The importance of CMOS sensor surface topography in the attachment, proliferation, and differentiation of human adult mesenchymal stem cells

Christa Y. Heyward
The importance of CMOS sensor surface topography
in the attachment, proliferation, and differentiation
of human adult mesenchymal stem cells

A Thesis
Presented to the Bayer School of Natural and Environmental Sciences
Department of Biological Sciences
Duquesne University

In partial fulfillment of the requirements
For the Degree of Master of Science

By:

Christa Heyward

Thesis Advisor: John S. Doctor, Ph.D.

Thesis Committee Members:
Mary Alleman, Ph.D.
Phil Campbell, Ph.D.
Mark Miller, Ph.D.
Name: Christa Heyward

Thesis Title: The importance of CMOS surface topography in the attachment, proliferation, and differentiation of human adult mesenchymal stem cells

Degree: Master of Science

Date: July 6, 2004

Approved: ____________________________________________

Dr. John S. Doctor, Advisor

Department of Biological Sciences

Approved: ____________________________________________

Dr. Mary Alleman, Committee Member

Department of Biological Sciences

Approved: ____________________________________________

Dr Phil Campbell, Committee Member

Carnegie Mellon University

Approved: ____________________________________________

Dr. Mark Miller, Committee Member

School of Health Sciences

Approved: ____________________________________________

Dr. David W. Seybert, Dean

Bayer School of Natural and Environmental Sciences
Acknowledgements

I would like to thank Dr John Doctor for his excellent guidance and mentorship, both in research and in life. I would also like to thank Dr. Phil Campbell whose aid made the completion of my thesis possible. I would like to thank Fernando Alfero who fabricated all of the CMOS sensors investigated and who was ready and able to answer all of my questions regarding the fabrication process. I would also like to thank Dr. Mary Alleman and Dr. Mark Miller for serving on my committee. Finally I’d like to thank the members of the Doctor Lab both past and present: Rabecca Waddell, Hina Qidwai, Jade Leung, Shelby Hott, Katie Gallagher, Sara Otaibi, and Joe Tiano.
# Table of Contents

Title Page.................................................................i
Signature Page ..........................................................ii
Acknowledgements.................................................... iii
Table of Contents........................................................ iv
List of Figures............................................................... vi

Abstract .......................................................................................................................... 1

Background....................................................................................................................... 2
  Tissue and biomedical engineering ........................................................... 2
  Dynamic qualities of bone ................................................................. 3
  Current methods of measuring bone mineral density ......................... 3
  Implants and bone ........................................................................... 5
  Human adult mesenchymal stem cells ........................................... 6
  The effects of surface topography on the adhesion of osteoblasts and
  Osteoblast precursor cells to biomaterials ........................................ 7
  Cell alignment on implant surface .................................................. 10
  Assessment of osteoblast proliferation and the effects of topography on
  Differentiation .............................................................................. 13
  Complementary Metal Oxide Semiconductor ................................ 17
  Photolithography .......................................................................... 19
  Titanium coating ........................................................................... 20
  Applications for the CMOS sensor .................................................... 23
  Significance of the CMOS sensor ....................................................... 25

Hypotheses ...................................................................................................................... 26

Experimental Approach ............................................................................................... 26
  Attachment ....................................................................................... 26
  Proliferation .................................................................................... 27
  Differentiation ................................................................................ 27

Manuscript title page....................................................................................................... 29
  Abstract ............................................................................................ 30
  Introduction ...................................................................................... 31
  Materials and Methods .................................................................... 34
    Human adult mesenchymal stem cells ......................................... 34
    Sensor Texture/Coating Types ...................................................... 34
    Assessment of cellular attachment .............................................. 34
    Cell proliferation assessment ...................................................... 35
    Calcium assessment ....................................................................... 36
List of Tables and Figures

Figure 1.  hAMSC grown on a biomaterial .......................................................45
Figure 2.  CMOS sensor design ........................................................................46
Figure 3.  Using photolithography to create surface features .........................47
Figure 4.  Creating CMOS sensor topographies ..............................................48
Figure 5.  CMOS sensor topography .................................................................49
Figure 6.  Cells attach to CMOS sensors ..........................................................50
Figure 7.  hAMSC attachment on 2mm x 2mm CMOS sensors .......................51
Figure 8.  hAMSC proliferate after three days of incubation .........................52
Figure 9.  hAMSC proliferate after three days of incubation .........................53
Figure 10. Fluorescence indicates that hAMSC proliferate after three days ......54
Table 1.  Viability of hAMSC ........................................................................ 55
Figure 10.  hAMSC cover the surface topography of CMOS sensors ...............56
Figure 11. Representative experiment showing calcium deposition ...............57
Figure 12. Etch depth effect calcium deposition ..............................................58
Figure A-1  Topography assessment by side ..................................................60
Figure A-2  Topography assessment by sensor ..............................................60
Figure A-3  Two-way ANOVA analysis of induced calcium deposition ...........61
Abstract

Osteoblasts and osteoblast precursor cells (mesenchymal stem cells) respond to the surface topography of metal implants by aligning along surface features in order to minimize shear stress. While several previous studies have investigated the morphology, attachment and proliferation of both osteoblasts and mesenchymal stem cells along grooved surfaces, the current study investigates the cellular response to depressions and peaks in surface topography, as well as surfaces that contain alternating combinations of depressions and peaks. The Complementary Metal Oxide Semiconductor (CMOS) sensor is a transducer fabricated from silicon and sputter coated with titanium. The CMOS sensor is designed to utilize piezoresistive strain gauges in the detection of stress on bone. To the left and right of the piezoresistive strain gauges are surfaces that contain either depression (dimple) or peak (pimple) topographies. The CMOS sensor topographies were prepared using photolithographic processing, a technique that creates surface topographies in a precisely controlled manner. This study assessed the attachment, proliferation, and differentiation of human adult mesenchymal stem cells (hAMSC) on the depressions and peaks of CMOS sensors as a preliminary means of selecting surface topographies that might promote the greatest bone in-growth in vivo and therefore display the greatest osteoconductive properties. The distance above and below the nominal surface plane (etch depth) for surface topographies examined was either 15, 37, or 60μm. LIVE/DEAD viability staining and electron microscopy revealed that cells attach on all CMOS sensor topographies. LIVE/DEAD viability staining was also used to determine that once attached, cells proliferate on all topographies after 3 days of incubation.
Regardless of differences in initial cell attachment, after 14 days in medium containing an osteogenic supplement that promotes hAMSC differentiation into osteoblasts, comparable amounts of calcium were detected on all topographies. Although the specific topography (depressions, peaks, or the combination topography with both depressions and peaks) had no impact on the amount of calcium deposited on CMOS sensors, there was a significantly greater amount of calcium deposited on topographies with an etch depth of 15µm when compared to the same topography with an etch depth of 60µm.

**Background**

*Tissue and biomedical implants*

The study of implant integration is a topic of great interest in the fields of tissue and biomedical engineering. Tissue engineering is the construction of living tissues whereas biomedical engineering is the use of man-made materials in living systems (25). There are several types of implants currently under investigation. Some implants involve the construction of tissue inserts such as heart valves or growth plates, and are made from seeding stem cells and growth factors onto a scaffold (24). Other implants involve the production of entire organs such as kidney or liver as a means of combating the scarcity of donated organs. In the field of biomedical engineering, extensive research is underway concerning the use of metals and plastics engineered for biological functions. Investigation of the Complementary Metal Oxide Semiconductor (CMOS) sensor falls into the category of biomedical engineering and involves the use of a silicon sensor sputter-coated with titanium intended for applications in the measurement of stress on the bone.
Dynamic qualities of bone

Bone is formed by the mineralization of extracellular matrix by osteoblasts, bone producing cells, resulting in a hard and durable structure that is also very porous(35). The bone contains channels of living cells that act to resorb and rebuild the bone during the lifetime of an organism. Osteoclasts, a cell type derived from the macrophage lineage of hematopoietic stem cells, break down old bone, whereas osteoblasts, derived from mesenchymal stem cells, build new bone by depositing extracellular matrix material called osteoid and then calcifying the osteoid with calcium-phosphate crystals called hydroxyapatite. As osteoblasts produce bone, they become trapped in the bone they form and are transformed into cells called osteocytes. These cells then go on to secrete reduced levels of extracellular matrix.

The constant remodeling of bone allows the skeleton to adjust to long-term variations in load(35). Mechanical stress influences the deposition and erosion of bone. For instance, subjecting the skeleton to mechanical loads, as in weight-lifting, stimulates the differentiation of hAMSC into osteoblasts and stimulates the deposition of bone. The breakdown and rebuilding of bone must be delicately balanced. When osteoclast activity exceeds that of osteoblasts, the level of calcium-phosphate is diminished causing the bone to become brittle resulting in the disease osteoporosis. Excessive bone formation by osteoblasts results in a disease called osteopetrosis.

Current methods of measuring bone mineral density

The measurement of Bone Mineral Density (BMD) is an important tool used to determine the likelihood of fracture and osteoporosis. BMD measures the amount of
calcium in regions of bone(4). There are several methods currently used to measure BMD, all of which involve exposing a patient to some form of radiation either in the form of X-rays, photons or ultrasound. All of these methods calculate BMD by comparing how well a specific type of radiation passes through the bone of a patient in relation to a standard curve for normal premenopausal women(4). These methods are based on the inference that deflection of radiation must correspond to the thickness and strength of the bone. In no way do these methods measure actual strength directly. Current techniques also fail to identify some people at risk for osteoporosis because measurements are based solely on BMD and do not take into account other factors such as the actual shape and size of the bone or determine whether there are small fractures called microdamage that put a patient at risk for a break in the bone. None of these methods are used to monitor bones as they heal inside a cast or the integration of grafted bone material to the bone of a patient.

BMD is the main factor used to determine whether a patient is at risk or has osteoporosis. Osteoporosis occurs when mineral deposition in the bone is minimal causing the bone to become more fragile and likely to break. Osteoporosis is a disease that affects forty-four million people in the United States(2). Twenty percent of patients that have osteoporosis and that break a hip will also die within a year. Better methods of detecting osteoporosis will improve identification of patients susceptible to fracture. Early detection will improve the quality of treatment such as the prescription of drugs that aid in bone formation and incorporation of load-bearing exercises into the daily routine of a patient.
Implants and bone

Regenerative medicine encompasses several diverse areas of research including the growth of tissues and organs such as bone, improving the rate of recovery, and understanding the process of implant integration. Previous research has lead to the development of engineered resorbable bone substitutes with the ability to provide a scaffold on which new bone can form while delivering cells and growth factors to voids in the bone. The optimal implant displays both osteoconductive and osteoinductive capabilities(13). Osteoconduction refers to the ability of a biomaterial to support tissue growth by allowing capillary and cell in-growth resulting in the formation of a three dimensional bone structure, provided fully differentiated and competent cells are available at the site of implantation(13, 30). Osteoinduction is the ability of a biomaterial to cause pluripotent cells in an environment lacking bone to differentiate into chondrocytes and osteoblasts resulting in the de novo formation of bone.

The formation of bone on the surface of an implant occurs in four phases(13). First, there must be a morphogenic signal present and this signal must exhibit specificity in its target site for delivery. Host cells must next respond to the signal. Once mesenchymal stem cells encounter the morphogenic signal, there must be a scaffold on which the cells can proliferate. Finally, bone formation at the implant surface requires a vascularized host bed to support the formation of tissues. Inflammatory and mesenchymal cells are the first cells to enter a location during the formation of bone. This is followed by angiogenesis and the differentiation of precursor cells into chondrocytes which occurs about three weeks post-implantation. Soon after osteoblasts, osteocytes, and chondrocytes
are readily synthesizing a collagen scaffold that will be mineralized into bone. After four weeks post-implantation, osteoclasts are present at the implant-bone interface and by 4-6 weeks bone marrow has formed.

Bone is a very dynamic structure. The formation of bone on the surface of an implant involves complex interactions between cells that secrete collagen, the main component of the extracellular matrix, non-collagenous signaling proteins that are present in the extracellular matrix, and osteoblasts, which mineralize collagen into bone. Integration between any implant and the surrounding bone tissue is essential for the long term success of the implant(17). Assessment of an implant’s ability to integrate into bone involves a thorough analysis of the cell/extracellular matrix/surface interface. While this study was not intended to assess the CMOS sensor’s potential for integration in vivo, it does investigate in vitro attachment, proliferation and differentiation of human Adult Mesenchymal Stem Cells (hAMSC) on CMOS sensors with depressions, peaks, or combination topographies with both depressions and peaks. Mesenchymal stem cells were chosen for this investigation because they are one of the first cells to migrate onto an implant surface and they will likely play a role in the production of bone necessary for the integration of the CMOS sensor to bone.

**Human Adult Mesenchymal Stem Cells (hAMSC)**

hAMSC are a population of cells derived from the bone marrow stroma(21) that possess the ability to self-renew their population, as well as differentiate into chondrocytes, adipocytes, muscle cells, and osteoblasts (bone producing cells) in vitro and in vivo under specified conditions(19, 29, 33). hAMSC differentiate into osteoblasts
when cultured in media containing an Osteogenic Supplement (OS) (100mM dexamethasone, 10mM β-glycerophosphate and 0.05mM ascorbic acid-2-phosphate)(33). Dexamethasone is a steroid that promotes cell differentiation, β-glycerophosphate is a glycerol derivative that acts as a source of phosphate, and ascorbic acid-2-phosphate and β-glycerophosphate are important for the mineralization of collagen. Commercial populations of hAMSC are available from a variety of bone sources including iliac crest or tabecular bone(29).

When cultured in medium containing an osteogenic supplement hAMSC populations to differentiate into osteoblasts that mineralize the collagen in their extracellular matrix(19, 29). Osteogenic differentiation is characterized by an increase in Alkaline Phosphatase (ALP) activity, the formation of cAMP, and increased expression of osteogenic marker genes such as collagen (type I), parathyroid hormone receptor, osteonectin, osteopontin, and osteocalcin. In vitro analysis of hAMSC differentiation on biomaterials intended for use in bone is a critical first step in the assessment of a biomaterials potential for osteointegration. In figure 1 hAMSC were seed on a tricalcium-phosphate based bone void filler. hAMSC treated with medium containing an osteogenic supplement for 14 days were viewed with scanning electron microscopy and differentiation was assessed by measuring alkaline phosphatase activity.

The effects of surface topography on the adhesion of osteoblasts and osteoblast precursor cells to biomaterials

Adhesion of osteoblasts on biomaterials relies on extracellular matrix proteins, (fibronectin, collagen, laminin and vitronectin), cytoskeletal proteins (actin, talin and
vinculin), and membrane receptors (integrins) as well as other additional proteins(5, 6). These proteins are regulated by the interaction between cells and the topographical features of a surface(5). Cell adhesion is important because it lays the foundation for cell growth, migration, and cell differentiation(20). Understanding how cells respond to surface characteristics such as topography, will allow for improvements in the integration of biomaterials after implantation.

Cell adhesion to biomaterials occurs in regions called focal contacts(5). Focal contacts are located at the cell surface and connect the extracellular matrix outside the cell to cytoskeletal elements inside the cell through a membrane-bound receptor such as integrin. Integrins play a central role in cellular adhesion by mediating a cellular response to surface topographies in the extracellular environment. Integrins govern interactions between the cell and the substrate for adhesion(20). The ability to bind various ligands is enhanced by the large number of members in the integrin family. Each member is a heterodimer composed of one alpha and one beta sub-unit. Integrins help to translate the attachment of external ligands into internal signals that regulate cellular responses such as cell growth and differentiation.

Integrins regulate cellular behavior by acting through signaling pathways shared with growth factors(11). When osteoblasts are cultured on textured surfaces integrins respond by regulating genes involved in the differentiation through the protein kinase C (PK-C) and MAP kinase pathways. These are the same pathways used by regulatory factors such as vitamin D and estrogen. One gene regulated by the interaction between integrins and the extracellular environment is TGFβ-1, a gene known for its promotion of osteogenesis. Increasing the amplitude of topographical features can increase the
response of integrins. In addition, a synergistic response occurs when osteoblasts are cultured on textured surfaces in the presence of regulatory factors.

The extracellular matrix plays an important role in mediating osteoblast adhesion to biomaterials(20). Fibronectin and vitronectin are extracellular matrix proteins that promote the formation of focal contacts and therefore mediate cellular attachment(5, 20). They bind to integrins in the extracellular matrix initiating a signaling cascade that results in the enhancement of osteoblast differentiation. Use of sera devoid of vitronectin and fibronectin significantly reduces levels of cellular attachment and spreading on patterned surfaces.

Surface roughness is a term used to describe both the amplitude and organization of topographical features on the surface of a biomaterial(7). Surface topography is a term that characterizes the undulations, steep gradients or pores which make up the landscape of a surface(9). Surface topography is usually described in terms of the distance above or below the nominal surface plane of a material (amplitude). The amplitude of topographical features with which osteoblasts or mesenchymal stem cells will come into contact is important because cells respond to discontinuities on the surface of a biomaterial by condensing and organizing the nucleation of actin which goes on to affect gene regulation as previously described(7). Surface topography is also important in cellular attachment, proliferation, and differentiation(5-7, 17).

Topographical features on the surface of an implant play a significant role in the ability of osteoblasts to attach, proliferate and differentiate at the site of implantation(17). Surface topography plays a significant role in cell adhesion(5, 7, 17). Surfaces with topographical features that have a high amplitude, distance above or below the nominal
surface level of the material, improve the alignment of cytoskeletal elements and improve cellular attachment over the course of several weeks when compared to tissue culture plastic(10). Cells appear elongated on surfaces with topographical features and this elongation results in a higher density of focal contacts along the edges of grooves.

*In vitro*, osteoblast “integration” refers to the adherence of osteoblasts to their substrate during the early stages of culture when osteoblasts are in direct contact with the substrate or, later on, the interaction between osteoblasts, extracellular matrix, and the substrate(10). *In vivo*, integration implies the creation of a cell/extracellular matrix/substrate interface. While osteoblasts initially exhibit a lower level of cellular adhesion on rough surfaces at the cell/extracellular matrix/substrate interface, adhesion increases with time so that by days 14 and 21 all rough surfaces had a significantly higher level of cellular adhesion than osteoblasts cultured on tissue culture plastic. Increased surface roughness is also associated with improved bone-to-implant shear strength(12).

Using rat bone marrow cells and human osteoblasts, Anselme and coworkers(7) determined that optimal surface roughness for the complete incorporation of an implant combines topographical features that extend a large distance above or below the surface of a material with a relatively low level of organization. This type of surface improves cell organization and promotes higher levels of mineralized extracellular matrix. Cells displayed the greatest response when topographical features were between 10 and 100µm. Chaotic surfaces with a large amplitude result in diminished levels of cell adhesion. This is likely due to osteoblasts inability to establish enough contact area with substrates.
Cell alignment on implant surfaces

Contact guidance is the phenomenon by which cells elongate in response to the topography of a substratum(26). Surface topography forces cells to modify their morphology that eventually results in changes in gene regulation(11). Cells respond to surface features by altering their shape, orientation, and polarity of movement(26, 34). The ability of cells to align along topographical features is governed by the depth and width of surface features(34). Contact guidance is the result of mechanical forces, such as stress or tension, on the filopodia of cells, which force the cells to reshape actin filaments and adjust to substrate topography. Cells align with substratum topography in order to minimize distortions to their cytoskeleton(26). Both the pitch of a grooved surface, defined as the distance across one groove and one ridge, as well as groove depth play a significant role in the alignment of osteoblasts along the surface of a material. Lu and coworkers found that groove widths of 24µm (or a pitch of 50µm) resulted in the most rapid cellular alignment on grooved titanium surfaces(23). They also concluded that topographical features similar to cell size (~20µm) exhibited the greatest effects on cell shape and orientation. Osteoblasts have been shown to align parallel to grooves as shallow as 5µm deep(5).

Oakley and Brunette(26) found that the alignment of cytoskeletal elements occurred in a chronologically ordered fashion: first microtubules, then micro-filaments, and finally focal contacts. Fibroblasts lacking microtubules fail to align along vary narrow grooves whose pitch was less than 1µm and required more time to align along topographical features than cells containing microtubules when the pitch was not as narrow (6µm or wider)(27). Microtubules could therefore be responsible for cellular
alignment on grooved titanium surfaces. Osteoblasts on grooved surfaces exhibit a smaller area and are not as flat as osteoblasts on smooth surfaces(26). Cells on smooth surfaces do not orient themselves in any particular direction. This is supported by the fact that cytoskeletal elements lack orientation when viewed with confocal microscopy(26).

Elongated cells aligned with the surface topography exhibit greater adhesive properties than more spherical cells(28). Eisenbarth and coworkers(17) showed that elongated cells have a higher density of focal contacts along the edges of grooves as well as better organization of the cytoskeleton and stronger actin fiber networks. Stronger actin fiber networks increase cell stiffness and allow cells to resist detaching shear forces. An increase is groove depth (Ra= 0.08 - 1.4µm) below the size of the cell (microroughness) results in an increase in the number of aligned cells. Eisenbarth and coworkers(17) also showed that cells on structured surfaces stop migrating 2 days after plating while cells on smooth surfaces continue to move and extend lamellopodia indicating that they have not formed focal adhesions protecting them from shear stress as the cells on structured surfaces have done. Cell alignment is also important in the prevention of scar tissue(32). Roughened and porous surfaces have been associated with the promotion of surface integration and biologic fixation however, rough surfaces may also result in random cell alignment that can give rise to scar tissue not seen on grooved surfaces.

Cell migration occurs more rapidly as groove depth increases indicating that grooved surfaces do not represent a barrier to cell migration(5). Focal contacts are evenly distributed on smooth surfaces in contrast to surfaces with topographical features where focal contacts are apparent at the extremities of cellular extensions which contact the
substratum. Smooth surfaces do not present enough discontinuities or the amplitude of discontinuities is too low to permit cell orientation and thus results in a lower level of osteoblast adhesion(10). Bigerelle and coworkers(10) concluded that human osteoblasts prefer surfaces with a high amplitude above the scale of cell size, roughly 20µm. Below cell size, cells adhere best when micro-roughness amplitude is low and the surface features are not ordered. Isoetched surfaces (described later) often exhibit few peaks or surface features at a scale below cell size but present precisely regulated topographical features at a scale above cell size. Bigerelle and coworkers(10) used isoetching to produce “bowl-like nets” with an etch depth of 0.3 - 3.3µm and demonstrated improved osteoblast adhesion on these topographies. These findings were used to select topographical designs for the CMOS sensor.

Assessment of osteoblast proliferation and the effects of topography on differentiation

Surface topography is important in the mediation of cell signaling pathways involved in the differentiation of hAMSC into osteoblast. Osteoblasts on smooth surfaces attach and proliferate however their expression of differentiation markers is minimal(11). Osteoblasts cultured on surfaces with topographical features exhibit decreased proliferation but display an increase in the expression of genes associated with differentiation such as collagen type I, TGF-β-1, and osteocalcin. In addition, studies show mouse calvarial osteoblasts increase their level of extracellular matrix protein after two days of culture on grooved surfaces compared to smooth surfaces(32).

Studies on the impact of grooved surface topographies on proliferation indicate a correlation between increased amplitude of topographical features and decreased levels of
proliferation. This is most likely due to the fact that these topographies significantly increase cellular differentiation when compared to flat surfaces. Decreases in proliferation are frequently coupled to an increase in cellular differentiation. When attachment and proliferation of osteoblast-like cells are compared between grooved surfaces and rough surfaces, grooved surfaces supported improved cell adhesion and proliferation(20).

While increases in peak amplitude have been associated with an increase in cell proliferation(10), most studies involve the analysis of osteoblast behavior on substrates that vary in surface roughness. Increases in surface roughness involve both an increase in the amplitude of topographical features as well as an increase in the randomness of the pattern in these features. Increasing the surface macro-roughness correlates with decreased levels of proliferation and alkaline phosphatase activity(5). Collagen synthesis and the synthesis of osteocalcin, however, increase with increased surface roughness.

There are several explanations for why topography plays an important role in osteogenesis(17). Increased depth of topographical features may permit the formation of multiple layers of cells resulting in higher cell density of osteoblasts, aid in the orientation of collagen fibers, or create alterations in cell shape that result in changes in gene expression(28). Increases in surface roughness and the amplitude of topographical features affect osteoblasts in many ways(11). The shape and orientation of osteoblast-like cells affects their differentiated phenotype(22). Increases in surface roughness are also associated with an increase in prostaglandin production resulting in enhanced osteogenesis(11). The amount of prostaglandin produced is dependent on the amplitude of topographical features. Inhibition of prostaglandin production prevents the
enhancement of differentiation seen on textured surfaces. The effect of surface topography on osteoblast differentiation depends on the maturation state of the cell. More mature cells are less sensitive to the effects of topography than an immature cell. Grooved substrata are capable of increasing the number of bone-like nodules formed in vitro by osteoblasts from rat calvarial bone when compared to nodule formation on smooth surfaces(28). In addition, the number of nodules increased with increasing groove depth. In vivo studies corroborate these results showing increased mineralization on titanium surfaces with a 30µm groove depth when compared to topographies with a 19µm groove depth.

Previous investigations of the connection between cell shape and the expression of genes involved in differentiation found that induction of a round shape promotes chondrogenic differentiation while induction of a flattened shape promotes differentiation into fibroblasts(16). Osteoblast precursor cells become polarized upon differentiation, meaning they display a specific direction of migration(16, 27). Grooved substrata promote the polarization of osteoblasts along their dorso-ventral axis enhancing their ability to differentiate. Topographies with deeper grooves may improve the alignment of cells by concentrating cell density in a particular area resulting in decreased proliferation. Not only does groove depth affect the orientation of cells it also affects the orientation of bone nodules and eventually the orientation of tissues. This will play an important role in the osteointegration of CMOS sensors into bone after implantation.

Prolonged culture of osteoblast-like cells can mimic cell density properties in tissues(22). Under high-density culture conditions, osteoblasts develop orthogonal multilayers in which the axes of cells in an entire cell layer are parallel but whose
orientation lies at roughly right angles to adjacent cell layers. Each cell layer orients its collagen fibrils accordingly creating significant tensile strength. Lamellar bone is composed of orthogonally arranged collagen fibers. Groove depth impacts the formation of orthogonal layers by orienting cells along the surface of the biomaterial. Among the cell layers, those osteoblast-like cells in contact with surface topography displayed a greater degree of alignment than those cells located at a distance from the surface. Increased mineralization reported on grooved titanium surfaces may be the result of improved orientation of collagen fibers along the axis of the groove. Not only do grooves affect the orientation of osteoblast-like cells within the grooves but they also affect the orientation of cells located near grooves on smooth surfaces through cell-cell interactions.

Analysis of osteoblast expression on commercially pure titanium revealed that genes that are differentially expressed on various surface roughnesses(12). The investigation assessed smooth surfaces, moderately rough surfaces produced by acid etching with hydrochloric, and very rough surfaces produced by plasma spraying with titanium oxide (TiO₂). While there was no significant difference in levels of cellular attachment and proliferation, the plasma sprayed topography produced the greatest change in gene expression due to surface topography. Genes whose expression was differentially expressed on rough titanium surfaces when compared to smooth titanium surfaces include the up-regulation of two phosphoprotein transcription factors, a molecular chaperone protein, and a metalloenzyme responsible for protecting cells against metal toxicity as well as the down-regulation of seven genes including interleukin 13 and a tyrosine kinase. Other genes that were differentially expressed include caspases, intermediate filaments, and stress response proteins. Greater differences in gene
expression were seen at 24hrs after plating than at 3hrs indicating that osteoblast response to surface topography occurs during long-term exposure.

**Complementary Metal Oxide Semiconductor (CMOS) sensor**

The CMOS sensor is a transducer made of silicon and titanium that may permit technicians to directly quantitate the strength of bone using strain gauges that are expanded or compressed in response to stress on the bone(14). Contrary to the previously mentioned methods of determining BMD, the CMOS sensor will determine bone density based on biomechanical properties. The CMOS sensor will allow for real-time examination of the status of healing bone and enhance therapeutic procedures on a patient-specific basis. Unlike the techniques discussed above, the CMOS sensor will assess bone density during mechanical stress.

The CMOS sensor remains dormant in the body until it is activated by a telemetry-based system capable of powering the CMOS sensor through a radio frequency signal(3). Data is relayed back to an external device by the transducer for interpretation. Investigation of the CMOS sensor so far has been divided into two aspects. The first is the mathematical modeling of a CMOS sensor capable of recording data at the bone/implant interface and the calibration of information generated by the CMOS sensor while applying stress to a material known to have mechanical characteristics similar to bone. This research is being carried out by Fernando Alfaro of the MicroElectroMechanical division of the Robotics Institute at Carnegie Mellon University. I have completed a thorough in vitro analysis hAMSC attachment, proliferation and differentiation on the CMOS sensor. hAMSC were chosen for this
analysis because mesenchymal stem cells are among the first cells to colonize the surface of implants in vivo (13).

Measurement of stress on the bone will provide a better understanding of the biomechanics at the tissue/implant interface. Current methods of evaluating bone strength involve the use of a radioactive source to determine the mineral content of bone, BMD, and only detect information on bone strength during a static state. BMD provides only a partial idea of actual bone strength and does not include information of the quality of bone present or the architecture of bone. The CMOS sensor would advance current understanding of bone/implant integration and provide real-time information on the strength of bone during physical activity. The CMOS sensor is designed to measure stress distributed along its axis. Not only could this technology be applied to the monitoring of osteoporosis, it can also be applied to the monitoring of prosthetic implants and wound healing such as that of critical sized defects treated with bioresorbable bone fillers.

The dimensions of the CMOS sensor are 2mm x 2mm or 1mm x 1mm with a thickness of 300 \( \mu \text{m} \). It contains piezoresistive strain gauges composed of a CMOS gate polysilicon layer (14). CMOS sensors contain both a positive polarity metal oxide semiconductor and a negative polarity metal oxide semiconductor that are activated at different times (1). This creates a small power source and therefore makes the CMOS sensor ideal for use as a biological transducer because of this battery function. The CMOS battery is activated by radio waves. Piezoresistors are beams that absorb and measure stress placed on the CMOS sensor. The voids between the beams are composed of a mesh that is biocompatible but does not allow bone and fibrous tissue to be synthesized in the voids. The left and right sides of the CMOS sensors are coated in
titanium to improve biocompatibility. The silicon strain gauges will not be coated with
titanium to prevent cells from migrating into this region as osteoblasts prefer not to grow
on silicon and are more likely to choose the titanium coated sides instead. Figure 2 is a
schematic diagram of the CMOS sensor design. Note the location of the piezoresistive
strain gauges and the areas covered by topographical features.

The importance of surface topography on implant integration has resulted in
several processing techniques that form desired topographical features. These techniques
include machine tooling, blasting surfaces with either sand, titanium oxide, alumina or
silica particles and acid etching surfaces using different concentrations of hydrochloric
acid(32). The two latter techniques frequently alter the surface chemistry of a biomaterial
possibly leaving undesired chemical contaminants behind. Blasting surfaces has also
been shown to give rise to random cell orientation upon attachment resulting in scar
tissue formation and thus impairing osteointegration. We used a photolithographic
technique to fabricate surface features in a highly controlled manner.

**Photolithography**

The topography to the left and right of the piezoresistive strain gauges is produced
using photolithographic processing to create topographical features on a silicon wafer.
Initially the wafer consists of flat template sensors, each with a piezoresistive stain gauge
in the middle. Photolithography, or isoetching, was first developed by the microelectronic
industry(18). This technology allowed for the production of micron and submicron
characteristics on the flat, single-crystal wafers that are used to produce microelectronic
circuits. This process was adapted for use in biomedical engineering. Osteoblasts display
enhanced proliferation on isoetched surfaces as opposed to topography created by machine-tooled or sand-blast processing(10). Figure 3 illustrates the steps involved in the processing of CMOS sensors. The process involves six step which result in the formation of the desired topographies. These steps include:

1. Silicon wafers are coated with a primer that supports strong adhesion of photoresist.  
2. Coating using a UV-sensitive polymer (photoresist).  
3. Exposure of the photoresist to UV light through a patterned mask.  
4. The pattern is developed by emersion in solvents which dissolve exposed areas only.  
5. Uncoated surfaces are removed by isotropic etching.  
6. Photoresist is removed using a strong solvent.  

Isotropic etching removes substrate in a manner that widens as it deepens. This ability allows for the production of larger and deeper surface features. Unlike most previous studies, my thesis examined a range of peak height or depression depths from 15 to 60µm (referred to as etch depth- the distance above or below the mean surface plane) Surface patterns included concave depressions (dimples, D), convex peaks (pimples, P) and alternating combinations of depressions and peaks (dimples and pimples, PD). This study investigated parameters such as the effects of different pitches (distance from one peak to the same location on the next peak), the effects of increasing dimple depth and pimple height, and the shape of surface features.

Photolithographic processing produced sensors with an etch depth of 15, 37, and 60 micrometers from a mean plane. In all cases the pitch equals twice the etch depth. Topographies created consisted of square peaks, rounded valleys and combinations of both peaks and valleys in an alternating fashion. Figure 4 shows the patterns created in the mask that are used to produce the 37.5µm and 60µm etch depth dimple/pimple topographies.
Titanium coating

Chemical composition of the surface of an implant is important in implant success because it determines the implants physio-chemical properties(36). These properties include surface energy (a measurement of the interaction between a solid and a liquid that dictates the hydrophobicity of the material), and surface charge. These material properties impact protein-surface interactions and ultimately cell surface interactions. Titanium is the most widely used bone implant material(34). This has resulted in intense investigation to improve the integration of titanium implants to bone. Titanium and its alloy (Ti6Al4V) are commonly used in various medical and dental applications due to their mechanical strength and inert character. Current implants have a relatively low life span and must be removed and replaced. Alteration of the surface topography is one technique used to extend the lifespan of implants by improving the level of integration.

Osteoblasts exhibit greater cell adhesion on hydrophilic substrates. Titanium provides a hydrophilic surface favorable for the attachment and proliferation of osteoblasts and osteoblast precursor cells(34). The surface of titanium is hydrophilic due to the formation of a layer of titanium oxide (TiO₂) on the surface of the metal after exposure to air as well as in biological fluids(36). This hydrophilic character contributes to the biocompatibility of titanium. Some of the biocompatibility attributed to titanium is the result of titanium’s ability to adsorb extracellular matrix components onto its surface conditioning the surface for the adhesion of osteoblasts or osteoblast precursor cells(11). Surface oxides are not stable in the biological milieu and the protonation and deprotonation of the titanium oxide in culture determines surface energy and surface charge and in turn impacts how the surface interacts with serum proteins(36). A protein
layer can form on titanium surfaces as early as ten minutes after exposure to serum containing medium.

Although titanium alloy is commonly used in orthopedic implants due to its strength, it was not chosen to coat the CMOS sensor because the CMOS implant does not have to support body weight, and aluminum and vanadium, found in the alloy, are known to be cytotoxic(8). While aluminum promotes osteoprogenitor differentiation, in long-term culture it inhibits the secretion of osteocalcin and prevents mineralization. The surface oxides of alloys are heterogeneous with high levels of aluminum oxide in some areas and increase vanadium oxides in others in addition to titanium oxide(31). At a physiological pH, aluminum oxide has a positive surface charge causing aluminum to adsorb fewer serum proteins than titanium, which has a negative surface charge, and resulting in a decreased amount of osteoblast attachment on alloy materials. Adsorption of serum proteins such as albumin and fibronectin on commercially pure titanium surfaces promotes osteoblast adhesion. Scotchford and coworkers(31) observed that commercially pure titanium bound twice as much fibronectin as aluminum surfaces and that osteoblasts preferred higher concentrations of fibronectin. In addition, vinculin, a protein associated with the formation of focal contacts, is more abundant on titanium surfaces than aluminum surfaces indicating that osteoblasts bound titanium better than aluminum.

Although little work has been done on effects of titanium on osteoblast gene expression, one study examined gene regulation using microarray analysis and identified several genes that are differentially expressed on titanium alloy versus tissue culture plastic(15). In osteoblast-like cells, proteins associated with lipids, membranes, and
vesicular transport undergo changes in expression on the different substrates. Actin-related proteins are up-regulated on titanium alloy indicating that there may be an increase in exocytosis associated with extracellular matrix secretion. Genes associated with apoptosis were down-regulated on titanium alloy surfaces. To date no studies comparing osteoblast gene expression on commercially pure titanium versus tissue culture plastic or other substrates have been completed. Investigation of osteoblast interaction with titanium surfaces resulted in the selection of commercially pure titanium to coat the surface of CMOS sensors in order to improve cellular attachment and proliferation over that of an uncoated silicon surface.

Applications for the CMOS sensor

The purpose of this study was to investigate possible surface features for use in the integration of a Complementary Metal Oxide Semiconductor (CMOS) sensor into bone for possible applications such as the assessment of stress on the bone and the biomechanics of bone. We investigated topographies on the CMOS sensor prepared using photolithography, a process that creates various surface features in a precisely controlled manner. Stress readings taken using the CMOS sensor may be used to monitor bone density and strength, especially in cases were patients are predisposed to developing osteoporosis, have bone non-union and are undergoing additional treatment such as the implantation of grafted bone, or need long-term monitoring at the interface between bone and a prosthetic implant. Measuring biomechanical stress during dynamic movement will provide physicians with a means of more accurately assessing bone strength, as well as increase the ability to predict fracture and adjust treatment accordingly.
When bone is damaged, stem cells respond by first proliferating and then differentiating into osteoblasts replacing cells lost due to injury(13). These cells may be utilized in securing the CMOS sensor to bone permanently. Fundamental to the operation of the CMOS sensor is its ability to firmly adhere to the surface of the bone. With the proper integration the mechanically solid implant should attain complete fusion with regenerating bone tissue. Similar to expansion grates on a bridge that expand and contract in response to changes in temperature, expansion and compression of piezoresistive strain gauges will be used to measure stress on the bone. The CMOS sensor must be firmly anchored to bone for the piezoresistive strain gauges to function correctly. When the ends of the sensors are anchored to bone, the strain gauges will be pulled apart or pushed together in response to stress on the bone. Readings collected via radio frequency interface will then be interpreted to provide a measurement of bone strength and possibly the likelihood of fracture.

Use of the CMOS sensor may become important in monitoring bone repair after orthopedic surgery because the CMOS sensor may be implanted as part of the surgical procedure. Utilization of the CMOS sensor does not involve exposure to harmful radiation and once the sensor is implanted and attached to the bone it does not require additional invasive procedures. The CMOS sensor may improve the ability to predict fracture because it can measure BMD during mechanical stress a feat not shared with other methods currently used to measure BMD. The CMOS sensor is non-degradable and could be used to assess bone density for long periods of time. Assessment of cellular attachment, proliferation and differentiation on the CMOS is the first step toward the clinical application of the CMOS sensor.
A key step in the investigation of the CMOS sensor is to establish whether bone progenitor cells (cells capable of differentiating into bone-producing osteoblasts) as well as osteoblasts will attach to the CMOS sensor. Measuring the attachment, proliferation, and differentiation of hAMSC on CMOS sensors in vitro will provide insight as to how cells might behave on the CMOS sensor in vivo. Osteogenesis on the CMOS sensor in vivo will depend on the delicate balance between attachment and proliferation to achieve adequate covering and the ability of those cells to differentiate into osteoblasts capable of producing enough bone to secure the sensor in place.

Significance of the CMOS sensor

The use of the CMOS sensor is novel in its attempt to detect in vivo bone stress via a wireless radio-frequency interface. Its small size allows for implantation using minimally invasive procedures, such as minor surgery. Procedures for implantation might include insertion during surgical repair or drilling a small hole in an area such as a vertebra. The CMOS sensor could also be attached to the bone during surgery using fibrin glue until osteoblasts can secure the CMOS sensor to the bone. If the CMOS sensor is introduced into the body through injection, radio-telemetry can be used to locate the CMOS sensor and determine its orientation.

The CMOS sensor is especially unique in that piezoresistive strain gauges embedded in the sensor move in response to stress on the bone allowing for feedback to guide medical treatment. Novel treatments might include implanting the strain gauge within bone grafts creating a long term means of monitoring bone regeneration.
Physicians could then use the information to adjust medications and other treatment measures.

Attachment of undifferentiated hAMSC on the CMOS sensor in vitro is indicative of whether the CMOS sensor will be secured to the bone in vivo. This is the first step in the investigation of how effectively the CMOS sensor will attach to the bone after implantation. Sensor topographies that promote cellular attachment, proliferation, and differentiation in vitro can then be tested for these properties in vivo. My research focuses on determining which topographies promote these properties using LIVE/DEAD viability staining, scanning electron microscopy, and the assessment of calcium deposition. Given the number of possible topographies, it is important to do an initial screen of the CMOS sensors for the ones that promote the greatest cellular attachment, proliferation, and differentiation.

**Hypotheses**

- Different CMOS sensor topographical features will affect the attachment of hAMSC to the surface of the CMOS sensor.
- Once attached, hAMSC will proliferate until they cover the surface of the CMOS sensor.
- Medium containing an osteogenic supplement will induce the differentiation of hAMSC and result in calcium deposition on the CMOS sensor.
- CMOS sensor topography will affect calcium deposition.

**Experimental Approach**
Attachment

Each CMOS sensor is composed of a piezoresistive strain gauge with surfaces containing topographical features to the left and right of the strain gauges. Topographical features were etched using photolithography. Sterile CMOS sensors were placed in 12-well plates as array as arrays of 12 sensors, 6 1mm x 1mm and 6 2mm x 2mm. The 1mm x 1mm sensors are shown in figure 5. There are two of each topography represented. From left to right the topographies are pimple/dimple, pimple, and dimple. Each well was seeded with hAMSC and incubated for 24hrs. Cellular attachment was assessed using a LIVE/DEAD viability stain in which live cells are stained green and dead cells are stained red. The number of live and dead cells on each side of a CMOS sensor was recorded independently. Statistical analysis was done to determine whether there was a difference in cellular attachment based on topography for the left and right sides of the CMOS sensor and also for CMOS sensors as whole (both sides added together). Differences in cellular attachment also had to be analyzed after determining whether there was a statistical difference in the number of cells attached to 1mm x 1mm versus 2mm x 2mm CMOS sensors. Differences in cellular attachment on 1mm x 1mm and 2mm x 2mm CMOS sensors were also assessed independently according to size.

Proliferation

Proliferation of hAMSC on CMOS sensors was assessed by comparing the number of live cells attached to the CMOS sensors at day 1 and day 4 using a two-tailed T-tests. To simplify analysis, CMOS sensors were divided into 1mm x 1mm and 2mm x 2mm groups and each group was assessed according to etch depth. The proliferation of
hAMSC on CMOS sensors was analyzed twice, once using data collected on the individual left and right sides and by using the totaled data for an entire CMOS sensor.

*Calcium deposition*

Calcium deposition on CMOS sensors was assessed after hAMSC were incubated with or without an osteogenic supplement for 14 days. After treatment, CMOS sensors were assessed for calcium deposition using a chemical assay. The effects of etch depth and topography on calcium deposition were then analyzed using 2-way ANOVA analysis where etch depth versus experiment and topography versus experiment were the grouping variables.
Manuscript in preparation:

The importance of CMOS sensor surface topography
in the attachment, proliferation, and differentiation
of human adult mesenchymal stem cells

Christa Heyward¹, Fernando Alfero³, Phil Campbell², John Doctor¹,
Gary Fedder³, Mark Miller⁴, and Lee Weiss²

¹Department of Biological Sciences, Duquesne University, Pittsburgh, PA. 15282
²Department of Biomedical Engineering, Carnegie Mellon University,
Pittsburgh, PA. 15213
³Robotics Institute, Carnegie Mellon University, Pittsburgh, PA. 15213
⁴Rangos School of Health Sciences, Duquesne University, Pittsburgh, PA 15282
Abstract

Osteoblasts and osteoblast precursor cells (mesenchymal stem cells) respond to the surface topography of metal implants by aligning along surface features in order to minimize shear stress. While several previous studies have investigated the morphology, attachment and proliferation of both osteoblasts and mesenchymal stem cells along grooved surfaces the current study investigates the cellular response to depression and peaks in surface topography as well as surfaces that contain alternating combinations of depressions and peaks. The Complementary Metal Oxide Semiconductor (CMOS) sensor is a transducer fabricated from silicon and sputter coated in titanium. The CMOS sensor is designed to utilize piezoresistive strain gauges in the detection of stress on the bone. To the left and right of the piezoresistive strain gauges are surfaces that contain either depression (dimple) or peak (pimple) topographies. The CMOS sensor topographies were prepared using photolithographic processing, a technique that creates surface topographies in a precisely controlled manner. This study assessed the attachment, proliferation, and differentiation of human adult mesenchymal stem cells (hAMSC) on the depressions and peaks of CMOS sensors as a preliminary means of selecting surface topographies that might promote the greatest bone in-growth in vivo and therefore display the greatest osteoconductive properties. The distance above and below the nominal surface plane (etch depth) for surface topographies examined was either 15, 37, or 60µm. LIVE/DEAD viability staining and scanning electron microscopy revealed that cells attach on all CMOS sensor topographies. LIVE/DEAD viability staining was also used to determine that once attached, cells proliferate on all topographies after 3 days of incubation. Regardless of differences in initial cell attachment, after 14 days in medium
containing osteogenic supplement which promote hAMSC differentiation into osteoblasts, comparable concentrations of calcium were detected on all topographies. Although specific topography, depressions, peaks, or the combination of both depressions and peaks, had no impact on the amount of calcium deposited on CMOS sensors, there was a significantly larger amount of calcium deposited on topographies with an etch depth of 15µm when compared to the same topography with an etch depth of 60µm.

1. Introduction

Bone is a very dynamic structure. The formation of bone involves complex interactions between cells that secrete collagen, the main protein component of the extracellular matrix, non-collagenous proteins that are present in the extracellular matrix, and osteoblasts, which mineralize collagen into bone. Integration between an implant and the surrounding bone tissue is essential for the long term success of the implant(17). Assessment of an implant’s ability to integrate into bone involves a thorough analysis of the cell/matrix/surface interface. This study aims to investigate cellular attachment, proliferation and differentiation of human Adult Mesenchymal Stem Cells (hAMSC) on Complementary Metal Oxide Semiconductor (CMOS) sensors with topographies consisting of depressions, peaks, or combinations of both. The response of human adult mesenchymal stem cells (hAMSC) on CMOS sensor topography is important because hAMSC are among the first cells to migrate onto an implant surface and they will likely play a role in the production of bone necessary to secure the CMOS sensor to bone.

Topographical features on the surface of an implant play a significant role in the ability of cells to attach, proliferate and differentiate at the site of implantation(5, 7, 17). Surfaces with topographical features that have a high amplitude, distance above or below
the nominal surface level of the material, improve the alignment of cytoskeletal elements and cellular attachment over the course of several weeks when compared to tissue culture plastic(10). Cells appear elongated on surfaces with topographical features and this elongation results in a higher density of focal contacts along the edges of grooves.

Studies on the impact of grooved surface topographies on proliferation indicate a correlation between increased amplitude of surface topography and decreased levels of proliferation. This is most likely a result of the fact that these topographies significantly increase cellular differentiation when compared to flat surfaces. Decreases in proliferation are frequently coupled to an increase in cellular differentiation.

Integration between the implant surface and the surrounding tissue is essential for the long-term success of an implant(17). There are several explanations for why topography plays an important role in osteogenesis. Increased depth of topographical features may permit the development of multiple layers resulting in higher cell density of osteoblasts, aid in the orientation of collagen fibers, or create alterations in cell shape that result in changes in gene expression(28). Osteoblast “integration” in vitro refers to the adherence of osteoblasts to their substrate during the early stages of culture when osteoblasts are in direct contact with the substrate or the interaction between osteoblasts, extracellular matrix, and the substrate(10). In vivo, integration implies the creation of a cell/extracellular matrix/substrate interface. While osteoblasts initially exhibit a lower level of cellular adhesion on rough surfaces at the cell/ extracellular matrix/substrate interface, adhesion increases with time so that by days 14 and 21 all rough surfaces have a significantly higher level of cellular adhesion than osteoblasts cultured on tissue culture plastic.
The purpose of this study was to investigate possible surface topographies for use in the integration of a Complementary Metal Oxide Semiconductor (CMOS) sensor to bone for applications such as the assessment of stress on the bone as well as the biomechanics of bone. We investigated topographies on the CMOS sensor prepared using photolithography, a process that creates various surface features in a precisely controlled manner. Stress readings taken using the CMOS sensor may be used to monitor bone density and strength, especially in cases were patients are predisposed to developing osteoporosis, have bone non-union and are undergoing additional treatment such as the implantation of grafted bone, or need long-term monitoring at the interface between bone and a prosthetic implant. Measuring biomechanical stress during dynamic movement will provide a means of more accurately measuring bone strength, as well as increase the ability to predict fracture and adjust treatment accordingly.

The current investigation of cellular attachment, proliferation, and differentiation found that hAMSC readily attach to the CMOS sensor and, once attached, they can proliferate until induced to differentiate. Regardless of the initial level of cellular attachment, there was no significant difference in levels of induced calcium deposition among the six topographies tested. This suggests that the functionality of the chip may ultimately determine which topography is chosen for further in vivo analysis. The ultimate integration of the CMOS sensor to bone will require a delicate balance between a surface topography that supports enough osteoblast precursor cell attachment and proliferation to cover the surface of the CMOS sensor while promoting the ability of the cells to differentiate into osteoblasts that deposit extracellular matrix.
2. Materials and Methods

2.1 Human Adult Mesenchymal Stem Cells (hAMSC)

hAMSC cryopreserved at passage 2 were purchased from Cambrex and cultured in Mesenchymal Stem Cell Basal Media (Cambrex). hAMSC were maintained at 37° C in a humidified atmosphere containing 95% air and 5% CO₂. Cells were passaged at subconfluent levels using 0.05% Trypsin/EDTA (Invitrogen). Cells were induced to differentiate into osteoblasts using osteogenic-inducing media which contains 100mM dexamethasone, 10mM β-glycerophosphate, and 0.05mM ascorbic acid-2-phosphate.

2.2 Sensor texture/coating types

CMOS sensors were produced at the Carnegie Mellon University Nanofabrication Facility using Deep Reaction Ion Etching (DRIE), a process that involves the coupling of low-pressure reactant gases that result in a plasma(3). Sensors were produced with one of three texture types: pimples (peaks), dimples (depressions), or both pimples and dimples. All topographies were fabricated at each of three etch depths: 15, 37 or 60µm. The pitch for each topography was twice the etch depth. Sensors tested were then sputter-coated with titanium.

2.3 Assessment of cell attachment

Individual titanium-coated sensors or arrays of several sensors were placed in a 12-well plate and sterilized using two 20 minute washes with ethanol, two 20 minute washes in sterile distilled H₂O, followed by drying overnight. Cells suspended in medium were added to the wells on top of the sensors and then the well plate was gently rocked to disperse cells evenly. Sensors were seeded at either 25,000 cells/sensor or 100,000 cells/array respectively. Cells were incubated for 24 hours and then rinsed in PBS and
assessed for cell attachment and viability using LIVE/DEAD staining as directed (Molecular Probes). Ethidium homodimer fluorescently stains the nuclei of dead cells red (excitation: 495nm and emission: 635) and Calcein AM is cleaved by an enzyme in live cells producing calcein which fluoresces green (excitation: 495nm and emission: 515). After incubation for 30 minutes, stained hAMSC were washed with PBS and CMOS sensors were mounted and viewed with a fluorescent microscope (Nikon Eclipse microscope fitted with a Diagnostics Instruments, Inc. digital camera) and images were captured using QED Camera Standalone imaging software and were then processed using Adobe Photoshop software. All cells on each titanium-coated sensor were counted and the CMOS sensor topography noted. Titanium-coated sensors were then fixed in 2.5% glutaraldehyde and processed for scanning electron microscopy (see scanning electron microscopy section).

2.4 Cell proliferation assessment

One titanium-coated sensor array was added to each individual well of a 12 well-plate and seeded with 100,000 hAMSC/array. Arrays were removed after 1 day and 4 days and stained using LIVE/DEAD stain as described. Arrays were then viewed using a fluorescent microscope and cell numbers assessed for each topography. Arrays were then fixed for scanning electron microscopy (see scanning electron microscopy section).

2.5 Calcium assessment

Calcium deposition was assessed using the Sigma Calcium Kit #587 colormetric chemical assay. One 2mm x 2mm sensor was secured to the bottom of each well in a 48-well plate using sterile vacuum grease. For each experimental trial, individual topographies were represented by six sensors. hAMSC were seeded at 12,500 cells/well.
hAMSC were then cultured in mesenchymal stem cell growth media for 7 days. On the seventh day, three sensors of each topography were then switched to medium containing an osteogenic supplement (100mM dexamethasone, 10mM β-glycerophosphate, and 0.05mM ascorbic acid-2-phosphate) and the remaining three sensors were incubated in mesenchymal stem cell growth medium as controls. 14 days after switching to osteogenic medium, all sensors were removed and placed in 100μl of 0.1N HCl. 10μl of the dissolved calcium in HCl from each individual sensor was added to 90μl of calcium reagent containing a 1:1 solution of Sigma Calcium Buffer and Sigma Calcium Reagent. Analysis of the colormetric assay was carried out on a Perkin Elmer HTS 7000 Bioassay Reader at a wavelength of 590nm. The calcium concentrations for the sensors were then determined by comparing their absorbencies with those of a calcium standard (Sigma360-5).

2.6 Scanning Electron Microscopy

hAMSC on CMOS sensors were fixed in 2.5% glutaraldehyde in PBS. For electron microscopy, glutaraldehyde was removed by three washes in PBS. Cells were then fixed in 2% osmium tetroxide for 30 minutes followed by washing with H2O. The samples were then dehydrated using an ethanol series (50, 70, 80, 90, 100, 100, 100%) and then critical point dried (Pelco CPD2). After drying, samples were mounted on glass slides and sputter-coated with gold (Pelco SC-6). All sensors were viewed with a Hitachi 2460 scanning electron microscope at an accelerating voltage of 15kV or 25kV. Images were captured using Quartz PCI (version 3) digital imaging software.
2.7 Statistical analysis

2.7.1 Attachment: Differences in hAMSC attachment after a 24hr culture period were assessed according to surface topography and etch depth. hAMSC attached to 2mm x 2mm CMOS sensors were analyzed using one-way ANOVA analysis on Prism software.

2.7.2 Proliferation: Outliers were removed from experimental data sets for both large and small sensors. The fold increase in cell number due to proliferation was assessed using a two-tailed T-test with Prism software. 1mm x 1mm sensors were assessed at 24 hrs and after 4 days to determine if cell numbers increased after three days of incubation (Day 1 v. Day 4). Identical analysis was performed on 2mm x 2mm sensors. Sensors were assessed using the numbers of cells attached to the left and right sides of the CMOS sensor individually and as whole sensors with the cells on each side totaled. Numerical data is represented as the mean of three or more separate experiments in which each separate experiment had an n≥3 sensors. Statistically significant differences were determined in all cases as having a P<0.05.

2.7.3 Differentiation: The effects of etch depth and topography on calcium deposition were then analyzed using 2-way Analysis of Variance (ANOVA) test and analyzed post hoc by Tukey HSD Test where etch depth versus experiment and topography versus experiment were the grouping variables. Only topographies with an etch depth of 15µm or 60µm were assessed for calcium deposition.

3. Results

This investigation assessed the interaction between hAMSC and a number of surface topographies on CMOS sensors. Cellular interaction with surface topography was
examined through the assessment of human adult mesenchymal stem cell (hAMSC) attachment, proliferation and differentiation on the CMOS sensors.

CMOS sensors are fabricated from a silicon wafer and specific topographies are created using photolithographic techniques. We studied three topographical features: dimples (depressions, D), pimples (peaks, P), and patterns in which pimples and dimples alternate across the surface (pimple/dimple, PD). Also investigated was the effect of etch depth, a measurement describing the distance of topographical features above or below the nominal surface level of a CMOS sensor. Etch depths of 15, 37, and 60 μm were used to assess attachment, proliferation and differentiation.

3.1 hAMSC attach to CMOS sensors

Attachment of hAMSC on CMOS sensor topographies was examined by seeding hAMSC on the CMOS sensors and incubating for 24 hrs. After 24 hrs, unattached cells were washed away and the remaining cells were stained using a LIVE/DEAD fluorescent viability stain and cell numbers were quantitated by visual inspection. After 24 hrs of incubation, hAMSC were able to attach on all surface topographies. Cells were imaged using scanning electron microscopy to assess cell morphology as well as determine location of cellular attachment (figure 6). Qualitative assessment of hAMSC attachment on CMOS sensor topographies 2mm x 2mm is supported by quantitative data in figure 7. The results are represented as the mean ± the SEM. The number of sensors analyzed for each topography is indicated in corresponding bar for that topography and was obtained through three or more independent experiments. For topographies with an etch depth of 15μm, there was no difference in the number of cells attached for the different
topographies as assessed using one-way ANOVA analysis. The same was also true for the 37.5µm etch depth. For the 60µm etch depth, however, the dimpled topography had a significantly lower level of cellular attachment than the pimpled topography and the combination pimple/dimple topography. When topographies were compared across all etch depths, using one-way ANOVA analysis, there was no significant difference in number of cells attached on any of the topographies after 24 hrs of culture. For additional statistical analysis of differences in cell attachment according to CMOS sensor topography and etch depth see appendix A.

3.2 Proliferation of hAMSC on CMOS sensors

We also investigated the potential for hAMSC to proliferate on CMOS sensors once attached. Proliferation was assessed by determining the number of hAMSC attached after 1 and 4 days of incubation. After 24hrs of incubation, hAMSC attached to CMOS sensors were either stained using LIVE/DEAD viability stain as described or washed and transferred to a new well plate. Only those cells that had attached after 1 day of incubation were transferred to the new well plate so that any increase in cell number on the CMOS sensors would be as a result of cellular proliferation. To simplify analysis, CMOS sensors were divided into 1mm x 1mm and 2mm x 2mm groups and assessed according to etch depth. Proliferation of hAMSC on CMOS sensors was assessed by comparing the number of live cells attached at day 1 and day 4 using two-tailed T-tests. Proliferation was assessed by counting cells attached to the left and right sides of each sensor (figure 8) and with both sides of a CMOS sensor added together to give the total number of cells attached per CMOS sensor (figure 9). The results show a statistically
significant increase in the number of hAMSC attached to CMOS sensors of all topographies after a proliferation period of three days. Fluorescent images taken on day 1 and day 4 also support an increase in cell number as well as indicate a high cell viability (figure 10). Fluorescent images indicating an increase in cell number were seen at all etch depths and images shown for the 60 µm etch depth are representative of the 15 and 37 µm etch depths as well (data not shown).

3.3 HAMSC viability

The viability of hAMSC on CMOS sensors was assessed after 1 and 4 days of culture. Table 1 shows the mean percent viability ± SEM for all topographies at day1 and day 4. The data were collected from 3 or more independent experiments on 2mm x 2mm CMOS sensors and represent the combined viability of cells on both sides of the CMOS sensor. The sensors column indicates the number of individual CMOS sensors assessed for attachment at days 1 and 4. The topographies analyzed were D (dimple), P (pimple), and PD (pimple/dimple) for all three etch depths, 15µm, 37.5µm, and 60µm. Table 1 shows that viability increased from day 1 to day 4 for all topographies assessed.

3.4 hAMSC deposit calcium on CMOS sensors

The potential for hAMSC to differentiate on CMOS sensors was assessed by measuring calcium deposition on each topography. Calcium deposition was assessed chemically and by the presence of hydroxyapatite or calcium-phosphate nodules using scanning electron microscopy (figure 11). Calcium crystals were difficult to find in SEM analysis due to the fact that mineralized calcium is located under hAMSC and therefore
could only be visualized in locations where there was a gap in the cell monolayer. Definitive analysis of calcium deposition was therefore further assessed by chemical assay for calcium. Incubation of hAMSC in medium containing an osteogenic supplement resulted in a statistically significant increase in the amount of calcium deposited on the CMOS sensor when compared to hAMSC incubated in control medium alone (p<.001) as seen in figure 12. 2-way analysis of variance (ANOVA) determined that there was no statistical difference between the pimple, dimple, and pimple/dimple topographies. There was, however, a statistically significant increase in calcium deposition on topographies that had an etch depth of 15 µm when compared to topographies with an etch depth of 60 µm (figure 13).

4. Discussion

The measurement of bone mineral density is an important tool used to determine the likelihood of fracture and osteoporosis. Bone Mineral Density (BMD) measures the amount of calcium in regions of bone(4). BMD is measured by exposing a patient to X-rays, photons or ultrasound. None of these methods are used to monitor bones as they heal inside a cast or the integration of bone grafts to the bone of a patient. The Complementary Metal Oxide Semiconductor (CMOS) sensor has the potential to measure bone density over long periods of time. The CMOS sensor may also measure bone mineral density during mechanical stress, a feat not shared with other methods currently used to measure BMD.

The current study examines the attachment of hAMSC to various CMOS sensor topographies. Initial cell adherence involves the use of contact guidance by cells to
establish focal contacts at the cell/implant boundary. Adhesion of osteoblasts on implants depends on extracellular matrix proteins, membrane receptors and cytoskeletal proteins. (5, 6). Increased amplitude of topographical features causes the condensation of cytoskeletal elements and organizes the nucleation of actin. Topographical features can result in the elongation and alignment of cells(17, 28). Elongated cells have a higher density of focal contacts along the edges of grooves as well as better organization or alignment of the cytoskeletal elements. A stronger actin skeleton then allows cells to resist detaching shear forces.

Etch depth and pitch play a role in the location of cells once attached. Cells on the CMOS sensors with an etch depth of 15µm are usually located on top of surface features. The space in between topographical features appears to be too small for cells to rest in this space. Cells on sensors with an etch depth of 60µm lie between the topographical features and are almost never found atop the peaks of surfaces with pimples or with pimples and dimples. Either cells that land on the peaks migrate off or the peaks fail to support cellular attachment possibly due to increased shear stress. Once attached cells on both the 15 and 60µm surfaces send out processes across the surface topography creating additional focal contacts.

The deposition of calcium on smooth versus grooved topographies has been studied previously(5-8). Collagen synthesis and increased production of osteocalcin and alkaline phosphatase have been correlated with increases in amplitude of topographical features(28). Perizzolo and coworkers(28) reported that not only did groove substrata increase the number of bone nodules formed in vitro by osteoblasts, but increases in the depth of grooves increased the number of nodules formed as well. The ability of CMOS
sensors to support the differentiation of hAMSC into osteoblasts was further investigated by measuring the amount of induced calcium deposition on the topographies. By 14 days in culture with an osteogenic supplement, there is no difference in the amount of induced calcium deposited among the different topographies. Cells most likely proliferate to cover the majority of available surface by the time they were induced to differentiate.

Chemical analysis of calcium deposition shows a statistically greater amount of calcium deposition on CMOS sensors cultured with an osteogenic supplement when compared to CMOS sensors cultured in control medium. Previous analysis of the differentiation of hAMSC into osteoblasts or the analysis of osteoblastic markers and bone nodule formation by osteoblasts found that the number of bone-like nodules increased with an increase in groove depth(28). Our results show that more calcium was deposited on CMOS sensors with topographies that had an etch depth of 15µm when compared to surfaces with the same topography but with an etch depth of 60µm. Other studies also indicate that osteoblast differentiation markers such as the production of osteocalcin and alkaline phosphatase also increase with increasing groove depth. While this study shows a significantly higher level of calcium deposition on surfaces with an etch depth of 15µm, our analysis of differentiation may be limited by the use of only one parameter for our analysis. We did not investigate the production of alkaline phosphatase or osteocalcin in response to various CMOS surface topographies.

Further studies are needed to assess the in vivo biocompatibility of the CMOS sensors as well as determine whether topography plays a role in the long-term integration of implants on the surface of the bone. Additional research is under consideration concerning the attachment of growth factors, such as fibroblast growth factor or
transforming growth factor beta as well as the addition of collagen to the surface of CMOS sensors in order to further improve the attachment, proliferation, and differentiation of hAMSC. Analysis *in vivo* will provide information about the in-growth of bone on the different surface topographies. Bone in-growth is necessary to secure the CMOS sensor in place for proper analysis of bone strength by the piezoresistive strain gauges.
Figure 1: The attachment, proliferation, and differentiation of hAMSC are frequently investigated in response to biomaterial surface chemistry and topography. Here hAMSC are grown on a tricalcium-phosphate based bone void filler biomaterial and assessed for differentiation after treatment with media containing an osteogenic supplement.
(Christa Heyward, Unpublished)
CMOS sensors were designed to be small, 1mm x 1mm or 2mm x 2mm, with textured surfaces on either side of piezoresistive strain gauges. 200µm holes provide a location for bone in-growth. A thin layer of titanium is sputter-coated over the surface topography in order to improve biocompatibility.
clean and prime surface

1. coat with resist

irradiate through mask

2. expose pattern to UV

3. develop pattern in resist

4. pattern resist layer

5. etch silicon

6. remove resist

Figure 3: Using photolithography to create surface features on the CMOS sensor. Hallgeen and coworkers. 2001. Photolithography involves the following steps in the formation of topographical features on the surface of a material: 1. Silicon wafers are coated with a primer that supports strong adhesion of photoresist. 2. Coating using a UV-sensitive polymer (photoresist). 3. Exposure of the photoresist to UV light through a patterned mask. 4. The pattern is developed by emersion in solvents which dissolve exposed areas only. 5. Uncoated surfaces are removed by isotropic etching. 6. Photoresist is removed using a strong solvent.
Figure 4: Creating CMOS sensor topographies. Isoetching is carried out in the Nanofabrication Facility at Carnegie Mellon University. A and B show masks used to create the 37.5µm and 60µm etch depth pimpled/dimpled topography. Solid areas are not etched as rapidly as areas where lines are close together. The solid areas will become pimples and areas where lines are close together will become dimples.
**Figure 5:** Sample CMOS sensor topography. CMOS sensors were analyzed as arrays of 12 sensors, six 1mm x 1mm (shown) and six 2 mm x 2mm, in order to facilitate their manipulation during testing. Each topography, pimpled/dimpled (A), pimpled (B), and dimpled (C) was represented by two 1mm x 1mm and two 2 mm x 2mm CMOS sensors. Shown here are topographies with an etch depth of 60µm. hAMSC on each side of the piezoristive strain gauges was counted independently and then totaled. Results in the figures that follow are based on cells/mm² of topography.
Figure 6: Cells attach to CMOS sensors after 24hrs of incubation. A) Dimple; B) Pimple; C) Dimple and Pimple have an etch depth of 15µm. D) Dimple; E) Pimple; F) Dimple and Pimple have an etch depth of 60µm. Images taken at 15kV and scale bar = 400µm.
**Figure 7:** hAMSC readily attach on all CMOS sensor topographies after 24hrs of culture. Results are depicted as the mean ± SEM. The number (n) of CMOS sensors assessed for each topography appears in that corresponding bar for that topograph. n was obtained through the analysis of three or more independent experiments. Differences in attachment on each topography were assessed individually for each etch depth and across all etch depths using one-way ANOVA analysis. * p < 0.05.
Figure 8: hAMSC proliferate after 3 days of incubation on CMOS sensors. The graphs represent the number of cells counted on each individual side of a CMOS sensor. Bars are the mean ± SEM. Filled bars represent day 1 and empty bars represent day 4. n = number of sides counted for each topography on its representative day. n appears either on or above each bar. Data are pooled from 3 or more independent experiments. P values were determined using a 2-tailed t-test and are listed above each day1-day 4 pair. The cut off for statistical difference was set at p< 0.05. Topographies are represented by D (Dimple), P (Pimple), or PD (Pimple and Dimple). A and B are CMOS sensors with an etch depth of 15µm, C and D have an etch depth of 37µm, and E and F have an etch depth of 60µm.
Figure 9: hAMSC proliferate after 3 days of incubation on CMOS sensors. The graphs represent the number of cells counted on each individual CMOS sensor. Bars are the mean ± SEM. Filled bars represent day 1 and empty bars represent day 4. n = number of sensors counted for each topography on its representative day. n appears either on or above each bar. P values were determined using a 2-tailed t-test and are listed above each day1-day 4 pair. The cut off for statistical difference was set at p< 0.05Data are pooled from 3 or more independent experiments. Topographies are represented by D (Dimple), P (Pimple), or PD (Pimple and Dimple). A and B are CMOS sensors with an etch depth of 15μm, C and D have an etch depth of 37μm, and E and F have an etch depth of 60μm.
Figure 10: Fluorescence indicates hAMSC proliferation after three days of culture. LIVE/Dead viability staining of hAMSC after 1 and 4 days of incubation revealed that cell number and viability increased as cells proliferated on CMOS sensors. Live cells stain green and dead cells stain red. Scale bar = 250µm. A) 60P day 1; B) 60P day 4; C) 60D day 1; D) 60D day 4; E) 60PD day 1; F) 60PD day 4.
<table>
<thead>
<tr>
<th>Topography</th>
<th>Day 1</th>
<th>Sensors</th>
<th>Day 4</th>
<th>Sensors</th>
</tr>
</thead>
<tbody>
<tr>
<td>15D</td>
<td>89±3</td>
<td>11</td>
<td>89±4</td>
<td>7</td>
</tr>
<tr>
<td>15P</td>
<td>86±5</td>
<td>11</td>
<td>91±4</td>
<td>8</td>
</tr>
<tr>
<td>15PD</td>
<td>93±2</td>
<td>10</td>
<td>93±4</td>
<td>7</td>
</tr>
<tr>
<td>37.5D</td>
<td>73±10</td>
<td>4</td>
<td>95±2</td>
<td>13</td>
</tr>
<tr>
<td>37.5P</td>
<td>65±20</td>
<td>4</td>
<td>87±6</td>
<td>5</td>
</tr>
<tr>
<td>37.5PD</td>
<td>83±7</td>
<td>4</td>
<td>82±7</td>
<td>6</td>
</tr>
<tr>
<td>60D</td>
<td>79±4</td>
<td>12</td>
<td>92±3</td>
<td>8</td>
</tr>
<tr>
<td>60P</td>
<td>80±5</td>
<td>13</td>
<td>87±3</td>
<td>8</td>
</tr>
<tr>
<td>60PD</td>
<td>83±4</td>
<td>12</td>
<td>86±3</td>
<td>8</td>
</tr>
</tbody>
</table>

**Table 1:** The viability of hAMSC after 1 and 4 days of culture was assessed using LIVE/DEAD viability staining and is given as the mean ± SEM for each topography. The data were collected from 3 or more independent experiments on 2mm x 2mm CMOS sensors and represent the combined viability of cells on both sides of the CMOS sensor. The sensors column indicates the number of individual CMOS sensors assessed for attachment at days 1 and 4. The topographies analyzed were D (dimple), P (pimple), and PD (pimple/dimple) for all three etch depths, 15µm, 37.5µm, and 60µm.
Figure 11: hAMSC cover the surface topographies of CMOS sensors after 14 days of culture in medium containing an osteogenic supplement. At this point they have initiated synthesis of extracellular matrix and begun to mineralize the matrix around them. A and B show the CMOS sensor topography underneath a layer of hAMSC while C and D illustrate collagen fibers and initial hydroxyapatite formation. For A and B the scale bar = 100µm and for C and D the scale bar = 5µm. Images were taken at 25kV.
Figure 12: Representative experiment showing the calcium deposition of hAMSC on CMOS sensors cultured in medium containing an osteogenic supplement deposited significantly more calcium than hAMSC on CMOS sensors cultured in control medium after 14 days of incubation. Filled bars are hAMSC grown in OS- media. Empty bars represent hAMSC grown in OS+ media. A) Calcium deposition on topographies with an etch depth of 15\(\mu\)m. B) Calcium deposition on topographies with an etch depth of 60\(\mu\)m. Surface topography is indicated by P (Pimple), D (Dimple), or PD (Pimple and Dimple). Results were analyzed using 2-way ANOVA and * indicates P<.001 and is considered to be a statistically significant difference. Bars show the mean ± SEM. n= 3 CMOS sensors.
Etch Depth Affects Calcium Deposition

**Figure 13:** Etch depth affects calcium deposition. Filled bars are CMOS sensors with an etch depth of 15µm and empty bars are CMOS sensors with an etch depth of 60µm. n = the number of sensors from three independent experiments analyzed for calcium deposition. n appears in the corresponding bars for a specific topography. P-values were determined using 2-way ANOVA analysis using etch depth and experimental trial as grouping variables. P-values are above corresponding topographies and the cut off for statistical differences was set at p< 0.05. Topographies are designated using D (Dimple), P (Pimple), or PD (Pimple and Dimple).
Appendix A

Statistical analysis of hAMSC attachment on CMOS sensors

Data collected for the attachment of hAMSC was analyzed for differences in attachment in response to different surface topographies. Outliers were removed from the experimental data sets for both 1mm x 1mm and 2mm x 2mm CMOS sensors for each topography. An independent T-test was used to assess whether there was a difference in cellular attachment on 1mm x 1mm versus 2mm x 2mm CMOS sensors. Differences in cellular attachment based on size were assessed both as cells attached per textured side of the CMOS sensor and for each CMOS sensor as a whole. For subsequent analysis, the number of cells attached to 1mm x 1mm and 2mm x 2mm were grouped together where t-tests indicated that there was no difference in cellular attachment based on the size of the CMOS sensor. If results from the t-test concluded that there was a difference in cellular attachment between 1mm x 1mm and 2mm x 2mm CMOS sensors analysis for each was done separately.

One-way ANOVA followed by a Tukey post hoc test was then used to determine the affect of topography on cellular attachment. Differences in attachment based on differences in topography were also assessed according to the number of cells attached on each textured side and total number of cells attached to a CMOS sensor. The topographies analyzed for attachment were represented by at least 3 CMOS sensors in at least 3 independent experiments. The results of the analysis of attachment can be found in appendix figures 1 and 2 (figure A-1 and A-2).
**Figures A-1 and A-2:** Statistical Assessment of cellular attachment on all CMOS sensor topographies using two-way ANOVA analysis as described in appendix-A.
The effect of topography on calcium deposition

We assessed whether the type of topographical feature played a role in the amount of calcium deposited on CMOS sensors. We used 2-way ANOVA with experimental trial and topographical feature (D, P, or PD) being the two grouping variables. Results of the analysis showed that there was no difference in calcium deposition among the three topographies for the 15µm or the 60µm etch depth (figure A-3). Variation in calcium deposition was completely attributed to variation in experimental trial. We also assessed the significance of etch depth in calcium deposition. 2-way ANOVA revealed that the 15µm etch depth had a significantly greater amount of calcium deposited when compared to the 60µm etch depth for all topographies tested (figure 7).

Figure A-3: 2-way ANOVA analysis of induced calcium deposition on CMOS sensors with an etch depth of 15 or 60µm. The two grouping variables for the 2-way ANOVA were topography (D, P, or PD) and experimental trial. Represents the dimpled topography, represents the pimpled topography, and represents the combination of dimples and pimples. Each topography is then divided into experimental trials 1, 2, and 3.
REFERENCES:


