

### ***Thin-film coat bonding***

Low-temperature curing, mechanically patterned polyimide layers have been extensively used by *Northrup* in further experimentation with PCR-chips, either to bond together silicon-silicon ([Northrup1995], [Northrup1998]) or silicon-glass ([Hsueh1995]) structures. This later approach has been recently followed by other teams [Poser1997]. Although the technique is useful and permits the use of a wider range of materials than anodic bonding, the mechanical steps introduced in the patterning of the polyimide layer raise implicit problems for the production line and the scaling down of the devices. These problems could be partially circumvented with the use of photo-curable polyimide layers, but, to date, their compatibility with PCR amplification has not been demonstrated. Another variant of thin-film coat bonding has been the use of potassium silicate as an intermediate layer for glass-to-glass bonding by another  $\mu$ -TAS pioneer, *Michael Ramsey* [Khandurina2000].

### ***Sealing caps: inlets and outlets***

Providing hermetic sealing to PCR-chips is an essential prerequisite to conduct PCR, since denaturation (and even extension) temperatures will invariably induce evaporation of the PCR mix. Nonetheless, it is also obvious that some means for insertion and extraction of the reagents are required. Although an outlet is not strictly required with the adequate sensors (quantitative PCR can be enough of a result to the analyst) it is nevertheless recommended in case that ulterior studies need to be conducted and, besides, the presence of an inlet is mandatory since most PCR reagents will not withstand room temperatures. Thus, PCR-chips cannot be manufactured with reagents already into place. If they were, even if they were constantly held at freezing temperatures after manufacturing, the procedure would not be recommended due to the specificity and efficiency reasons already stated (see p.79) and because it would intrinsically limit the span of reactions that could be carried out with each chip (because every PCR must be custom tailored to the amplicate and template it deals with).

Even though the need for insertion/extraction systems is such a self-evident case in PCR-chips, approaches to the subject have been excessively vague and variable, or even casually ignored in reports, almost to the point of producing non-concluding results and leaving the issue, in contrast to

other PCR-chips aspects, still widely open. For instance, *Northrup's* original use of polyethylene tubes displayed easy capping characteristics (by physically clamping the tube), but was not reliable due to its encasing in silicone and to the fact that PCR evaporating material became stuck at the tube walls, thus diminishing efficiency. Hence, the encased tube approach has been generally discarded, posing the problem of how to cap the open input/output holes. In this sense, several capping strategies have been used. *Wilding et al.* ([Wilding1994], [Cheng1996a], [Cheng1996b], [Shoffner1996], [Wilding1998]) used mechanically pressured rubber washers to effectively seal their devices. Other teams [Poser1997] have gone back to gluing tube inlets and outlets, as in the case too, although necessary there, of continuous-flow PCR [Kopp1998]. Still, other teams have resorted to the usual capping (in eppendorf tubes, see p.68) with mineral oil ([Burns1996], [Northup1997], [Northup1998]) or to the use of acrylic biocompatible (and supposedly PCR-friendly) adhesive tapes ([Taylor1997], [Lin2000a], [Lin2000b], [Zhan2000]), while some have sacrificed sealing efficiency by removing the whole glass cover, sealed with varnish, for sample insertion and extraction [Murakami2000].

### **Thermocycling elements**

Thermocycling elements have been also developed in a number of ways, but they can be readily classified into two different groups: integrated and external cycling elements. Integrated heaters were first developed by *Northrup* using thin-film deposited polysilicon mounted onto a silicon nitride membrane ([Northrup1993], [Northrup1995]). This basic scheme has been later followed by other authors, typically using platinum [Zhan2000] or aluminum [Burns1996] thin-film deposited resistances. Other materials, such as indium tin oxide (ITO) have also been studied as thin-film heater and sensing elements, due to the transparent nature of ITO [Hsueh1995]. On the other hand, external cycling elements have been widely used for several reasons. The obvious one is the reduced design complexity and time they imply, since they do not need to be integrated and can be pasted at desired locations on the chip. Additionally, the most frequently used devices for cycling (see [Wilding1994], [Shoffner1996], [Cheng1996a], [Cheng1996b], [Wilding1998], [Taylor1997], [Lin2000a], [Lin2000b]), Peltier cells, exhibit dual operating regimes, rapidly heating or cooling their surface depending on the sense of the applied voltage (see *Materials and Methods*, p.305), and can thus provide better cooling times than passive-cooled integrated thin-film systems. Unfortunately, Peltier cells cannot be efficiently integrated in silicon, since the most efficient combination of PN junctions ( $\text{Bi}_2\text{Te}_3\text{-CuBr}$ )

cannot be easily deposited using CVD techniques [Fauquet1989]. Other external cycling devices include the development of an external thermocycler with three separate heat-blocks [Murakami2000] for rapid, mechanical cycling, or the use of three fixed heat-blocks in the continuous-flow PCR scheme [Kopp1998].

### Materials

Although there is a general trend in searching for new materials in PCR-chips and, in general, in the  $\mu$ -TAS field, the three major building blocks of PCR-chips have already been mentioned (plastic, glass and silicon) and each of them presents relevant advantages and disadvantages. Plastic (be it either polyethylene [Findlay1993], polycarbonate [Anderson2000] or another biocompatible layer) has the advantage of being a long used material in the biochemical arena that can be produced at low cost for disposable cartridges and at the microscopic scale using silicon chips as moulds [Becker1999]. However, the use of plastic hampers many of the technological steps required to integrate active circuitry and sensors onto the chips, forcing them to become passive cartridges in active macroscopic apparatus. In this sense, glass comes as an intermediate level on the material scale. Glass is also a deeply studied and used material regarding biocompatibility, and it allows some forms of micro-technology processes (as layer sputtering or chemical etching), but it still prevents, or sets hurdles onto, other integration processes (such as active circuitry, including photodiode sensors). Another relevant advantage of glass is its greater transparency (with respect to most plastics and, evidently, to silicon), although glass is not altogether transparent at some of the most useful UV wavelengths in molecular biology (i.e. denaturing UV light for sterilization).

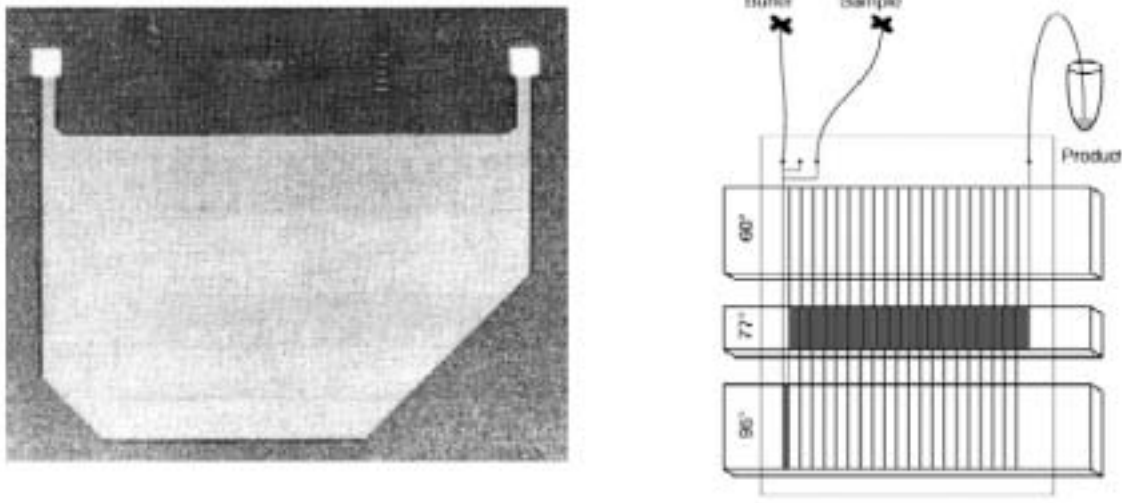
Finally, silicon has been, by far, the most widely used material in the field of PCR-chips. The reasons for this extensive use are many, but it seems clear that the availability of countless technological processes and the possibility of integrating almost all the necessary analytical tools into the chip make it a strong candidate for producing PCR-chips. In addition, chip production at large-scale also yields low-production costs (although not as low as plastic) and can even be accommodated to produce disposable (or shortly reusable) devices. Moreover, silicon is a better heat conductor than either glass or plastic, and has available a whole range of depositable materials of varying heat capacities that, together with deep silicon etching [Poser1997], can provide well-defined heat regions in the chip. Nonetheless,

silicon poses some strong problems for conducting PCR in chips. Apart from its natural opacity to UV and visible light wavelengths (which hinder detection if it is not carried out with integrated sensors [Burns1996]), silicon by itself has been reported as a strong inhibitor of PCR ([Shoffner1996], [Cheng1996a]), although other sources [Northrup1993] report no inhibition whatsoever. In fact, contradiction among the PCR-friendliness of the different Si-based layers described to date (mainly silicon dioxide (SiO<sub>2</sub>) and silicon nitride (Si<sub>3</sub>N<sub>4</sub>)) abounds in the literature, as on the success of silanization procedures and the use of adjuvants (like BSA) to improve yields. For instance, *Northrup* stated in his seminal paper that no silicon related materials were inhibitory to PCR [Northrup1993], but he silanized his chips "just in case". A more severe review of the inhibitory properties of silicon-related materials was carried on by *Schoffner's* team ([Shoffner1996], [Cheng1996a]) and concluded that pure silicon was a moderate inhibitor of PCR, whilst silicon nitride and silicon dioxide were more tolerant surfaces (silicon oxide being the best-rated surface). At the same time, they conducted experiments with silanizing products and obtained different results (with SurfaSil, previously described as a silanizing layer, being clearly inhibitory to PCR when untreated), but concluded that silanized surfaces provided unreliable results due to the own nature of silanizing processes and recommended thick (over 1000 Å) silicon oxide layers as the optimal PCR-friendly surface. These results have repeatedly been contradicted by the successful use of silanes by *Northrup* ([Northrup1995], [Northrup1998]) and *Poser* [Poser1997], but they have also been corroborated by repeated use of SiO<sub>x</sub> in other studies ([Wilding1998], [Burns1996], [Taylor1997], [Lin2000a], [Zhan2000]). In the case of adjuvants, BSA has been reported as a necessary adjuvant in several assays ([Taylor1997], [Lin2000a], [Murakami2000]) although its mechanisms of action are not altogether clear. It has been proposed that BSA may compete with polymerase for nonspecific adsorption at the chip walls, which becomes relevant in chips due to their increased surface/volume ratio, allowing larger amounts of polymerase to be available to the reaction [Taylor1997]. Nonetheless, the addition of BSA has also reportedly been disregarded as useful [Northrup1998].

### System designs

PCR reservoir designs have varied little and, until recently, have been even less commented upon. After the initial rectangular pool of *Northrup* [Northrup1993], most designs have followed the layout proposed by *Wilding et al.* [Wilding1994] (see Figure 45). A notable exception to this rule is the

serpentine-like layout advanced by *Manz* in his continuous-flow PCR system [Kopp1998]. Albeit its success, the design by *Wilding* has been lately criticized ([Lin2000a], [Lin2000b], [Zhan2000]) due to the extraction problems it poses, which shall be discussed in a later section (see p.131, p.142). *Lin et al.* have resorted to serpentine-like reservoirs, although it is not clear if the linear geometry produces any positive or negative effect on PCR kinetics. Conversely, *Zhan et al.* reverted to the rectangular pool used in the initial work of *Northrup*, an approach also taken by others [Taylor1997]. *Northrup* himself has regularly maintained the rectangular shape, although he introduced an innovative motif in design [Northrup1995], consisting on a dual heater (a chamber made by two etched and bonded pieces of silicon, each with its own thin-film heater) that prevents vapor condensation on the cover, in a similar way as conventional thermocycler hot-plates prevent evaporation within the eppendorf tube (see p.68).



**Figure 45** - *Wilding's* basic PCR-chip design and *Manz's* group serpentine-like constant-flow PCR-chip. Source: [Cheng1996a], [Kopp1998].

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## 2. OBJECTIVES

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### 2.1. GENERAL OBJECTIVES

Back in 1997, when the here-related doctoral work began, the time seemed ripe for the integration of the necessary technological setup and processes that should lead to a complete, multifunction and versatile DNA  $\mu$ -TAS. This was, at least, the state of affairs that could be readily glimpsed from a layman's point of view, following a quick survey of the existing literature in the field. At that time, PCR [Northrup1993] and gel electrophoresis [Woolley1994] chips had already been demonstrated in the laboratory and, all the while, jigsaw economic movements were being played fast on the DNA-array arena [Stipp1997]. Therefore, and in the light of the perceived situation, the present doctoral work was launched to carry out extensive research into the field, and with the main aim of developing and acquiring at CNM-IMB the necessary know-how to:

- Establish a common-ground technological setup for the creation of customized DNA  $\mu$ -TAS based on different and combined analytical processes.
- Standardize as much as possible the involved technological processes with regard to industrial production requisites.
- Generate a series of working and testable prototypes for the assessment of device functionality, versatility and inherent or derived advantages over conventional instrumentation systems.

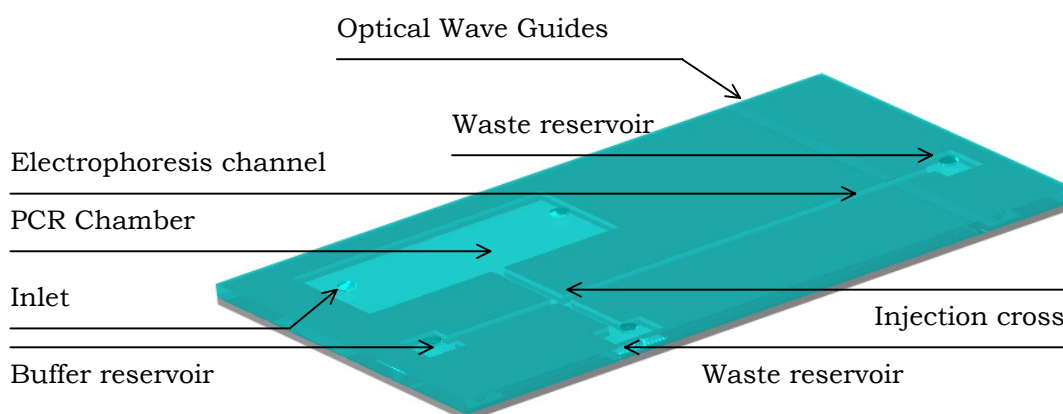
#### 2.1.1. STATEMENT OF OBJECTIVES

After some research into the, at that time, state of the art of DNA-chips, this manifestly generic set of objectives was considerably concretized, generating the more specific set of goals that is listed hereafter:

- To generate an independent and integrated DNA generic analysis system based on PCR and gel electrophoresis techniques (see Figure 46) and, to this effect:

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- To conduct the necessary research for the development, adaption and integration of the involved technological processes.
- To produce working prototypes in a proto-industrial batch-processing environment.
- To create the basic experimental setup for the functional test and evaluation of the manufactured devices.
- To conduct a thorough evaluation of the prototypes, in order to assess their prime advantages and capabilities, and yield positive feedback on the design process.



**Figure 46** - A DNA  $\mu$ -TAS system as proposed in the MUDA project. The chip integrates PCR reservoirs, heaters and control circuitry, together with optical wave guides for LIF detection. Heaters and control circuitry are placed on the backside of the PCR-chamber (not shown).

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The inherent shift and focusing of this more specific set of objectives was not without cause. On the one hand, PCR and electrophoresis were possibly the most extensively used techniques in the domain of molecular genetics and, besides their capability to bear sequencing assays (see p.46), they provided the necessary backbone for an extensive set of common analytical procedures (genetic fingerprinting, SNP detection, etc.). On the other hand, the other (at the time) mainstream market niche of DNA-chips (i.e. hybridization chips) was already entering a commercial stage, with its technological principles firmly established (and much patented) and large companies looming over the field. Hence, although a serious analysis and some prospective work on the subject were carried out, hybridization chips were deemed an untimely objective for technological setup development at a non-profit research center like CMN-IMB. Therefore, with this particular set of objectives, the necessary steps were taken to provide complementary

funding for this research and, to this effect, project MUDA<sup>7</sup> (Microsystems for Ultra-high speed DNA Analysis) was submitted to the CICYT<sup>8</sup> and ultimately granted in the late 1997. The goals of the MUDA project were a concretion of the doctoral objectives enumerated above, focusing on the development of a PCR-plus-electrophoresis DNA analysis system with integrated control circuitry and laser-induced fluorescence (LIF) detection (see Figure 46). But, although parallel in many respects, the goals of both the MUDA project and this doctoral work should not to be mistaken, even though, as will be detailed in due course, the project's role as the primary funding source for this thesis conditioned many of the research decisions and alternatives here taken. In this sense, a key aspect of the aforementioned divergence in objectives was the fact that, whilst MUDA project objectives were, to a large extent, fixed, the goals of the here reported doctoral work did evolve over time, as research shed light on different aspects DNA-chips and their inherent technology.

### **2.1.2. TECHNOLOGICAL HURDLES AND REDEFINITION OF OBJECTIVES**

Although the above set of objectives may, in retrospect, seem unduly overoptimistic, it was not considered thus (or not that much) at the time. In fact, it does hold true that the purely technological stages required to produce such a monolithic device were not insurmountable (albeit some issues, like the integration of wave-guide elements, posed by themselves many compatibility problems with other technological processes). Instead, what is made manifest by the candor shown in objectives design is the inability to foresee the many and multifarious challenges and hindrances such a kind of project was to face from its early beginnings, a set of problems that has been extensively detailed elsewhere [Erill2000b] and that is sketchily summarized in the following section. These quandaries stem mainly from the multidisciplinary nature of a project that involves handling and work with biological material at its core and, hence, requires critical optimization in biochemical, biological and materials technology. As research advanced, it became progressively clear that such a multidisciplinary approach could not be accounted for by a single technological group, but this conclusion, so obvious a posteriori, could only be obtained with the benefit of hindsight, a gift that life often bestows only upon experience.

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<sup>7</sup> MAUD - TIC97-0569 / Microsistemas para Análisis Ultra-rápido de DNA

<sup>8</sup> Comisión Interministerial de Ciencia y Tecnología



## **Technological hurdles**

Not considering the main silicon-technology problems posed by the integration of different active systems (electrophoresis, PCR, optical detection, etc.) in a monolithic device, the development of the project was plagued from its outset by problems arising from the biochemical arena. The main problem came from the different substrates and reagents demanded both by PCR and electrophoresis, suggesting that the two processes had to be somehow physically separated even in a monolithic system [Erill2000b]. At this point, it was decided to split research in two parallel branches: PCR and electrophoresis chips, putting a strong emphasis on the compatibility of the technological processes used in order to allow an ulterior feasible integration of the two modules. Within this framework, electrophoresis and PCR chips were developed, and both electrophoresis and PCR (albeit with very low efficiency) were demonstrated in chips [Erill2000b]. Nevertheless, there were still fundamental hurdles to be considered in many major areas, such as detection in electrophoresis chips or optimization and experimental setup development in PCR-chips. The multifaceted nature of these hurdles, when combined with the limited man-labor the operating budget conferred and the determined aim of yielding fully-operational, working demonstrators (which implied dedicated optimization work) at the end of the present research, led to an ultimate concretion of efforts and to the final declaration of objectives that conforms the main showcase of this doctoral work.

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## **2.2. SPECIFIC OBJECTIVES**

In essence, it was decided to center all efforts in the development of functional PCR-chips, setting temporarily aside gel electrophoresis devices, in order to obtain working PCR-chip prototypes that could be characterized, optimized and brought to a pre-industrial production stage. In practice, this goal was to be distributed among the following specific objectives:

- To develop, ready, adapt and standardize the necessary technological processes for the production of PCR-chips, maintaining a close, but more relaxed, watch on compatibility with electrophoresis chips.
- To validate PCR amplification in passive (without integrated heaters) PCR-chips.

- To develop a custom experimental setup for PCR-chips test and functional optimization.
- To optimize PCR amplification in chips using passive PCR-chips and the aforementioned experimental setup.
- To envision, study and develop the technological processes for the production of active PCR-chips and to fit them to the ongoing processes of passive PCR-chips.
- To adapt (or make anew) a custom experimental setup for test and functional optimization of active PCR-chips.
- To validate and optimize PCR amplification in active PCR-chips.
- To make a comparison study of yields and versatility between active and passive PCR-chips.

This final set of objectives, together with the work-plan they imply and the previous work on basic design and technological development, conform the basic outlines of the doctoral work here reported. Although it may not be explicitly pointed out hereafter, it must be noted that many design and technological constraints and options can only be fully understood in the light of previous developments both in PCR and electrophoresis chips.

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### **3. BASIC DESIGN AND TECHNOLOGICAL DEVELOPMENT**

Although PCR and electrophoresis chips (and the different subclasses of PCR-chips) differ substantially in many respects, there is a set of common features and design strategies that apply to them all, and this fact is of relevant interest in this work due to the ultimate goal of integrating these different kinds of chips in a monolithic device. Hence, the following section covers some broad aspects of design and process methodology that were early assessed in the development of DNA-chips at CNM-IMB. Many of these features are shared by PCR and electrophoresis chips (or between active and passive PCR-chips in later cases) and, thus, some of the compromise strategies adopted hereafter can only be understood in the light of this sharing. The bulk of this initial process development has already been partially reported [Erill2000b] and, for further particulars, the reader is referred to this previous account if, in some respects and for reasons of economy of space, the decision-making or technological processes are too sketchily depicted here. Finally, as it is prone to happen in any developmental process, the different turning points and strategies followed during the development of this doctoral research do not turn up to be all neatly arranged in a chronological manner, as they usually come about from dead ends and trial-and-error paths. However, they are exposed here in a seemingly way for the sake of clarity, although the reader is warned that such a false chronological account may occasionally render a little incongruence along the road.

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#### **3.1. SUBSTRATE TECHNOLOGY**

The very basic outline for both PCR and electrophoresis chips was made plain by previous work on the subject (see p.48). DNA analysis chips required a substrate into which reservoirs and capillary manifolds could be defined. Additionally, the systems should provide the means for hermetic sealing, coupled with inlets and outlets, and, in some cases, for the integration of particular technological gadgets (such as heaters in PCR-chips or electrodes in electrophoresis chips). What was not so evident, judging on the available literature, was which of the possible technological substrates provided the best platform to develop DNA-analysis microsystems. The choice of substrate is a fundamental one, since it determines the technological toolset available and, as it will be seen, places strong constraints on key developmental issues. Hence, the selection of a

substrate for developing DNA-analysis chips was a foremost pre-requisite for launching the present work and, consequently, it was the first issue to be addressed. The following is a reasoned account of this selection process.

### 3.1.1. SILICON OVER GLASS

As mentioned before, glass and silicon exhibit many intrinsic advantages in the development of PCR-chips (see p.102). This basic picture is undoubtedly altered by the inclusion of electrophoresis chips, but the main lines of reasoning described earlier still apply. In some respects, glass is an ideal candidate for the production of DNA-analysis chips, since it presents many inherent advantages over silicon. For instance, glass is a ready substrate for conducting electrophoresis, since it is a strong electrical insulator, a fact that allows the outright application of kV order voltages for separation procedures. Silicon, in contrast, is a known semiconductor and quite an efficient conductor in the kV range. On the other hand, glass is translucent to both visible and a wide range of UV wavelengths, making it a suitable substrate for the straight coupling of optical detection and stimulation devices. Regarding biocompatibility, glass has a long history of laboratory use and, although it can be a source of undesirable ions in some applications, it is usually regarded as a convenient biocompatible material.

Nevertheless, glass does also carry some disadvantages. For instance, glass is a much poorer heat conductor than silicon, a fact with key implications in PCR-chips. Moreover, although they are mainly translucent, glass substrates convey background fluorescence noise that can hinder fluorescence detection at certain wavelengths. However, the main drawback of glass resides in the technological toolkit it bears. Although glass can be easily micro-machined to produce channels and reservoirs, its amorphous nature poses some restrictions on the types of three-dimensional structures it can yield. Furthermore, many sputtering techniques for depositing electronically interesting layers are averted by the use of glass, since many sputtering methods rely on temperatures beyond the glass melting point. Finally, glass is not an active semiconductor substrate, precluding the integration of active circuitry within chips, be it either data conditioning or sensing circuitry (as in the case of integrated optical diode detectors).

Bearing all these facts in mind, the choice between these two rivaling technologies was made on the grounds of practical estimations for technology set-up times and, mainly, of the intermingling limitations and

advantages on further development posed by each technology. In this respect, it must be noted that CNM-IMB enjoys a potent infrastructure for silicon processing, with a class-100 clean room facility and a wide set of standardized processes that range from conventional thin-film depositions, oxidations and CMOS processes to advanced micro-machining and encapsulation processes. Even though CNM-IMB facilities could also be used for the main aspects of glass micro-machining technology, the fact is that, to date, the required technological process setup to accommodate glass micro-machining has not been developed. Hence, although glass exhibited some interesting advantages for an easy start-up on the path towards DNA-analysis chips (biocompatibility, transparency, electrical insulation, etc.), setup times for glass basic processing were estimated to be well over one year, clearly undermining much of its initial boon. Moreover, and more fundamentally, glass technology limited the extent to which active circuitry and sensors developed in the silicon arena could be integrated in subsequent devices, a fact that was perceived as a major drawback in a silicon-oriented research facility like CNM-IMB.

As a final remark, it must be noted that plastic, another candidate technology for the production of DNA-analysis chips, was disregarded from the onset. There were two main reasons for this overlooking. On the one hand, plastic micro-fabrication techniques were not approachable with the equipment available at CNM-IMB facilities and the necessary setup costs for such a technology exceeded by far the budget limitations this doctoral work operated upon. On the other hand, in the case of plastic, restrictions on the functional integration of new components were even more acute than in the case of glass.

### **3.1.2. SILICON SUBSTRATES**

In light of all this, it was decided that the best option was to face the different limitations of silicon for DNA-analysis chip development (passivation issues both for biocompatibility and electrical insulation, optical detection strategies, etc.) since, if overcome, they would lead to a scalable platform for the integration of additional functionalities in DNA-analysis chips, and they would also contribute to the existing know-how at CNM-IMB in silicon processes related to insulation and biocompatibility issues. In this respect, P-type 100-silicon wafers (instead of N-type) were chosen as the candidate substrates, since electrophoresis procedures would

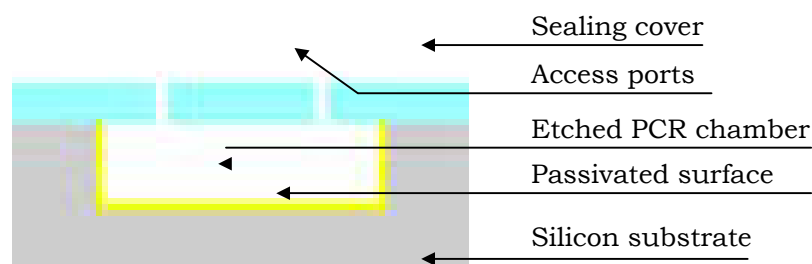
benefit from more resistive (P-type) silicon and the 100-orientation guaranteed an easy subsequent integration of electronic circuitry.

Once the substrate technology had been specified, there were several technological issues that had to be addressed in order to develop DNA-analysis chips. Although the following discussion is centered on the case of PCR-chips, electrophoresis chips also determined some of the technological options undertaken, as it will be pointed out hereafter.

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### 3.2. THE BASIC BAUPLAN

As already mentioned, the basic *bauplan* for producing PCR-chips was easily deduced from previous work in the field ([Northrup1993], [Wilding1994]). A simple, passive PCR-chip (see Figure 47) basically required an etched reservoir (possibly with a treated or passivated surface), capped with some sort of sealing mechanism and access holes (or ports) for reagent insertion and extraction.



**Figure 47** - Basic *bauplan* for a PCR-chip.

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Although such a basic blueprint does not present a priori insurmountable problems in silicon micro-machining, there were some intermingled key technological issues that, both for practical and economical reasons, had to be dealt with in order to generate a feasible and scalable technological line of attack on PCR-chips. This was all the more so when electrophoresis chips, with their own characteristic requisites, were also addressed.

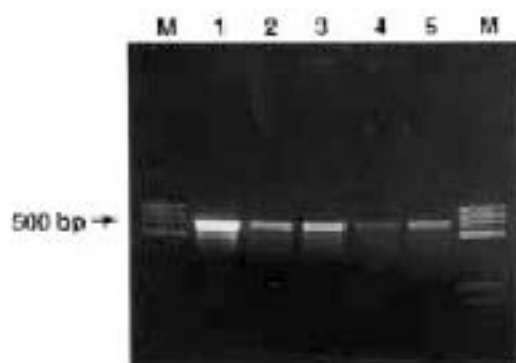
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### 3.3. SURFACE PASSIVATION AND SEALING

#### 3.3.1. PASSIVATION ISSUES

Even though, at the time this research started, almost all the work done on PCR-chips had been carried out in silicon ([Northrup1993], [Wilding1994],

[Northrup1995], [Shoffner1996], [Burns1996]), the most serious approach to biocompatibility issues [Shoffner1996] had already revealed that silicon might pose serious inhibition problems in PCR, and hinted at the use of silicon dioxide as the best PCR-friendly material for conducting PCR (see Figure 48). Another alternative, advanced by *Northrup* and other researchers [Northrup1993], was the treatment of the silicon surface with silanizing agents, but this option faced reproducibility problems [Shoffner1996] and it also brought shadows on possible fabrication yields, since it introduced chemical steps outside the stringent boundaries of conventional silicon-processing techniques. On the other hand, silicon dioxide (and preferably thermal  $\text{SiO}_2$ ) was also the obvious candidate surface for developing electrophoresis chips, since it is a well-characterized and potent electrical insulator that could theoretically allow the application of the kV order voltages necessary for conducting electrophoresis on a chip. Hence, it was decided to establish silicon dioxide as the main surface passivation layer, a fact that would have retroactive implications in the design and elaboration of sealing mechanisms.



**Figure 48** - Agarose gel of PCR-chip products. M lines correspond to *HaeIII* ladders, while line 1 is a thermocycler-run control and lines 2, 3, 4 and 5 correspond, respectively, to native silicon, thermally grown  $\text{SiO}_2$ ,  $\text{Si}_3\text{N}_4$  and CVD deposited  $\text{SiO}_2$ . Source: [Shoffner1996].

### 3.3.2. SEALING METHODOLOGIES

Surface passivation and sealing are intimately linked issues in the development of PCR (and electrophoresis) chips, for the obvious reason that the sealing cover must be somehow bonded to the surface passivation layer. Several techniques for sealing can be readily thought of. The obvious one, approached by *Northrup* in his seminal work [Northrup1993] was to simply glue a glass cover to the chip surface using standard silicone rubber as the intermediate layer and inserting polyethylene tubes to provide access ports (see p.96). Nevertheless, easy as it may seem, this technique has

fundamental flaws when approaching a mass-production scheme, and must be handled with extreme care to avoid invasion of the PCR-chamber by silicone. A more methodological approach for using intermediate layers as bonding agents was addressed using Mylar polyester film, but the results [Eri12000b] yielded poor and non-efficient bonding, with frequent invasion of the PCR chambers by the Mylar film and degradation of the bonding when exposed to DNA denaturing (~100 °C) temperatures (see Figure 49). Although, in retrospect, it seems sensible to consider that other alternatives (like photo-curable polyamide coatings) could have been tried out, they have not been reported to date in PCR-chip technology and it is not altogether clear if they would withstand denaturing temperatures or the more stringent conditions of autoclave (a useful methodology for chip reuse, which is a helpful advantage during the prototyping stage; see *Materials and Methods*, p.316), nor if they would pose any compatibility problems with PCR.



**Figure 49** - Non-homogenous bonding and invasion of the PCR chambers by Mylar film.

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### **Anodic bonding**

Once the use of intermediate polymer layers was discarded, alternative bonding techniques were sought in the literature. Usual alternatives in silicon processing are glass-silicon ([Quenzer1992], [Legtenberg1994]) or silicon-silicon [Field1990] bonding at low temperatures, but both these approaches imply spinner deposition of a borosilicate intermediate layer (typically  $\text{Na}_2\text{O}:\text{SiO}_2$ ) that would require a complex setup at CNM-IMB for spinner deposition in deep etched wafers and which has unknown effects on both PCR and electrophoresis. Silicon-silicon direct bonding can also be obtained at high temperatures without intermediate layers [Barth1990], but, although silicon-silicon bonding had been approached by *Northrup* in his dual-heater



scheme [Northrup1995] (see p.103), using silicon as a cover lid blocked the way for fluorescent or otherwise optical detection schemes in electrophoresis and PCR, and was considered an excessive risk in initial development of these devices, since it precluded visual inspection (and thus possible optimization) of the same processes.

A tried alternative for bonding glass and silicon wafers was available at the time at CNM-IMB. Anodic bonding, first developed in 1969 [Wallis1969], is a method for bonding glass and silicon wafers at low temperatures (see *Materials and Methods*, p.293). However, the technique is very sensitive to impurities (see Figure 50) and changes in its coupling layers, and a systematic characterization was required in order to deal with the silicon dioxide passivation layer necessary both in PCR and electrophoresis chips.



**Figure 50** - Electrophoresis cross-injection chip with bonding anomalies. Micrometer impurities lead to millimeter non-bonded areas.

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### ***Characterization of anodic bonding***

Anodic bonding of SiO<sub>2</sub> passivated silicon wafers had been previously reported in the literature [Kanda1990], but it required the use of higher temperatures that ranged well around the glass melting point, a fact that might pose problems in the definition of channels and reservoirs. In addition, a systematic analysis of the silicon dioxide thickness range anodic bonding could handle had not been conducted, and it had to be assessed if the necessary thickness for electrical isolation of electrophoresis chips (above 1  $\mu\text{m}$ , see [Erill2000b]) could be effectively bonded.

In a preliminary bonding assay, two possible problems were addressed. First, it was checked whether the narrow channels of both PCR and electrophoresis chips could be invaded by glass softened at the higher temperatures of SiO<sub>2</sub>-passivated wafer bonding, an effect that often occurs in wafers with extensive non-etched regions and narrow etched bands. Secondly, it was assessed if the relatively short amount of bonding surface of PCR-chips (the major part of the surface of a PCR-chip corresponds to the PCR reservoir and is not available for bonding) was enough to report efficient bonds. The experiment was done with bare (non-passivated) PCR and electrophoresis chips that were anodically bonded under SiO<sub>2</sub> bonding conditions (see *Materials and Methods*, p.295), producing satisfactory results in both instances.

The second issue concerning anodic bonding characterization was also critical, since it would determine the maximum silicon dioxide layer thickness that could be used in the chips. Although in PCR-chips there were not explicit prerequisites for SiO<sub>2</sub> layer thickness, a general rule of thumb (the thicker the better [Shoffner1996]) had already been introduced in the field and the general trend was to use maximal silicon dioxide layers to minimize any inhibitory interactions with the subjacent native silicon. Additionally, SiO<sub>2</sub> layer thickness in electrophoresis chips was of the utmost importance, since it would limit the strength of the electric fields that could be applied to the system and, thus, put an upper limit on the resolution and efficiency of these devices (see p.34).

Hence, a battery of tests with incremental oxide layer thickness was carried out in order to determine the maximum *bondable* SiO<sub>2</sub> layer. Silicon dioxide layers of 1200, 2400 and 4800 Å were grown by wet chemical oxidation (see *Materials and Methods*, p.287) and anodically bonded using silicon dioxide bonding parameters (see *Materials and Methods*, p.295). The results revealed that effective bonding did occur with layers up to 2400 Å thick. Chips with 1200 Å thick layers could withstand both hot water baths and lateral mechanical stresses, while the quality of bonding diminished perceptibly at 2400 Å. Most significantly, 4800 Å layers yielded mostly poor and non-reproducible bonds and, although some chips thus bonded did withstand the stringency controls above mentioned, it was decided that 2400 Å was the maximum effective passivation oxide thickness for anodically bonded devices. Although such an oxide layer sufficiently covered the passivation requirements of PCR-chips, it fell dramatically short

for electrophoresis chips. This last issue was successfully circumvented by further research on the bonding of thick oxide layers (up to 2  $\mu\text{m}$ ) capped with an anodic-bonding friendly non-doped polysilicon layer that allowed effective bonding while maintaining the device electrical isolation, leading to successful electrophoresis assays [Erill2000c].

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### **3.4. ACCESS HOLES**

#### **3.4.1. ACCESS HOLES STRATEGIES**

As previously seen (see p.100), the necessity for access ports is self-evident both in PCR and electrophoresis chips. At the time the present research started, the conventional approach to provide access holes in PCR-chips made without intermediate-layer covers was to use ultrasonically-drilled and polished wafers that can be readily obtained by sending mask specifications to the glass-wafer provider. Unfortunately, this single step multiplies by five the price of the glass wafers and, due to the limited budget this doctoral work operated on, it was decided to discard pre-drilled glass wafers, thus allowing a greater number of technological variation assays on other critical issues.

#### **Glass wafer access holes**

Having discarded pre-drilled glass wafers, the next available option was to manually drill the glass wafers at CNM-IMB or nearby facilities. Different methods can provide the necessary drilling. Chemical etching, for instance, produces smooth holes, but it required a lengthy setup process of glass lithography at CNM-IMB, an option that had already been discarded when dealing with substrate selection (see p.111). Laser and sandblasting drilling techniques, although available at nearby facilities, induce damage in bonding surfaces and can liberate loose particles under operation [Diepold1996], a fact that clearly impeded their use in the present research. Finally, ultrasonic drilling does theoretically provide neat drills, but when it was assayed at CNM-IMB (see *Materials and Methods*, p.295), the resulting glass wafers were not suitable for anodic bonding, even after extensive rinsing with de-ionized water and immersion in 10%HF baths.

Not having available the necessary polishing machinery for surface homogenization after drilling, and considering that the documented results for post-drilling chemical surface polishing [Diepold1996] did not suggest a

straight overcoming of the impurities of the ultrasonic drilling process, a last attempt was carried out to drill holes on already bonded glass wafers. Unfortunately, this procedure resulted both in the filling of channels and reservoirs with abrasive debris (see Figure 51) and, if badly timed, on the drilling of the subjacent silicon substrate [Erill2000b].



**Figure 51** - Electrophoresis cross-injection chip with two correctly drilled holes and a badly timed hole that perforates the silicon substrate. Stuck abrasive debris can be seen as clearer areas in the channel structure.

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### Silicon wafer access holes

In view of the impossibility of manually drilling access holes onto the glass wafer, it was decided to address the question from an opposite perspective: drilling the access holes on the silicon substrate by means of silicon micro-mechanization techniques. Although the method seems a bit counterintuitive (for instance, it does not allow direct observation of the channels and reservoirs during insertion and extraction) and complicates somewhat the technological process, drilling holes on the silicon wafer offered the advantage of fully controlling the production process at CNM-IMB, using first-hand known techniques, instead of relying on external and expensive facilities (like pre-drilled glass wafers), a fact that helps also to speed up redesign processes. Even though this approach was unheard of at that time, it was being simultaneously developed by other researchers [Taylor1997], although their lead has not been followed to date.

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## 3.5. DOUBLE-SIDE PROCESSING

Switching to silicon wafer access holes bears implicit a major change in the underlying technological process. Access holes need to be etched onto

silicon and, obviously, this implies the use of a patterned mask to etch the holes at the desired locations on the silicon surface. Unfortunately, spinner deposition of the necessary photo-curable resists (see *Materials and Methods*, p.288) cannot be achieved with reliable accuracy once the silicon wafers have been etched beyond 5-10  $\mu\text{m}$ , a depth that was clearly exceeded by both electrophoresis channels (50 to 100  $\mu\text{m}$  deep, see [Erill2000b]) and PCR reservoirs (100-200  $\mu\text{m}$  deep). The obvious alternative, to first etch the holes and then proceed with reservoir and other processes, was not feasible either, since it posed similar resist depositing problems and, additionally, it limited subsequent processes (like LPCVD) that are not prone to be carried out in perforated wafers. Hence, it was deduced that providing access holes through the silicon wafer implies both the use of double-side wafer processing and that holes should be opened at the last processing steps.

### **3.5.1. IMPLICATIONS OF DOUBLE-SIDE PROCESSING**

By its own, the use of double-side processing techniques conveys some major changes in the underlying technology. For instance, 300  $\mu\text{m}$  thick double-side polished wafers (instead of conventional 500  $\mu\text{m}$  one-side-only polished ones) had to be used, since they exhibit the maximum thickness the available double-side aligner at CNM-IMB can manage, and special alignment motifs had to be incorporated to allow double-side processing (see *Materials and Methods*, p.290). These issues have repercussions on chip design. For example, switching to 300  $\mu\text{m}$  wafers limits the depth of functional PCR-chambers (since a too thin membrane on the backside will not withstand the pressures exerted in insertion/extraction and washing procedures, see p.131). This limit on chamber depth, in turn, requires a compromise on the amount of wafer surface devoted to PCR chambers if equivalent volumes of fluids are to be handled. Other consequences of switching to double-sided processing are its influence on etching procedures and their optimization (which will be detailed hereafter) and the switch from  $\text{N}_2$ -flow drying to oven-evaporation drying of wafers to prevent wafer rupture after perforation.

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## **3.6. ETCHING PROCEDURES**

Etched structures, like channels and reservoirs, are a basic (not to say *the* basic) issue in both PCR and electrophoresis chips and, hence, they were addressed and optimized from the early beginnings of this work. In silicon

processing, there are two main standard options for etching wafers (and deposited layers): wet chemical etching and dry etching. As their names suggest, these techniques work on very different principles. Wet chemical etching makes use of chemical agents (etchants) that, in immersion, display selective etching behavior against some particular materials and/or crystallographic orientations. On the other hand, dry etching consists in the physical bombarding with electromagnetically accelerated particles to provide surface erosion (etching), although the most popular dry etching technique, RIE (Reactive Ion Etching), aims at getting the best from both worlds by using active chemical ions as the bombarding agents. As it is to be expected, both techniques yield quite different results and carry implicit advantages and disadvantages. Sketchily, conventional wet etching techniques are of easier setup, are typically more selective and classically produce trapezoidal shapes in 100-silicon, displaying selective (anisotropic) behavior towards silicon crystallographic orientation planes (a fact that can be a nuisance or a very welcome advantage in different applications [Ristic1994]). Conversely, dry etching techniques are typically harder to setup and less chemically selective, but they exhibit quasi-vertical etching profiles independently of the subjacent crystallographic orientation.

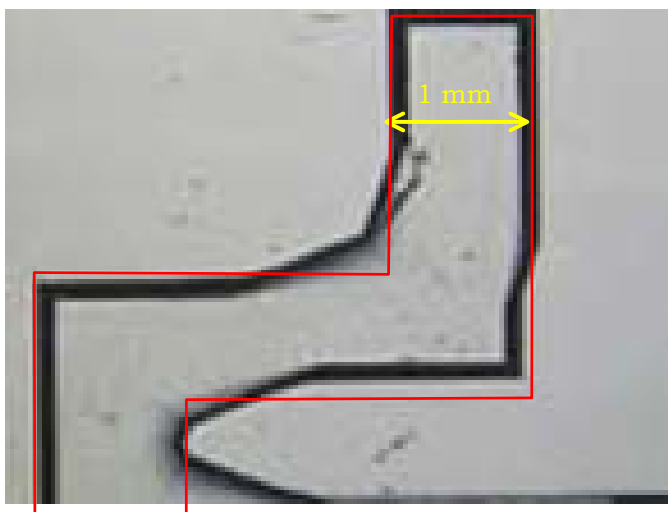
### **3.6.1. SELECTION OF ETCH PROCEDURES**

PCR and electrophoresis chips pose very different requisites on etching procedures. In electrophoresis devices, cross-injection schemes require regular and well-defined channel intersections at the microscopic scale, a feat that cannot be achieved with chemical etchants due to their weird behavior at convex surfaces. On the other hand, PCR chips require substantially deeper structures (beyond 200  $\mu\text{m}$ ), but are more tolerant to chemical etch anisotropic aberrations since their typical structures lie in the millimeter range (see Figure 52). Although using a single technique for both PCR and electrophoresis chips would have been the ideal choice, the depth of PCR-chip chambers was a limiting factor for RIE use, since around 200  $\mu\text{m}$  etching rates were on the verge of what could be RIE-etched using conventional  $\text{Si}_3\text{N}_4$  or  $\text{SiO}_2$  masks. Availing such etch depths with the RIE technology that was available at the time at CNM-IMB required the use of aluminum (or other metal) coatings as masks for the RIE process and, unfortunately, since CNM-IMB clean-room facilities accommodate both standard CMOS and micro-machining processes, such an initial use of aluminum would prevent wafers from entering oxidation furnaces (due to contamination problems), a key step in passivation of the devices. Hence,

since electrophoresis devices clearly demanded RIE resolution etches and PCR-chips called for deep, oxidation compatible etching, it was decided [Eri12000b] to use different approaches on PCR and electrophoresis chips, bearing in mind that, in principle, both processes could be made compatible by simply granting greater surface area to PCR reservoirs and backing to single RIE etches.

### 3.6.2. ANISOTROPIC WET CHEMICAL ETCHING

Due to the deep nature of the desired etches, isotropic etchants, like  $\text{HNO}_3:\text{NH}_4\text{F}$ , were discarded in favor of the more versatile anisotropic agents. At that time, two different anisotropic wet etching techniques were available at CNM-IMB for bulk micro-machining: potassium hydroxide (KOH) and tetramethylammonium hydroxide (TMAH). Although they do offer similar yields in anisotropic etching and material selectivity, KOH was discarded in favor of TMAH because the later does not contain any metallic ions (like  $\text{K}^+$  in KOH) that would lead to furnace contamination on subsequent oxidation steps.



**Figure 52** - Typical TMAH etch structural aberrations at convex surfaces. Detail of a serpentine-like PCR-chip.

#### Characterization of TMAH etching

TMAH attacks are usually carried out by immersing a wafer in a hot TMAH solution, yielding maximum  $60 \mu\text{m}/\text{h}$  etch rates ([Ristic1994], [Williams1996], see *Materials and Methods*, p.291 for details). To produce selective etching

at one region of the wafer, a silicon dioxide or silicon nitride mask layer is grown and patterned using conventional photolithographic techniques and chemical etching. Since the process of growing the mask layer usually also involves the backside of the wafer, TMAH attacks were conventionally conducted by simple immersion of the wafer in TMAH solution.

### ***Etch procedures and double-side processing***

When it was decided to switch to double-sided processing for the opening of access holes, the simple solution insertion method was seen as an easy way to cut down process times by simultaneously attacking both the front and back sides of the wafer. However, after some trials it was discovered that double-sided attacks induced also a lateral etching of the wafer, which, due to the prolonged nature of the deep attacks, resulted in a debilitation of the wafer structure at its extremes, yielding bristle PCR-chip wafers that often broke up under uncharacteristic low stresses [Eri112000b]. To circumvent this problem, TMAH etch procedures were modified, introducing a protecting PVC tool to protect the non-attacked side from the TMAH solution and to prevent (with a constant N<sub>2</sub> efflux) TMAH intrusion after perforation (see *Materials and Methods*, p.293). This new setup dramatically decreased the effects of TMAH on wafer debilitation, but it did not come for free. On the one hand, TMAH processing times were doubled, since two separate attacks were required. On the other hand, the PVC tool required a full centimeter of wafer radius for effective cramping of the wafer, thus limiting the wafer surface available for design, even though a similar (or even greater) safety distance had to be assumed to produce good yields with a double-sided simultaneous attack.

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## **3.7. MASKS**

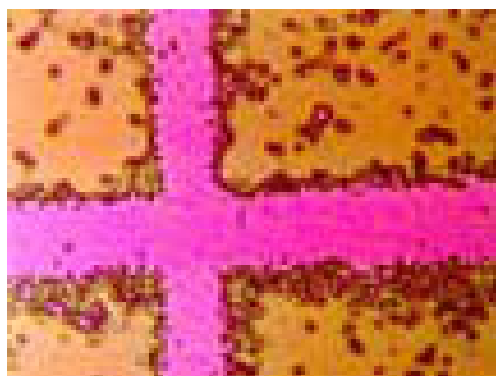
A last silicon technology issue that had to be dealt with at the early stages of this doctoral work concerns the development and optimization of a low-cost large-motifs mask process. Traditional silicon technology makes use of very small mask motifs (below 6 mm<sup>2</sup>) that are sequentially repeated across the mask or the chip by a stepper. However, PCR and electrophoresis chips require relatively large motifs (in terms of micro-technology, sitting in the centimeter scale). A standard rule of thumb in silicon technology is: bigger means costlier, and this statement does also readily apply in the case of photolithographic masks. Acknowledging double-side processes and the



possibility of creating active PCR-chips, the mean number of masks per run was estimated to range from 4 to 9, posing strong limitations on the number of technological runs that could be carried out with conventional masks due to limited available budget. Hence, as in the case of glass versus silicon access holes, it was decided to pursue some initial research lines devoted to obtain cheaper, large-motif mask processes that, although they could pose some restrictions on design, would permit research on a further number of technological and design options.

### 3.7.1. SLIDE FILMS

A first approach to cheap mask design was to use laser-jet slide films, printed with a standard Laserjet Series 4 (*Hewlett-Packard*, 300 dpi), even if the use of 300 dpi resolution (meaning roughly 85  $\mu\text{m}$  diameter dots) imposed some restrictions in design (harsher in the case of electrophoresis chips). The main problem with slide-film masks was the non-uniform ink deposition of the laser printer (see Figure 53). This problem was aggravated by the nature of the patterns and the type of photo-curable resists (negative) typically used at CNM-IMB (see *Materials and Methods*, p.288), which led to dark-field masks with predominant black (badly ink-covered) regions. In addition, slide films cannot be used in conventional steppers, since they are not rigid and cannot automatically be manipulated. Instead, they have to be carefully deposited by hand. Manual alignment was done on a S.E.T stepper at UAB class-10,000 clean room facilities, yielding poorly reproducible results, adding contamination factors and posing hard alignment problems in multi-step (not to mention double-side) processes.



**Figure 53** - Corrupt oxide layer in a cross-injection electrophoresis chip after patterning with a 300 dpi laser printer slide-film.

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