FUNCTIONAL FLUIDICS
DEFINING Red Blood Cell Health

NOVEL HYPOXIA-INDUCED SICKLING ASSAY FOR ASSESSMENT AND MONITORING OF SICKLE CELL DISEASE TREATMENTS AFFECTING HEMOGLOBIN POLYMERIZATION

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INTRODUCTION

Sickle Cell Disease (SCD) pathology is caused by polymerization of deoxygenated hemoglobin S (HbS) within RBCs. Resultant RBC "sickling" promotes vaso-occlusion and leads to an array of clinical complications including vaso-occlusive episodes (VOEs), acute chest syndrome, stroke, and others. The amount of sickling depends on the size of polymerized fraction and polymerization kinetics, which can be significantly affected by treatments, e.g., by hemoglobin-modifying drugs. There is a need for rapid assays to monitor therapeutic response. Objective: Develop a hypoxia-induced RBC sickling assay as a potentially CLIA-waived test for use in drug development research and clinical monitoring of SCD therapies affecting Hb oxygen affinity (e.g., Hb modifiers, HbF inducers, PK activators, etc.).

❖ Challenges

SCD therapies affecting Hb oxygen affinity must ensure adequate oxygen delivery while reducing RBC sickling-associated complications. Conditions that favor oxygen delivery, such as high Hb concentration or lower Hb-O_2 affinity for faster and more efficient oxygen off-loading, would also favor RBC sickling. Conversely, therapies that reduce sickling through left-shifting of O_2 binding curve by Hb modification or through changes in 2,3-DPG concentration may decrease O_2 delivery for patients that already suffer from impaired blood flow and O_2 delivery.

❖ Need

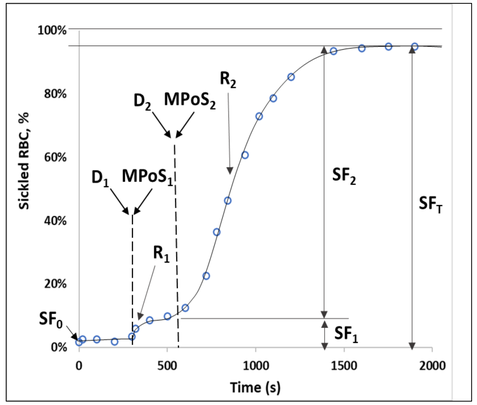
There is a pressing need for tests that assess baseline RBC polymerization risk and monitor response to SCD therapies affecting Hb-O_2 affinity. Current approaches predominantly use gas exchange to deoxygenate blood samples, which takes makes it difficult to achieve precise control over the rate and depth of desired hypoxic conditions.

❖ Solution

Enzymatic systems have advantages offering easier control over the oxygen consumption rates and the level of resultant deoxygenation (hypoxia) in the medium. Presented approach uses Protocatechuate Dioxygenase/Protocatechuate Acid (PCA/PCD) oxygen scrubber system, with the PCD nonheme iron center catalyzing the conversion of PCA and molecular oxygen in 1:1 stoichiometry into ß-carboxy-cis,cis-muconic acid. Additional attraction of the PCA/PCD system is that, unlike other enzymatic oxygen scrubbers, the reaction does not generate any reactive oxygen species (like peroxide or superoxide).

❖ Dynamic Sickling Assay (DSA)

- Hypoxia is induced in microfluidic channels
- RBC sickling is assessed through time-lapsed imaging analysis.
- Medium PaO_2 is simultaneously measured by O_2 probe. Hypoxia is induced by the addition of PCD to blood sample containing PCA at T=0
- Hypoxia is induced up to 0% final O_2
- Pre-hypoxia sickled RBCs quantified at T=0
- Presented sample contained two fractions of sickling RBC, with SF_T representing the total fraction of RBC that sickle
- Each fractions is described by Sickling Fraction size (SF), sickling Rate (R), delay in sickling (D) and Morphological Point of Sickling (MPoS) – PaO_2 corresponding to 5% sickling of the fraction

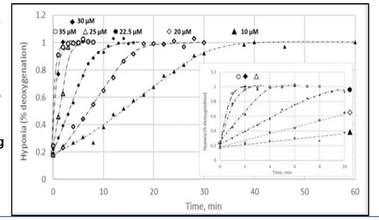


❖ **Conflict of Interest Statement:** Tarasev, Gao, Mota, Ferranti, Edenstrom, Zaidi, and Hines are employees and Tarasev, Gao and Hines are also shareholders of Functional Fluidics., a company developing methods for assessing functional properties of blood cells. This work was fully funded by Functional Fluidics.

DSA: FEATURES

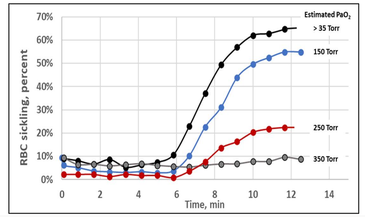
❖ Wide range of deoxygenation rates

- Modify deoxygenation rates in a range from seconds to hours
- Assay can be performed in both a quasi-equilibrium mode (common) and in a kinetic mode
- Kinetic assay can model Hb polymerization and RBC sickling as it would occur *in-vivo*, in capillary network



❖ Wide range of final hypoxia levels

- Method allows to modify the severity of hypoxia (partial oxygen pressure) at the end of the assay
- Allows *in-vitro* modelling of *in-vivo* oxygen environment (e.g., in different organs or tissues, or changes due to changes in condition)



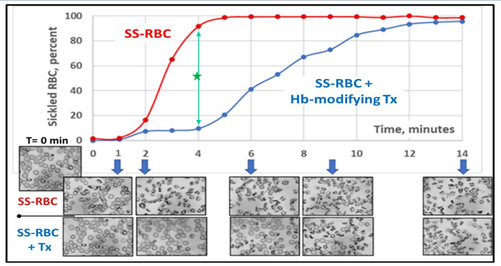
❖ Follow-up features

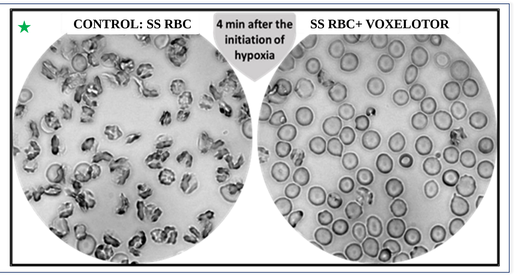
- Repeated sickling after one or more cycles of reoxygenation
- Sickling of adhering RBC (e.g., on VCAM1)
- Sickling under flow or under shear
- Simultaneous monitoring of kinetics of O_2 release from Hb (hemoximetry)
- Differentiation of sickled RBC forms (crescent, holly leaf, granular) and transition states (e.g., cell crenation)

DSA: ILLUSTRATIVE APPLICATION

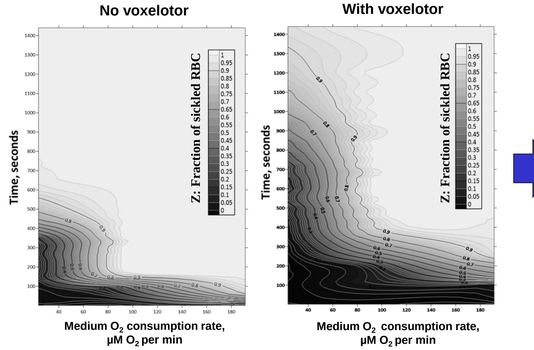
❖ Decreased sickling due to *in-vitro* treatment with Hb-modifying drug (voxelotor)

- Untreated HbSS sample
  - Delay ≈ 80 s (time to get PaO_2 to a level inducing RBC sickling)
  - Single phase sickling curve (only one sickling rate)
  - About 98% RBC are sickled at zero percent oxygen
- HbSS sample *in-vitro* treated with GBT440:
  - Two subpopulation with different propensity to sickle
  - 10% of RBC showed sickling similar to that of untreated cells,
  - 90% exhibited significant increased delay in sickling (about 260 s)
    - Sickling occurring at a much-reduced rate
    - Cumulative fraction of sickled RBC did not change significantly (> 97%).





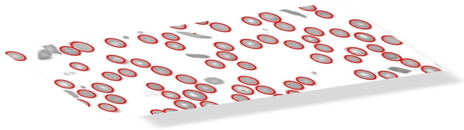
❖ Data collection and analysis using 3D surfaces and contour maps



alternative axis

Medium actual PaO_2 measured at each time at each set oxygen consumption rate

- Effect: incubation with Hb-modifying drug (voxelotor)
- Fractions of sickled RBC was determined over time at different rates of medium O_2 consumption
- 3D surface calculated through interpolation and presented as a counter map



- Conditions for induced hypoxia/medium deoxygenation can be optimized based on particular application's needs to select best conditions for detection of:
  - Differences in RBC sickling due to *in-vitro* drug treatment (pre-clinical drug development)
  - Changes in patient blood samples before and during *in-vivo* treatment (drug development clinical and treatment monitoring)
  - Changes in RBC sickling propensity between patient baseline and severe pathology conditions including SCD-related vaso-occlusive complications (e.g. pain crisis)
  - Changes in RBC properties as a result of gene editing (both done in-vitro and as a result of gene-editing patient treatment)