rest power when the bulb is ready (usually 275).

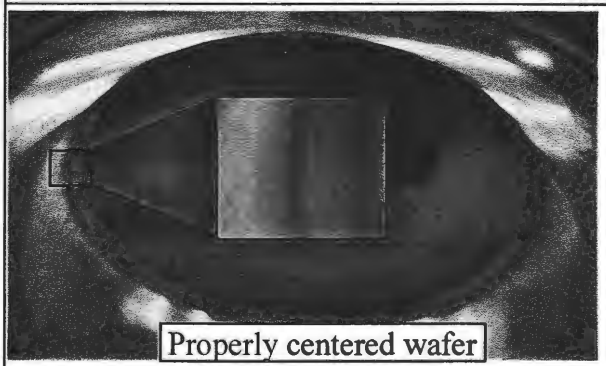
See Dr. Tuttle if problems persist.

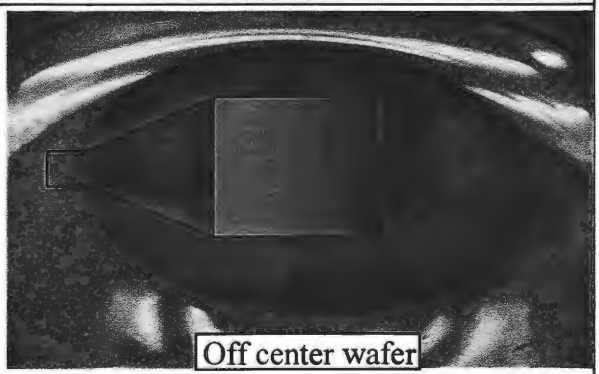


55 Place the large spinner circle on the spinner chuck.

56 Place a wafer in the middle of the spinner chuck then start the spinner using the foot pedal. Open the lower cabinet of the wet bench and adjust the spin speed to 4000 rpm and the spin time to 40s.



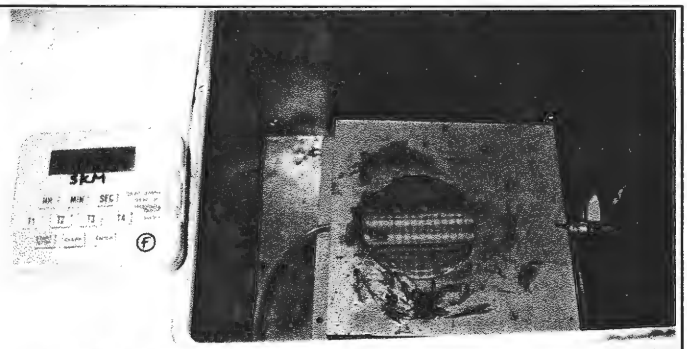




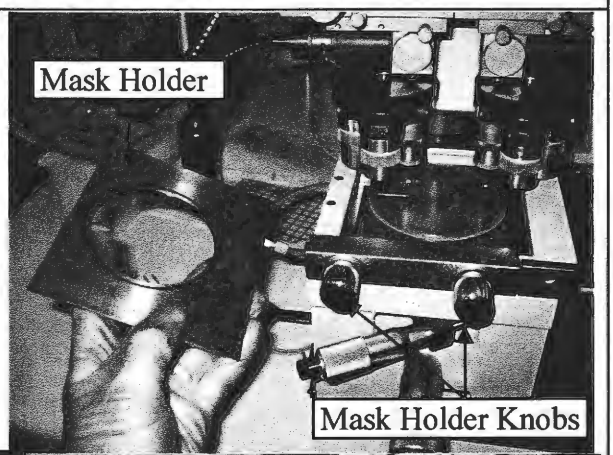
57 Wafers must be centered on the spinner circle to ensure full coverage. With a wafer in the middle of the spinner, start the spinner with the foot pedal. To ensure the wafer is in the center examine the edges of the wafer while it is spinning. The wafer will look solid around the edges while it is spinning if it is centered – if it is not centered it will appear fuzzy.

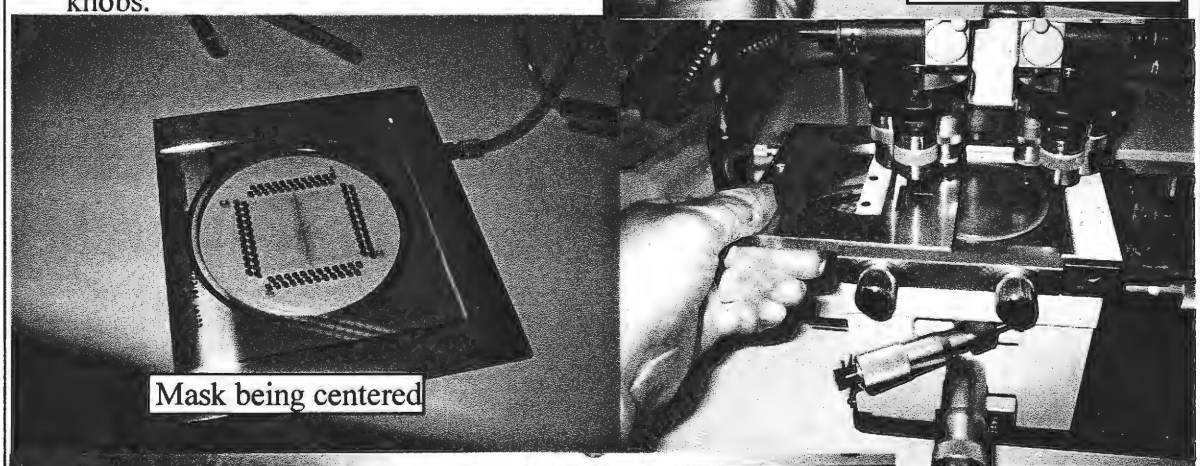
58 Pour a 3cm diameter pool of photoresist into the center of a motionless wafer. Step on the foot pedal to start the wafer spinning and allow the wafer to come to a complete stop (40s).

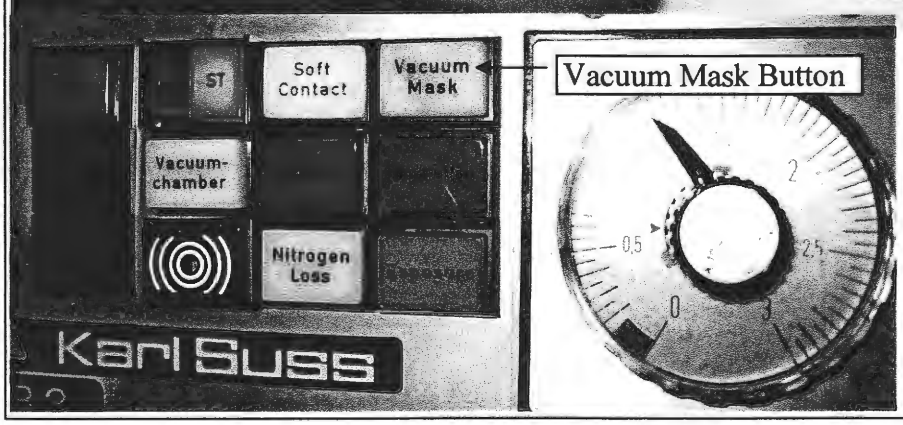
59 Remove the wafer from the spinner and place it on the hot plate for 60s. Remove the wafer and place it in a wafer carrier.



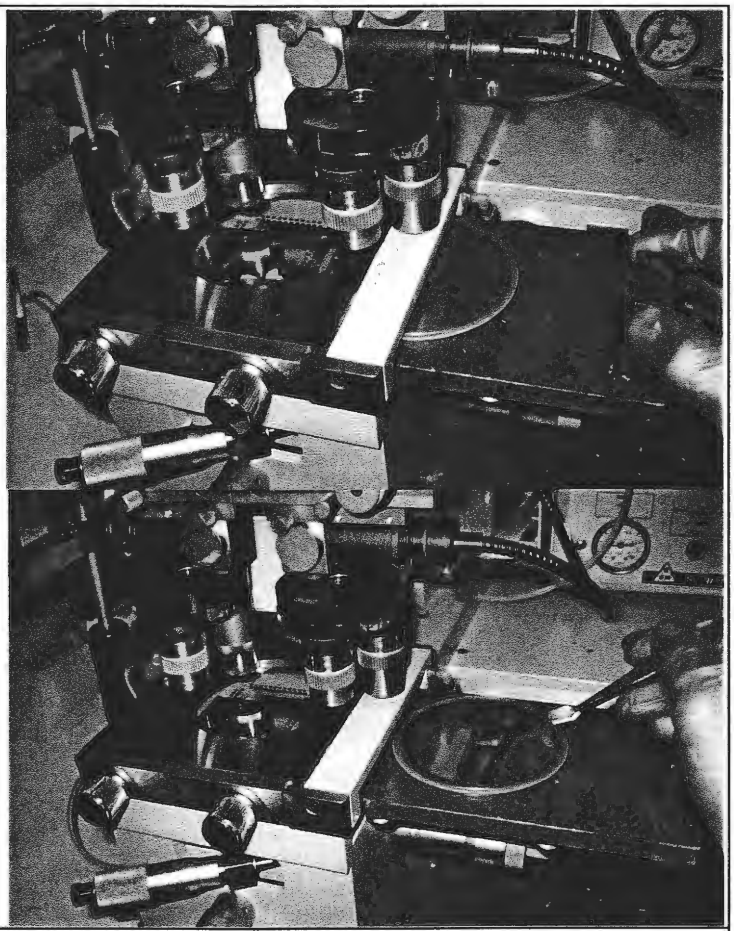
60 Unscrew the two mask holder knobs on the mask aligner and slide out the mask holder. Turn it upside down and center mask #1 in the open circle area of the holder. Hold the mask in place while pushing the "Vacuum Mask" button on the aligner's front panel until it lights up. Test the mask to make sure it is securely attached to the mask holder. Slide the holder back into the aligner and tighten the two mask holder knobs.



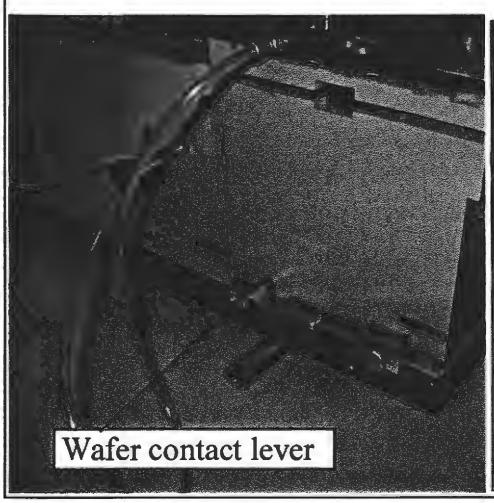


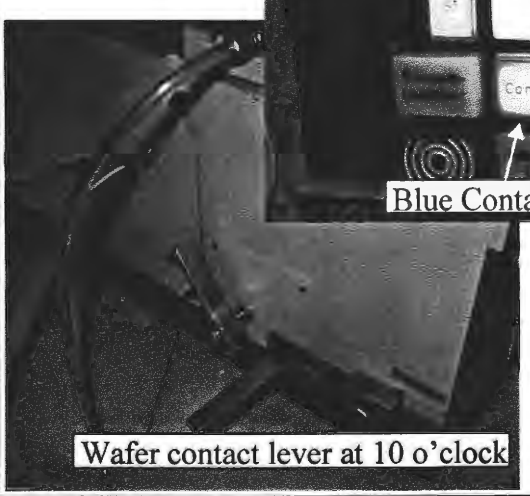


61 Slide out the wafer holder and center a wafer on the chuck. Align the flat side of the wafer towards yourself. Slide the wafer holder back into the aligner.



62 On the left side of the aligner, locate the wafer contact lever and slowly flip it towards the backside of the aligner. When it reaches about "10 o'clock" you should see the wafer come into contact with the mask. Continue rotating the lever until you hear a loud click and the blue "Contact" button lights up on the aligner's front panel.

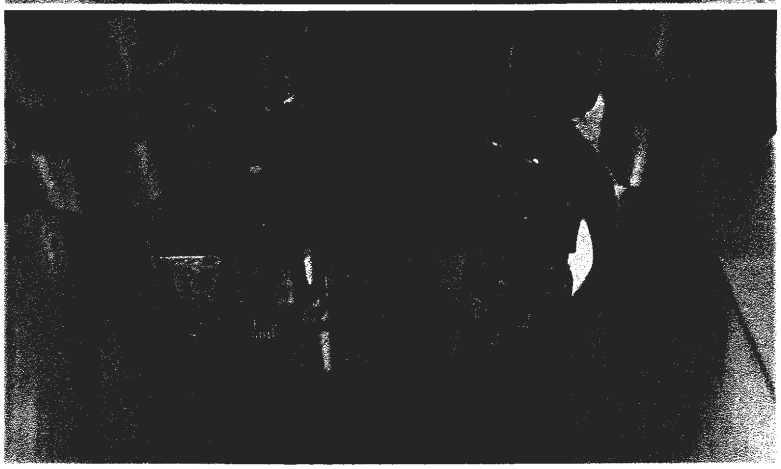




63 Notice the double pattern of the mask in the picture of the wafer that is not in contact with the mask. This pattern disappears as the mask and wafer are brought into contact.

This wafer is not in contact with the mask.

When the wafer contact lever is at 10 o'clock the wafer should come into contact with the mask and the double pattern should merge into one pattern.



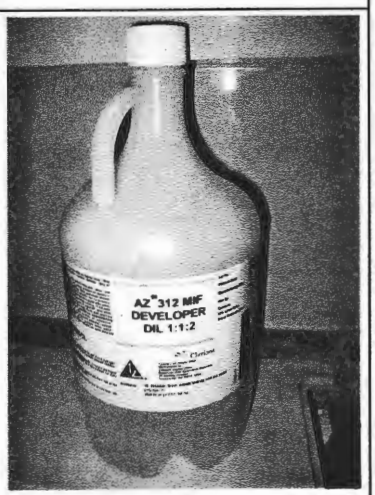
64 Set the time of the exposure using the large dial on the aligner's front panel; turn the large outside knob to adjust the time and the small inside knob to adjust the time scale. Set it to expose the wafer for 102 seconds.

65 Press the green "Exposure" button on the aligner's front panel to start the exposure. The entire top portion of the aligner will move forward over the wafer area in order to expose the wafer to UV light.

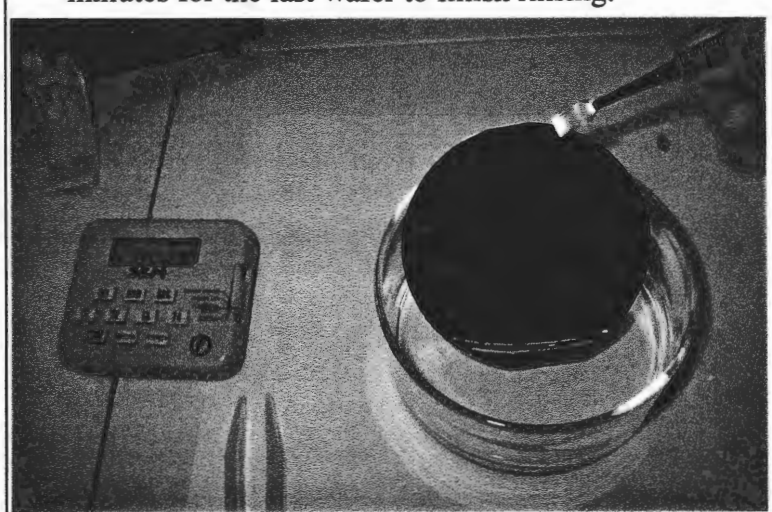
You will notice a little red arm in the time dial that displays how much time is left for the current exposure.

When the exposure is complete, the top unit will slide back to its original position. Turn the wafer contact lever towards the front of the aligner in order to separate the wafer from the mask. Slide out the wafer holder, remove the exposed wafer, and place it in a wafer carrier.

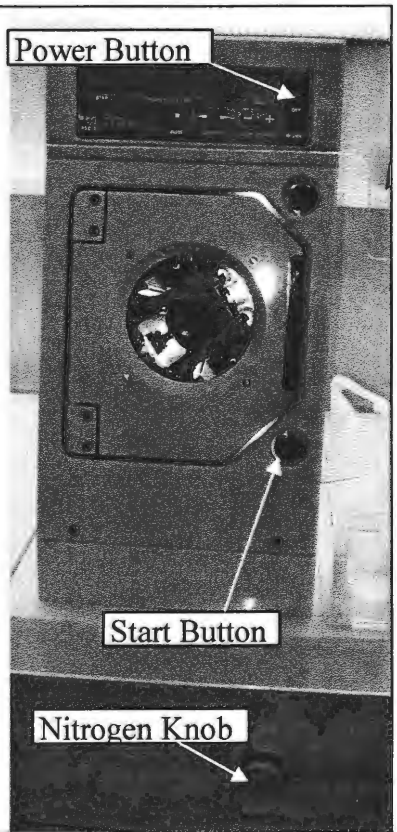
66 Fill a glass dish to about 1cm high with "AZ312 MIF dil 1:1.2". This developer contains tetramethyammonium hydroxide (TMAH) and is extremely dangerous. Open the cascade rinse tub and place the lids to the right of the tub. Turn on the water by turning the blue knob clockwise a few turns. Turn on the nitrogen by turning the yellow know clockwise just slightly – make sure the nitrogen is adjusted to a slow rate.



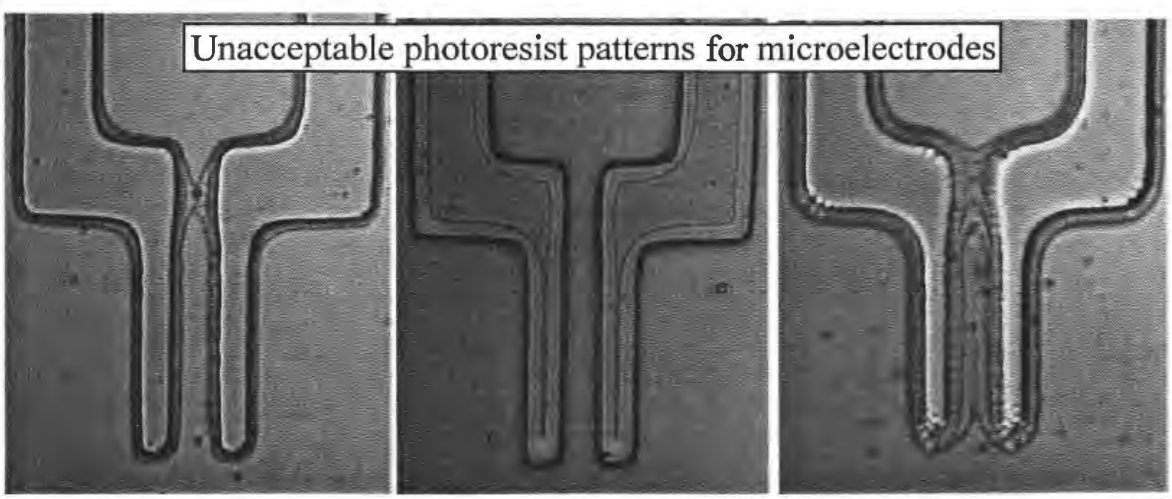
Submerge the wafer in the developer and slightly agitate for 85 seconds. Remove the wafer and place it in a wafer carrier that has a handle attached. Place the carrier in the casecade rinse tub and when full of wafers allow 3 minutes for the last wafer to finish rinsing.



67 Remove the wafer carrier from the cascade rinse tub and remove the handle. Take the wafer carrier to the NSF lab next door. Turn on the nitrogen to the spin rinse dryer by turning the yellow knob, just below the spin rinse dryer, counter-clockwise a few turns (you will hear a quiet hiss of nitrogen). Turn on the power to the spin rinse dryer and allow it to run through its startup tests; it will display "idle" when it is ready. Open the door and place the wafer carrier inside the spin rinse dryer with the H-bar of the carrier towards the back of the dryer. Close the door and press the "start" button. This should take about 6 minutes to complete and the dryer will display "idle" when the dry cycle is complete.



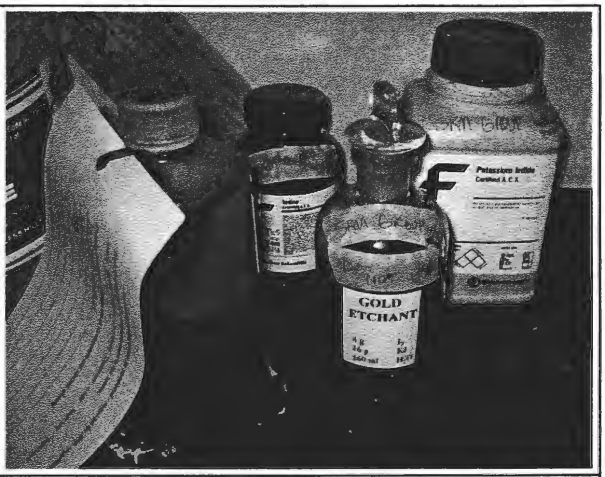
68 Inspect the photoresist pattern under a microscope and proceed if the pattern is acceptable. The pictures below are all considered UNACCEPTABLE; the microelectrodes should have a space in between them (similar to the dark outline of the middle picture). If not, remove the photoresist in a 5 minute acetone bath followed by a 3 minute cascade rinse and a spin rinse dry cycle.



69 Retrieve the gold etch from the hood in the NSF lithography lab. If there is not any etchant remaining make some more in the empty gold etchant bottle using the following recipe:

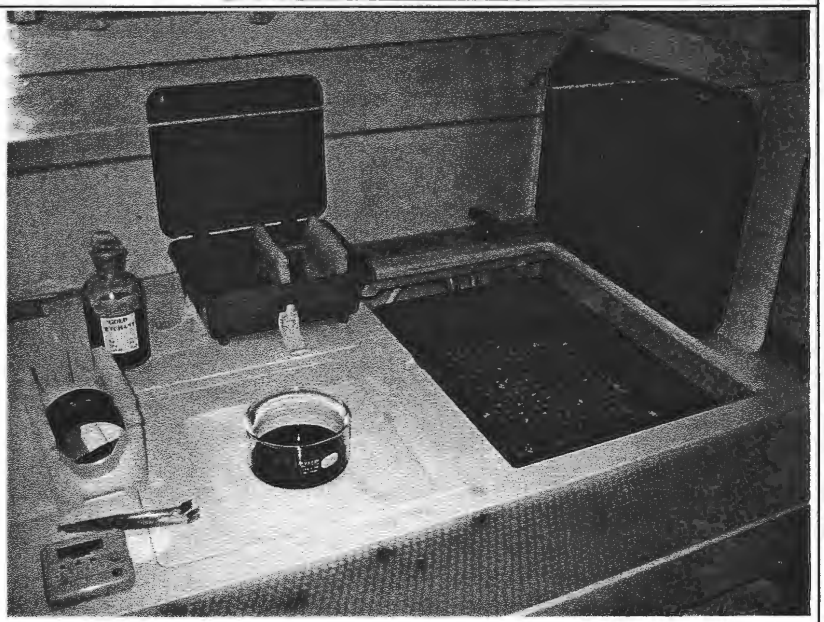
- 40 ml de-ionized water
- 4 g potassium iodide
- 1 g iodine

Mix these ingredients and let sit overnight.



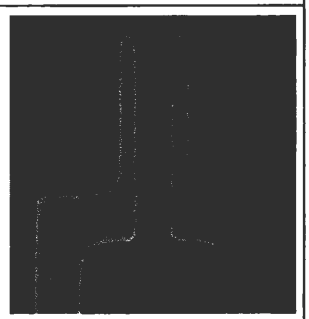
70 Fill a glass dish to about 1cm high with gold etch. Open the cascade rinse tub and place the lids to the right of the tub. Turn on the water by turning the blue knob clockwise a few turns. Turn on the nitrogen by turning the yellow know clockwise just slightly – make sure the nitrogen is adjusted to a slow rate.

Submerge the wafer in the gold etch and agitate for 90 seconds. Remove the wafer and cascade rinse for 3 minutes. Remove the wafer from the rinse and place on a clean room wipe next to the sink. Dry the wafer with the nitrogen gun located near the sink.



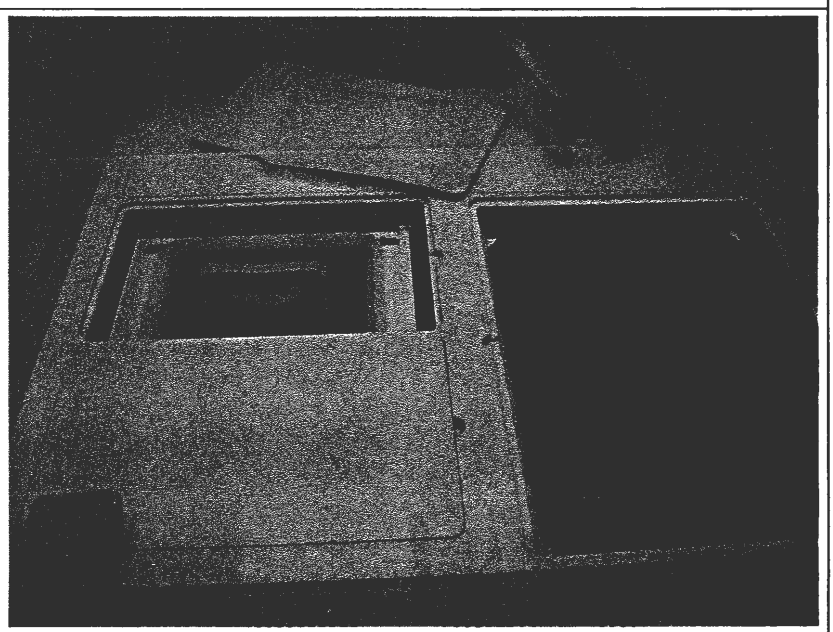
71 Wipe the face of the wafer lightly with a clean room wipe to remove a filmy residue left by the gold etch.

72 Inspect the wafers under the microscope in the NSF lithography lab. The microelectrodes should look similar to this photograph.



73 Remove the photoresist in a 5 minute acetone bath, followed by a 3 minute cascade rinse, and a spin rinse dry cycle.

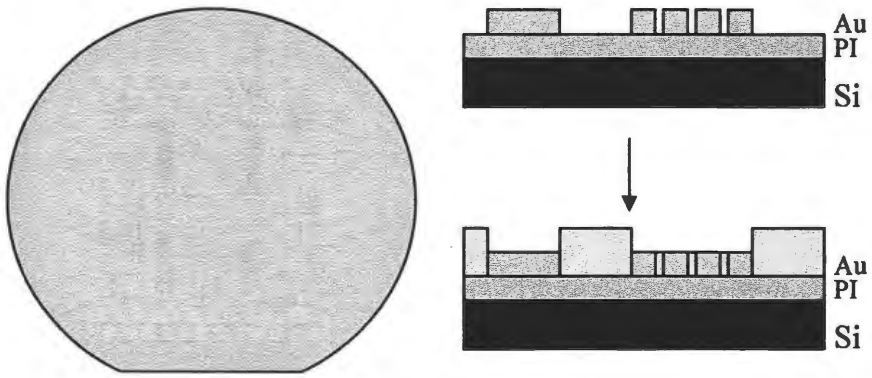
74 Logout of any equipment and make sure everything is put away and cleaned up.



Patterning the Top Polyimide/Groove Layer

Locations: Room 180A ASC I (old clean room), NSF lithography lab, and NSF lab

Top View **Side View**



Materials:

- PI2771 Polyimide
- AZ312 MIF dil 1:1.2 developer
- 4" glass dish
- Wafer transport box
- Metal tweezers (2)
- Mask #2
- Acetone and Methanol spray bottles

Equipment:

- Spinner (Room 180A ASC I or NSF lithography lab)
- Hot Plate (Room 180A ASC I or NSF lithography lab)
- Karl Suss i-line Mask Aligner (NSF lithography lab)
- Cascade Rinse Tub
- Microscope (NSF lithography lab)

Notes:

- The process to pattern the PI2771 polyimide is humidity dependent; the ideal humidity is 40% +/- 10%. The NSF lithography room humidity level cannot be controlled therefore in the summer the humidity level sometimes rise to almost 80%; when this is the case the spinning and the first bake must be done in Room 180A ASC I – this room usually has a lower relative humidity.
- PI2771 is also very finicky about the amount of time in between each step do not start this section if you cannot finish it in one sitting.
- You must let the PI2771 polyimide sit out overnight in order to warm up to room temperature; this prevents condensation from contaminating the polyimide.

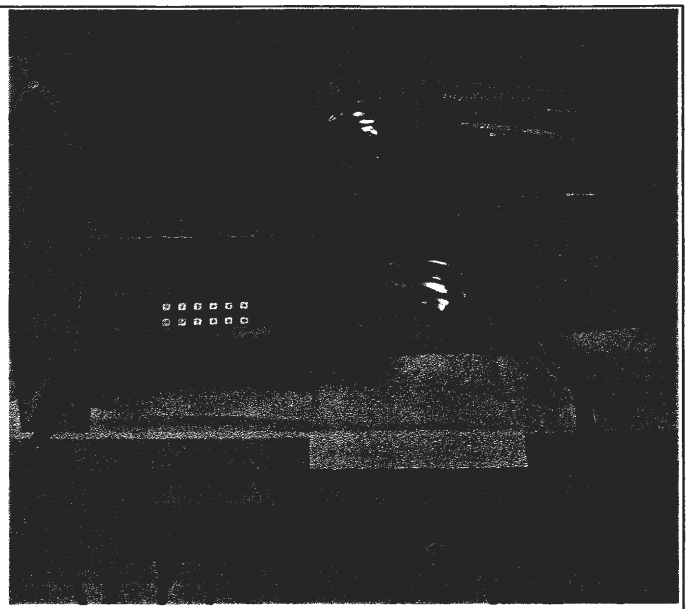
Procedure:

75 Log onto the spinner and the mask aligner on the computer in the NSF lab using Lab Logger. Put on a lab coat and safety goggles.

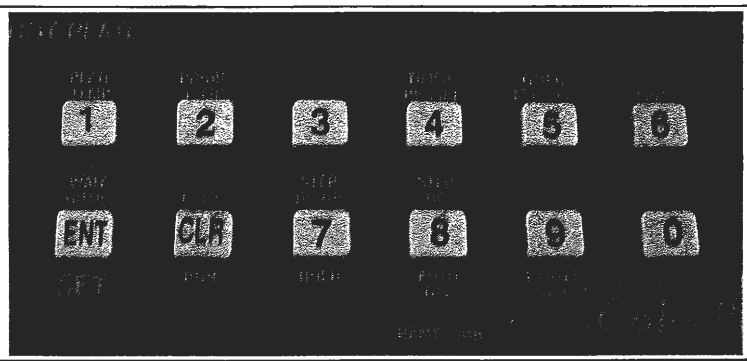
76 The following steps are for the spinner and hot plate located in room 180A ASC I. If you are using the spinner and hot plate in the NSF lithography lab please follow steps 51, 52, 55, and 56 while changing the parameters to match the steps in this section.

77 Startup the mask aligner (see step 54)

78 Turn on the power strip and make sure the spinner, hotplate, and vacuum pump are all plugged into it.

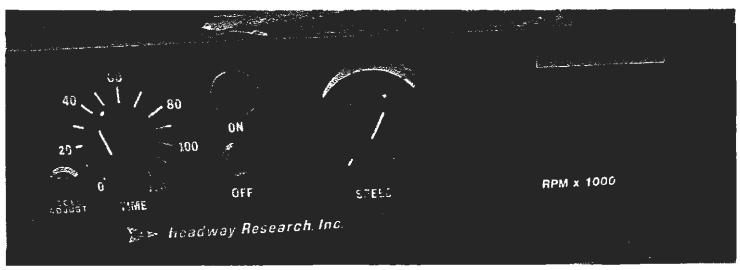


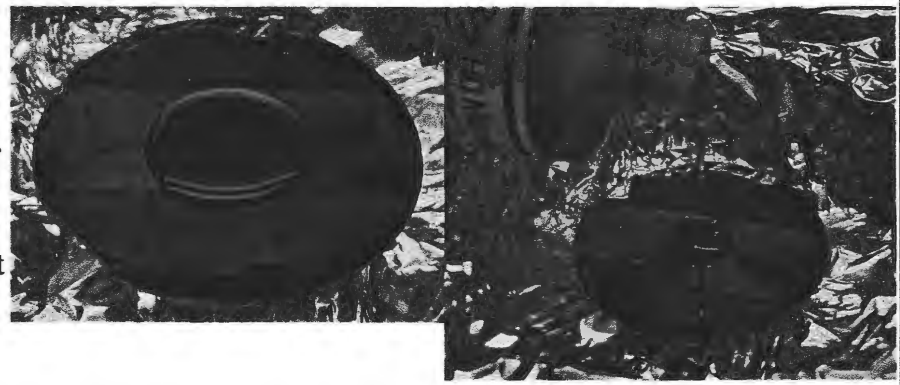
79 Turn on the hotplate and set it to 115°C by pressing these buttons: "Set" then "Plate Temp" then 1, 1, 5 then "ENT". This hotplate will alternate a display of the set point temperature and the current temperature.



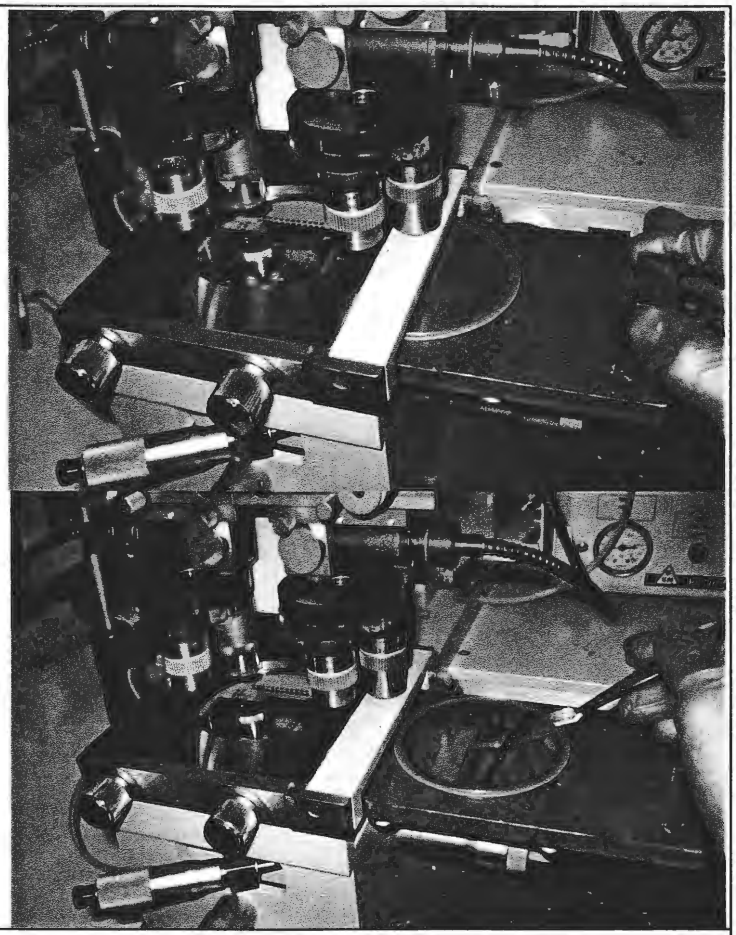
80 Cover the spinner with aluminum foil and place the large spinner chuck on the spinner.

81 Turn on the spinner and set the "Time" to the 50 second line – this is actually closer to 60s. Start the spinner spinning with the foot pedal and count how long it spins. Adjust the "time" knob as necessary to get a spin time of 60s

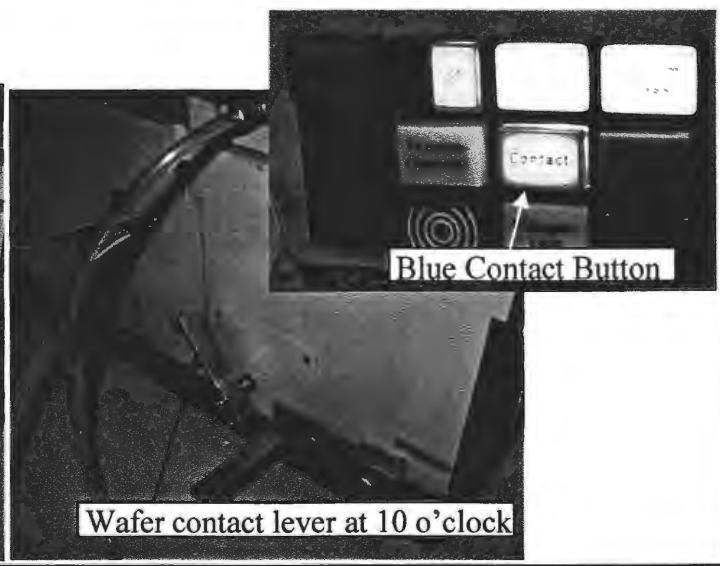


82	When the hotplate has reached 115°C, center a wafer on the chuck and turn the spin speed down to zero.
83	Pour a 2cm diameter pool of polyimide into the center of the wafer and wait a few seconds for it to spread out. Do not use more polyimide than is necessary to coat the wafer. 
84	Start the spinner with the foot pedal and slowly turn the spin speed knob clockwise to increase the speed of the spinning wafer. Do this so that the wafer reaches 3500 rpm after 30s of spinning. The wafer should then continue to spin at 3500 rpm for 30s more.
85	After the spinner has come to a stop, pick the wafer up with metal tweezers using a second pair of tweezers to hold the wafer steady. Touch as little of the wafer as possible. Place the wafer on the hot plate for 120 seconds. Remove the wafer and place it in a wafer carrier. Coat the rest of the wafers. Place the wafer carrier in a transport box and close the lid – the PI2771 polyimide is photodefineable and therefore is sensitive to UV light. Take the wafers to the NSF lithography room.
86	Load mask #2 into the mask aligner (see step #60)

87 Slide out the wafer holder and center a wafer on the chuck. Align the flat side of the wafer towards yourself. Slide the wafer holder back into the aligner.

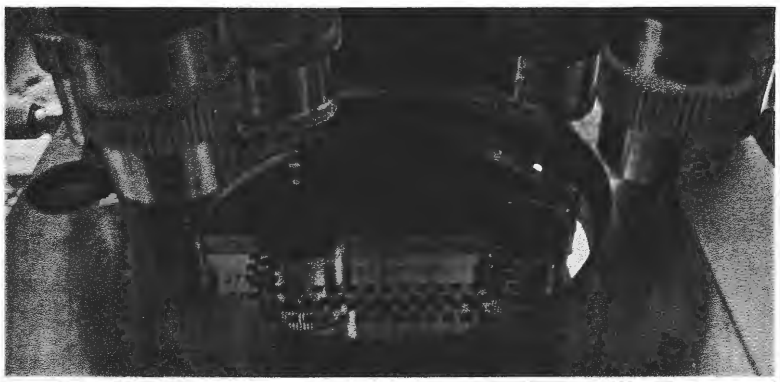


88 On the left side of the aligner, locate the wafer contact lever and slowly flip it towards the backside of the aligner. When it reaches about "10 o'clock" you should see the wafer come into contact with the mask. Continue rotating the lever until you hear a loud click and the blue "Contact" button lights up on the aligner's front panel.



89 Notice the double pattern of the mask in the picture of the wafer that is not in contact with the mask. This pattern disappears as the mask and wafer are brought into contact.

This wafer is not in contact with the mask.



When the wafer contact lever is at 10 o'clock the wafer should come into contact with the mask and the double pattern should merge into one pattern.

90 Set the time of the exposure using the large dial on the aligner's front panel; turn the large outside knob to adjust the time and the small inside knob to adjust the time scale. Set it to expose the wafer for 132 seconds.

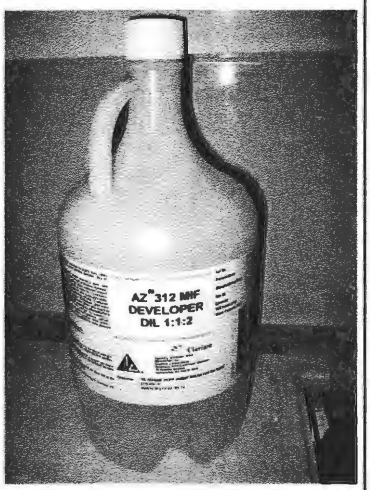
91 Press the green "Exposure" button on the aligner's front panel to start the exposure. The entire top portion of the aligner will move forward over the wafer area in order to expose the wafer to UV light.

You will notice a little red arm in the time dial that displays how much time is left for the current exposure.

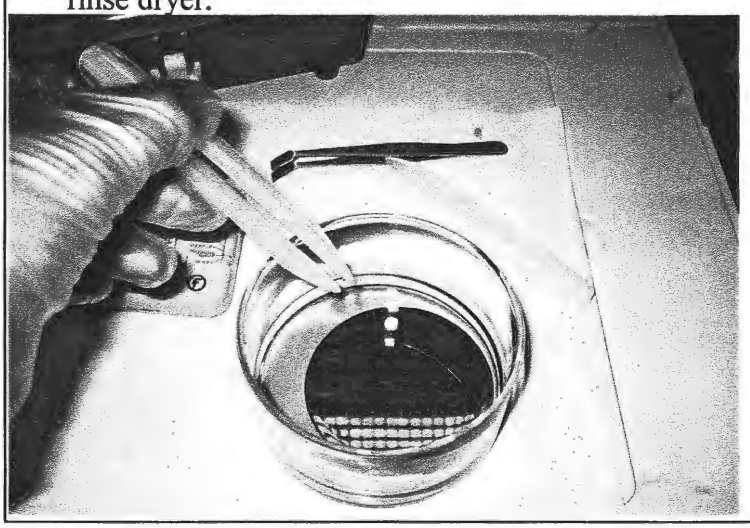
When the exposure is complete, the top unit will slide back to its original position. Turn the wafer contact lever towards the front of the aligner in order to separate the wafer from the mask. Slide out the wafer holder, remove the exposed wafer, and place it in a wafer carrier.

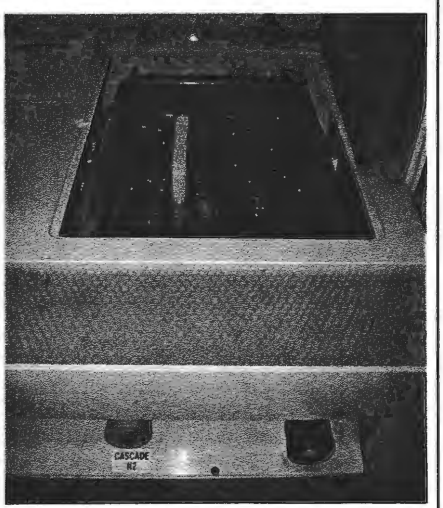
92 Finish the remaining wafers. Let each wafer sit at room temperature for 30 minutes before proceeding to the next step

93 Fill a glass dish to about 1cm high with "AZ312 MIF dil 1:1.2". This developer contains tetramethyammonium hydroxide (TMAH) and is extremely dangerous. Open the cascade rinse tub and place the lids to the right of the tub. Turn on the water by turning the blue knob clockwise a few turns. Turn on the nitrogen by turning the yellow know clockwise just slightly – make sure the nitrogen is adjusted to a slow rate.

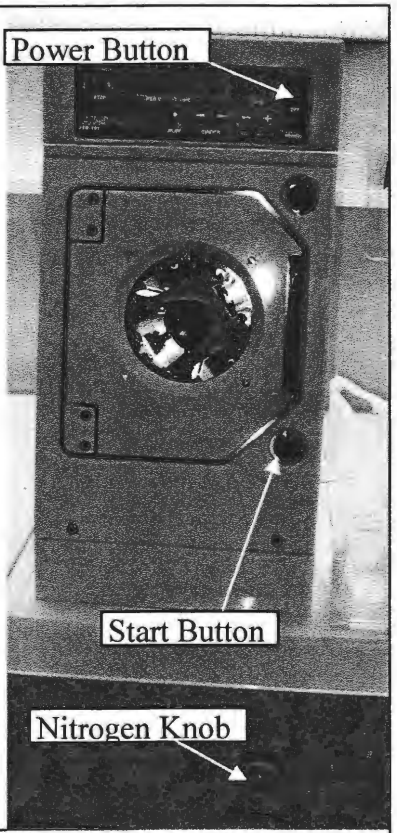


Submerge the wafer in the developer and aggressively agitate for 100 seconds. You will see the pattern forming around the contact pad area. Remove the wafer and place it in a wafer carrier that has a handle attached. Place the carrier in the casecade rinse tub and only allow each wafer to be in the rinse for 3 minutes. After this time remove the wafer and place it in a wafer carrier with a clean room wipe underneath to soak up the water that drips. When all the wafers are finished take this wafer carrier to the spin rinse dryer.

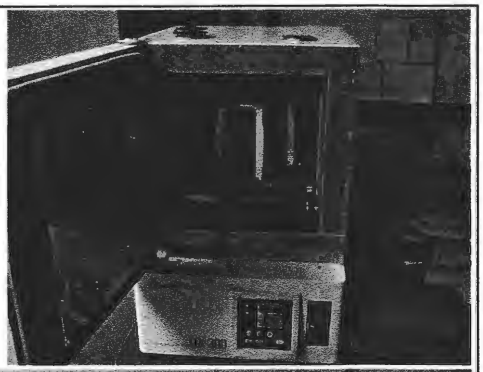




94 Turn on the nitrogen to the spin rinse dryer by turning the yellow knob, just below the spin rinse dryer, counter-clockwise a few turns (you will hear a quiet hiss of nitrogen). Turn on the power to the spin rinse dryer and allow it to run through its startup tests; it will display "idle" when it is ready. Open the door and place the wafer carrier inside the spin rinse dryer with the H-bar of the carrier towards the back of the dryer. Close the door and press the "start" button. This should take about 6 minutes to complete and the dryer will display "idle" when the dry cycle is complete.



95 Place the wafer carrier into a transport box and take to room 111 ASC I.



96 Place the wafer carriers into the curing oven.

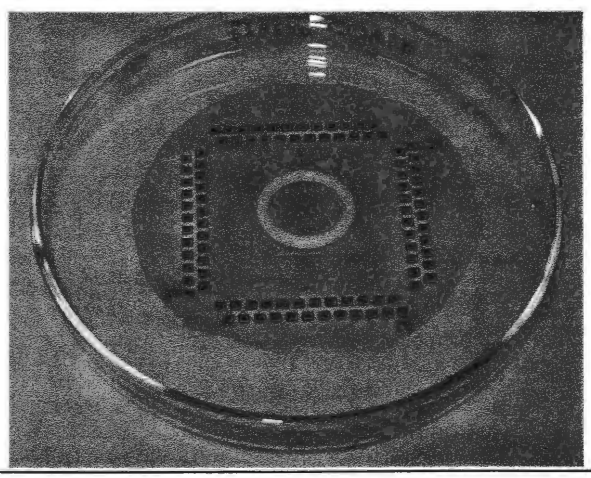
97 Set the oven to go to 240°C and to turn off after 2 hours with the following procedure

- Turn Power on with black switch
- Use the arrow keys to turn the red light to the left of "Auto-Stop" on.
- Press "timer" then press "enter"
- The "set temp" display will blink, here you can set the temperature using the arrow keys. When you have set the desired temperature press the "enter" button
- Now the "set temp" display will blink again, here you can set the desired time, in hours, until the oven will shut off. When the time is set press "enter".
- Press "Start/Stop" twice to start the program, and the oven will automatically shut off.

98 After the oven has cooled, remove the wafer carriers and place them into wafer transport boxes.

99 The microelectrode devices are complete and are now removed from the wafers by scoring around the device with a razor blade. The devices usually peel off of the wafers very easily.

100 These microelectrode devices must now be prepared for cell culture. They will look like this when they are done.



Appendix B: Detailed ICC Procedures

Horseradish Peroxidase ICC Protocol – Retrofitted for Cells - From Dr. Sakagucki's Lab – Updated June 2005

Day 1 (The Beginning):

Verify the presence and appearance of cells before beginning ICC procedures

Make 50mM KPBS for washes done on both days.

- About 130ml/small Petri Dish will be needed
- Prepare a filtered supply of this buffer

50mM KPBS:
4 L ddH_2O
36g NaCl
23.4g K_2HPO_4
9g KH_2PO_4

Make up 0.3v% Peroxide solution for incubation (Step 2).

- Dilute stock Peroxide solution (usually 30v%) with 50mM KPBS.
  - 30% H_2O_2 must be < 1 month old (Ignore this for cell staining)
  - Example: Dilute 1 ml of 30% H_2O_2, with 100ml KPBS to obtain 0.3% H_2O_2
- Approximately 5mL of solution is need per small Petri Dish
- Note: If using HyVee / Wal-Mart H_2O_2 the concentration is 3% not 30%

Note: All solution washes must be done w/ extreme care. Cells can be easily displaced and lost during the procedure. Remove and add all solutions to the edge of dish away from coverslips using a Pasteur pipette.
All subsequent washes should be done immediately after the previous solution has been removed because cells should not be allowed to dry.

1) Gently aspirate or Pipette-off the culture media

2) Gently rinse cultures in 0.1M PO4 Buffer 2 X 1 min

3) Fix cells in 4% Para-fix 1 X 20 mins

- – cool to room temp.
- Remove fix; Dispose in satellite waste area

4) Wash 50mM KPBS 3 X 7 min.

- Gently Rinse do not displace any cells!
- Fresh buffer, < 1 week old
- Wash in Small Petri used to hold the film-coverslip-assembly.
- 5ml/coverslip (No need to measure).

5) Incubate 0.3% H_2O_2 1 X 30 min.

- Removes endogenous Peroxidase activity in cells

While incubating with H_2O_2, make up the diluent that will be used for in the blocking (step 6) and primary antibody incubation (step 7) steps.
Approx. 5mL/coverslip need for blocking step and 200µL/coverslip for incubation on Parafilm

Diluent is KPBS, 1g/v% BSA (Bovine Serum Albumin), and 0..4v% Triton-X 100.

Note: Normal Blocking Serum (NBS) comes from the animal that the secondary antibody is made in. (i.e. - If secondary is a Biotinylated Rabbit anti-Rat IgG, then Normal Rabbit Serum (NRS) must be used)

6) Block NBS 1 X 30min

- If using different 1° antibodies, do **not** make cocktails that contain the different NBS's; make individual NBS's.
- Apply 5mL/slide, completely covering each section.
- Close chamber and do not move during incubation.
- Excess NBS can be stored in the fridge.

Normal Blocker Solution(NBS)/Diluent for Antibodies
10mL KPBS
0.1g BSA (In chemical fridge)
40µL of Tri-X (cut pipette tip and leave in solution)
150µL Normal Blocking Serum

While You Wait:
Prepare Primary Antibody Solution

Thaw frozen antibody aliquot and dilute with the NBS Solution (see above) to desired concentration (varies with Antibody).
(i.e. - H222 estrogen receptor antibody is used at 1:1000. Aliquots are 10µL in glycerol at 1:2. Therefore, 1 aliquot (10µL) diluted with 5 ml NBS = 1:1000 dilution.)

Prepare KPBS-TrX Solution

Add **200 µL Triton-X 100** to each liter of remaining 50mM KPBS and refrigerate.
This will be used for washes on day 2.
Approx 100ml of KPBS-TrX solution will be used per Petri dish.

7) Incubate 1^{o} Antibody 1 X 20 hrs at 4°C

- Apply 200µL/coverslip of antibody or blocker on Parafilm in the shallow lid ("top") of large Petri dish. Label all drops to indicate type of incubation (+ or -, see below).
- Remove Coverslips from NBS
- Place coverslips with cells down on the drops of antibody or blocker (if a control).
- Do not apply primary antibody to negative controls (NCs). NCs remain incubating in NBS.

- Run the following **control slides:**

Primary antibody / Secondary antibody	
- / -	Examines the effect processing may have on the tissue. No abs. applied.
- / +	Tests the non specific binding of the secondary antibody
+ / -	Examines the effect of primary antibody with no amplification/visualization

A negative means that during incubation Normal Blocking Serum was used instead of the antibody. Positive means that the corresponding antibody was used.

- Place wet Kim wipe in the deep lid of the large Petri dish and cover.
- Label and refrigerate left over primary.
- Primary is good up to one month after diluting.

Day 2 (The Middle):

Prepare 0.1M Sodium Acetate (NaAc): 13.6 gNaAc/L ddH_2O

Will use about 60mL per film

1) Wash KPBS-TrX Solution 10 X 5 min.

- Remove slides from Parafilm and place in small Petri dishes filled with KPBS/TrX.

Between Washes

Prepare secondary antibody

- Use the NBS Solution prepared on the 1st Day (For each 10 ml KPBS add 0.1g BSA, 150 µL NBS, and 40 µL Trtiton-X 100) as diluent for secondary antibody.
- Secondary antibodies are used at concentrations of 1:500/1:600 see antibody data base or previous titration's before using.

2) Incubate 2^o Antibody 1 X 2 hrs.

-Wipe dry edges and back of slides and place in humid chamber.
-Apply 500µL/slide.
-Close chamber and do not move during incubation.

Before Incubation is Over

Take out one 4mL aliquot of DAB (di-Amino-Benzidine) from freezer this will be more than sufficient.
Thaw tube/aliquot in beaker of warm water.

DAB stock is made up at 20 mg/mL of 0.1M NaAc -- Sonicated and aliquoted into plastic screw top tubes at 4 or 6ml/tube. These tubes are frozen down at -20 C and stored in a box in the chemical freezer. DAB is light-sensitive, so aliquots are wrapped in aluminum foil.

DAB is hazardous -Causes eye, skin, and respiratory tract irritation. Light sensitive. Hygroscopic (absorbs moisture from the air). Check the tube for cracks and wear gloves when handling aliquots or solutions.

During the Following Washes

Prepare **9g/L NaCl** Solution

Weight out need NaCl and dilute in ddH_2O
i.e.- Need 100mL of NaCl solution, so weight out 0.9g of NaCl
10mL per Petri dish is more than sufficient.

Prepare DAB Solution (In the Chemical Hood)

DAB Solution Ratio: | 1mL DAB: 1.25g $NiSO_4$: 50ml of 0.1M |

Add $NiSO_4$ to 0.1M Sodium Acetate (NaAc) and mix in a brown bottle
When DAB stock is thawed, add to the solution while stirring.
Approximately 20 ml or so should be held back in a small brown bottle for addition of H_2O_2 prior to the reaction.

Prepare Bleach Solution
A strong bleach solution (approx 1-2 liters, 50% bleach and 50% water) should be ready for decontamination of glassware, tubes, lab bench, etc.

3) Wash **KPBS-TrX Solution** **8 X 5 min.**

- Dump off secondary and replace slides in rack. Wash in small Petri dishes

4) Wash **0.1M NaAc** **4 X 5 min.**

5) React **DAB Solution** **1 X 6 min.**

- Add 25µL 30% H_2O_2 to the small aliquot of DAB sol'n.
- Do not allow the reaction to proceed longer than 6 min.
    - Let Reaction Proceed Longer if cell staining is faint…check this under LM

Clean Up

Put all tubes, stir bars, bottles, etc. contaminated with DAB into the bucket of bleach solution. Pour pure bleach into the staining dish to decontaminate. Cover and leave overnight. (Remember to wash 24 hours later).. A brown precipitate will form, leave it in this overnight.
Next day dump bleach decontamination solution in sink all of DAB should have reacted thus decontaminating labware.

6) Wash **0.9% NaCl** **4 X 5 min.**

- Termination of DAB reaction.
- Solution is 9 g/L.

7) Dehydrate **Ethanol Series** **Dip Coverslips**

- Dehydrate through EtOH series
- 70%, 85%, 95%, 100%, 100% (Histology Only)
- Dip each coverslip several times in each EtOH concentration
- From lowest to highest concentration
- Let Dry
- Mount onto microscope slide

8) Adhere Coverslip to Microscope Slide

- Reduce the possibility of air bubbles as most as possible.
- Place a drop of GelMount on small cover slip
- Remove film from original coverslip: Use tweezers to pull of from corner after pushing under the film to unstuck it from the original coverslip.
- Place film with cells down onto drop of GelMount.
- Place another drop of GelMount on microscope slide then flip coverslip with film and gel onto the drop so that the film is sandwiched between coverslip and microscope slide.
- Label experimental slides with the antibody being used.
- Place in deli fridge for 10min
- Remove from fridge cover sides of coverslip with clear nail polish