

immobilized on the surface (e.g., via covalent bonds, non-specific interactions and/or specific binding interactions). Preferably, the region to be coated is defined by a physical boundary that acts as a barrier to confine the deposited fluid to the pre-defined region (e.g., a surrounding ledge or depression, a boundary formed of patterned materials deposited or printed on the surface, and/or a boundary formed via an interface with a surrounding region that varies in a physical property such as wettability) so as to form a fluid containment region.

**[0158]** In certain preferred embodiments, antibodies or other binding reagents (preferably proteinaceous binding reagents) are immobilized on carbon ink electrodes by non-specific adsorption. It may be advantageous to allow the assay reagent solution to dry on the electrode during the immobilization procedure. Preferably, the immobilization procedure further comprises blocking un-coated sites on the surface with a blocking agent such as a protein solution (e.g., solutions of BSA or casein), washing the surface with a wash solution (preferably a buffered solution comprising surfactants, blocking agents, and/or protein stabilizers such as sugars) and/or drying the surface.

**[0159]** In a preferred immobilization procedure of the invention, imprecision due to variations in the ability of different assay reagents to adsorb on a surface such as a carbon ink electrode are reduced by immobilizing via a specific binding interaction involving a first and second binding partner. Such an immobilization technique is less likely to be affected by small variations in the properties of the surface. By way of example, antibodies may be patterned by patterned deposition of antibody solutions (the first binding partner) on a surface coated with an antibody binding reagent (the second binding partner, e.g., an anti-species antibody, protein A, protein G, protein L, etc.). Alternatively, assay reagents labeled with the first binding partner (preferably, biotin) may be patterned by patterned deposition of the assay reagents on a surface coated with the second binding partner (preferably, anti-biotin, streptavidin, or, more preferably, avidin). Most preferably, the second binding partner is deposited in the same pattern as the assay reagents. By analogy, the method can be adapted to use any of a variety of known first binding partner—second binding partner pairs including, but not limited to, hapten-antibody, nucleic acid—complementary nucleic acid, receptor-ligand, metal-metal ligand, sugar-lectin, boronic acid—diol, etc.

**[0160]** Accordingly, one embodiment of an immobilization method of the invention comprises forming an assay domain comprising an assay reagent by: i) treating a pre-defined region of a surface (preferably, a carbon ink electrode surface) with a solution comprising a second binding partner so as to form an adsorbed capture layer (or, alternatively, a covalently bound layer) of said second binding partner (preferably, avidin) within the pre-defined region of said surface; (ii) treating the capture layer in the pre-defined region with a solution comprising the assay reagent, wherein the assay reagent is linked to or comprises a first binding partner (preferably, an assay reagent that is labeled with biotin) that binds the second binding partner. Preferably, a micro-dispensing technique is used to pattern the second binding partner and/or the assay reagent into the pre-defined region (more preferably both are patterned). More preferably, the pre-defined region is defined by a boundary (preferably

erably defined by a dielectric layer patterned on the surface) adapted to confine small volumes of fluid to the pre-defined region.

**[0161]** The treating steps may comprise allowing the solutions to dry on the pre-determined regions. Between binding the second binding partner and binding the assay reagent, it may be advantageous to wash the surface with one or more wash solutions to remove excess unbound second binding partner. The wash solutions, preferably, comprise surfactant and/or blocking agents. After immobilizing the assay reagent, it may be advantageous to wash the surface with one or more wash solutions to remove unbound assay reagent. The wash solutions, preferably, comprise surfactants, blocking agents and/or protein stabilizers such as sugars. Useful blocking agents include standard blocking agents of the art (BSA, casein, etc.) but also include blocking reagents comprising the first binding partner (for example, free biotin) so as to block free binding sites on the immobilized layer of the second binding reagent. The wash steps may employ the wash techniques of the invention that employ concentric tubes for adding and removing wash solution. The surfaces are optionally dried after preparation for long term storage.

**[0162]** Preferably, the amounts of the second binding reagent and assay reagent applied to the pre-defined region are equal to or less than that required to saturate the surface. By choosing amounts roughly equal to the amounts required to saturate the surface, it may be possible to minimize both the amount of excess unbound reagent and the amount of unbound sites and thus avoid the need for washing or blocking steps. In an alternative embodiment, the amount of the assay reagent is kept below the amount of available binding sites in the capture layer to ensure that the binding capacity is determined by the amount of assay reagent added and not by amount of immobilized second binding partner (thus reducing the effect of variability in the efficiency of, e.g., the adsorption of the second binding partner).

**[0163]** The method may be applied to forming a plurality of assay domains comprising assay reagents immobilized in a plurality of pre-defined regions. In this case, the method is simply repeated for each of the pre-defined regions. Preferably, at least two of the assay domains comprise assay reagents that differ in selectivity for analytes of interest. When forming a plurality of assay domains, it is particularly advantageous to block the final product with a blocking reagent comprising the first binding partner (but not the

**[0164]** analyte specific components of the assay reagent) to block excess binding sites on immobilized second binding partners; this procedure prevents assay cross-talk due to excess assay reagent on one pre-defined region diffusing and binding, via first binding partner-second binding partner interactions, to a different assay domain. For example, after using the two step procedure of binding avidin and then a biotin-labeled antibody, the surface may be blocked with free biotin. Alternatively, after using a two step procedure of binding Protein A (or other Fc binding receptor) and then an antibody against an analyte of interest, the surface may be blocked by using a different antibody or, more preferably, an Fc fragment of an antibody.

**[0165]** It has been observed that in some cases assay reagents adsorbed on a surface such as a carbon ink may, over time, slowly dissociate from the surface. This disso-